Expression, biological activity and the diagnostic potential of leptospiral sphingomyelinases

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CERTIFICATE

This is to certify that the thesis entitled **"Expression, biological activity and the diagnostic potential of leptospiral sphingomyelinases"** submitted by **Ms. Reetika Chaurasia** bearing registration number **12LAPH09** in partial fulfillment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

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4. AS 804	Analytical Techniques	3	Pass
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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Expression, biological activity and the diagnostic potential of leptospiral sphingomyelinases"** has been carried out by me under the supervision of **Prof. Manjula Sritharan** at Department of Animal Biology, School of Life Sciences. The work presented in this thesis is a bonafide research work and has not been submitted for any degree or diploma in any other University or Institute.

Date:

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- A strict Academician
- Dedicated for Science
- A good Critic
- And nice women as a Person

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This Thesis Is

Dedicated To My Beloved

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LIST OF ABBREVIATIONS	
ATCC	American Type Culture Collection
BCIP / NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate / Nitroblue Tetrazolium
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DAPI	4′,6-Diamidino-2-phenylindole
DFM	Dark Field Microscopy
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylene Diamino Tetra Acetic Acid
EEPD	Exo-Endo Phosphatase Domain
ELISA	Enzyme - Linked Immunosorbent Assay
EMJH	Ellinghausen - McCullough Johnson Harris
FITC	Fluorescein isothiocyanate
g	Gram
h	Hour
Kb	Kilobase
kDa	Kilo Dalton
LB	Luria-Bertani
LERG	Leptospirosis Epidemiology Reference Group
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight
MAT	Microscopic Agglutination Test
mg	Milligram
mL	Milliliter
mM	Millimolar
µM	Micromolar
MOI	Multiplicity Of Infection

NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
nm	nanomolar
NTP	Nucleoside triphosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PUO	Pyrexia of Unknown Origin
RBC	Red Blood Cell
RNA	Ribonucleic acid
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
TAE	Tris-Acetate-EDTA
TEMED	Tetramethyl ethylenediamine
TLC	Thin Layer Chromatography
Tris	Tris - (Hydroxymethyl) aminoethane
UV	Ultra Violet
WHO	World Health Organization

ABSTRACT

Leptospirosis is a zoonotic disease that affects humans and animals worldwide. The disease, causing high morbidity and mortality is more prevalent in tropical regions as the humid environment favours its transmission. The disease is caused by pathogenic *Leptospira* that enters a mammalian host through the skin and conjunctiva, either by direct contact with an infected host or from contaminated environment. The organisms preferentially reside and multiply in the proximal convoluted tubules of the kidney and are periodically shed in the urine of infected hosts. In humans, who are often accidental hosts, the disease is acute and usually self-limiting as the mammalian host mounts a strong humoral immune response that kills the pathogens. However, in about 5 -10% of the patients, the disease can progress to the severe form called the Weil's disease, characterised by the involvement of the liver, lungs and kidneys. Jaundice, pulmonary haemorrhage and renal dysfunction are seen in the severe form that is often fatal. Clinically, since the disease presents symptoms such as muscular pain, headache and fever that overlaps with other tropical diseases such as common flu, malaria and dengue, the disease is difficult to diagnose based on clinical manifestations. Limitations in laboratory tests, that includes the cumbersome microscopic agglutination test (MAT) and the expensive ELISA-based commercial kits, the disease is often under-diagnosed. The focus in our lab has been the identification of pathogen-specific leptospiral proteins. And the development of simple ELISA-based diagnostic tests using these antigens.

Sphingomyelinases are virulence factors produced by pathogenic *Leptospira* spp. They account for the lytic activity of these organisms by virtue of their hemolytic and enzymatic activities. Whole genome sequencing identified five sphingomyelinase genes encoding the sphingomyelinases Sph1, Sph2, Sph3, Sph4 and SphH in the genome of *Leptospira interrogans* serovar Lai. Among them, Sph2 is structurally and functionally characterized and established as a true sphingomyelinase. Currently, with the availability of the genome sequences of additional leptospiral genomes, considerable information has made comparative genomics possible; the non-pathogenic *Leptospira biflexa* serovar Patoc carries hemolysin genes in its genome but lacks the *sph* genes. *In silico* analysis, however, must be supported with experimental studies to characterise these molecules.

The objectives of the current study included studying the expression of sphingomyelinases by pathogenic *L. interrogans* serovar Pomona in axenic cultures and upon infection of Vero cells and to evaluate the diagnostic potential of this protein and the hemin binding protein HbpA, a well-studied antigen in our lab. The serovar Pomona harboured four *sph* genes and lacked the *sph4* present in serovar Lai. Sph2 of serovar Pomona was bigger due to the presence of an additional stretch of 25 amino acids. It is likely that there are differences in its regulation of expression, as basal levels were relatively higher than that seen in serovar Lai; salt addition, however enhanced the expression of this molecule in both the serovars. A notable observation was the high hemolytic activity of the spent growth medium, indicated that serovar Pomona secreted an active sphingomyelinase molecule. The cell damaging effect of the sphingomyelinases were studied using scanning electron microscopy and flow cytometry. Upon infection of Vero cells with live leptospires, adherence of the pathogen followed by damage to the host cell surface was evident. Within two hours, pores were formed in the host cell and by four hours, marked decrease in the cell viability was measured by flow cytometry.

Further studies linked these observed changes to the sphingomyelinases. When the leptospires were pre-incubated with anti-sphingomyelinase antibodies, there was protection initially, with evidence of apoptotic changes occurring with time. There was increased expression of Sph2, evident from the 44-fold increase in the transcript levels of *sph2* and the expressed protein. A 42 kDa sphingomyelinase was secreted into the growth medium of infected Vero cells. The smaller size of the molecule indicated it is a processed functional form of sphingomyelinase that exhibited both hemolytic and sphingomyelinase activities. The growth medium of the infected cells, devoid of live organisms caused the lysis of a fresh batch of Vero cells. This finding and the lytic effect of recombinant sphingomyelinase unambiguously establishing the effect of the sphingomyelinase on the Vero cells.

Screening for antibodies against the leptospiral protein HbpA is well established in our lab. Here, the specificity of detection was increased by cloning and expressing a 34 kDa fragment of HbpA. The first clinical study focused on the detection of antibodies against $HbpA_{34}$ and sphingomyelinase, specifically $rSphCD_{210}$, carrying the common domain present in all the four sphingomyelinases of serovar Lai. The performance of

these tests was compared with the gold standard MAT and the commercial PanBio ELISA kit. Using MAT as gold standard, it was found that there was better sensitivity and specificity of $SphCD_{210}$-ELISA (92%, 90%) and $HbpA_{34}$-ELISA (95%, 89%), when compared to LipL41-ELISA (84%, 76%) and PanBio IgM-ELISA (56%, 95%). The sensitivity of $HbpA_{34}$ as an antigen was also reflected by its performance in the lateral flow device for the sero-diagnosis of leptospirosis.

A salient contribution of this study was the demonstration of the presence of HbpA, sphingomyelinase and the other pathogen-specific LipL32 and LipL41 in the urine of leptospirosis patients. They were absent in the urine of dengue patients, thereby establishing the usefulness of screening urine for a confirmatory diagnosis of leptospirosis. It is well known that leptospires migrate to the kidneys and are shed in the urine of infected animals and humans. Thus, this non-invasive method of urinary screening will be of clinical significance. These leptospiral antigens were identified both by ELISA and Western blotting. Immunoblots showed the presence of the 42 kDa sphingomyelinase in some patients and a 58 kDa band in a few other patients. It is possible that the environmental signals influence the expression of these specific sphingomyelianses. Further studies are required to characterise these bands representing the sphingomyelianses. These patients also showed high levels of antibodies against leptospiral whole cell sonicates of serovar Lai and Pomona, with much lower titres with the non-pathogenic serovar Andamana. Using ten serum samples with high titres of antibodies, immunoblotting was done using whole cells sonicates of pathogenic serovar Lai and Pomona. Six leptospiral proteins were purified and identified by MALDI-TOF MS/MS analysis as biotin-requiring enzyme (LA2432), acyl-CoA dehydrogenase (LA2639), serine hydroxyl methyl transferase (LA1409), argininosuccinate synthase (LA4165), ferritin-like protein (LA3598) and the molecular chaperone GroES (LA2654).

Overall the current study highlights the biological activity and clinical significance of the leptospiral sphingomyelinases, making possible the development of screening tests for this disease, more so, using urine that will offer a non-invasive method of diagnosis of leptospirosis.

CHAPTER – I

REVIEW OF LITERATURE

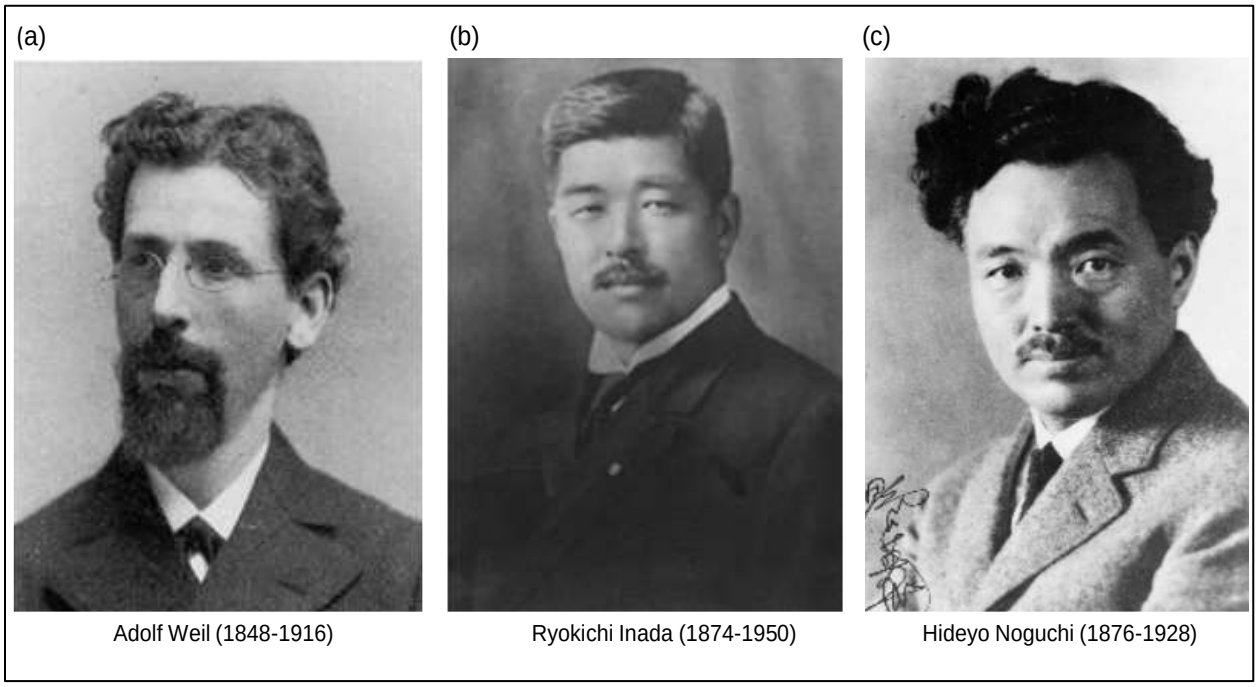
1.1. Introduction

Leptospirosis is a widespread zoonotic disease, with the tropical and subtropical countries being the most affected as humid environment favors the transmission of the disease (Bharti *et al.*, 2003). Annually, there are 1.03 million cases and 58,900 deaths due to leptospirosis worldwide (Jimenez *et al.*, 2018). The causative organisms are the pathogenic members of the spirochetal bacteria belonging to the genus *Leptospira*. Rodents, wild and domestic animals serve as carriers and promote transmission of the disease; more than 160 species of animals carriers are reported (Gallagher. S & Dunn. N, 2018). Humans are accidental hosts. The infected animals shed the organisms through their urine into the immediate environment, thus promoting indirect transmission, while direct transmission by contact can also occur (Sritharan, 2012). Pathogenic *Leptospira* enter the human host through cuts and abrasions in the skin and conjunctival tissues. They remain in the circulating blood for about a week and when antibodies against leptospiral antigens begin to rise, they migrate to tissues, preferably liver, lungs and kidneys. The disease is often self-limiting with the host mounting a strong humoral immune response that helps to kill these pathogens. However, in about 5 -10% of the patients, the disease can progress to the severe form resulting in haemorrhage in several organs including the liver, lungs and kidneys, a condition referred to as Weil's disease. Pulmonary haemorrhage is increasingly being recognized as a major complication in developing countries (Dolhnikoff *et al.*, 2007).

The disease is clinically difficult to diagnose as the symptoms overlap with other diseases like common flu, malaria and dengue, commonly seen in tropical countries. Differential diagnosis of leptospirosis is thus challenging, not only due to lack of defined clinical presentations but also because of limitations in the available screening tests. Culture, often a gold standard for bacterial infection is not easy to achieve and the laboratory tests are often based on detection of specific antibodies, as the mammalian host mounts a strong humoral immune response in leptospiral infection. Among the antibody-based tests, the microscopic agglutination test (MAT), based on the agglutination of live organisms by anti-leptospiral antibodies had emerged as the 'gold standard'. Despite the high specificity of this test, there are several limitations of MAT and efforts are made to replace it with the easy-to-perform ELISA-based tests using pathogen-specific leptospiral antigens

1.2. Historical perspectives

Adolf Weil, a German physician made the first public presentation of the clinical manifestations of leptospirosis in 1886 (Weil, 1886). Direct demonstration of the pathogen was made by Stimson, who showed the presence of these organisms in autopsy specimens of renal tubules collected from a patient who died of yellow fever. He called them *Spirocheta interrogans* by virtue of the hooks seen at their ends resembling a question mark (Stimson, 1907). Unfortunately, his observations went unrecognized and the two Japanese scientists, Inada and Ido who presented the causative organisms as



(Adler, 2015)

Figure 1.1. Pioneers in the field of leptospirosis. Adapted from (Adler, 2015).

Spirochaeta icterohaemorrhagiae and also demonstrated the role of rats in the transmission of the disease to humans (Kobayashi, 2001). While it was later called *Spirochaeta icterroganes* (Uhlenhuth, 1916), Noguchi proposed the genus *Leptospira*, meaning 'thin spiral' organism that is morphologically different from other genera of spirochetes (Noguchi, 1918). The awareness of leptospirosis increased within a decade after the discovery of leptospires along with the identification of several serovars during this period (Kmety & Dikken, 1993).

Leptospirosis is an occupational disease. It is therefore called as “akiyami‟ or “autumn fever‟ in Japan, “rice harvest jaundice” in China, “swineherd’s disease” in Europe and “sewerman’s flu” in the United States (Faine, 1999). In India, while reports of febrile jaundice were reported (Chowdry, 1903), organisms demonstrated in urine and liver samples (Barker, 1926), confirmatory presentation of leptospirosis was reported from Andamans (Taylor & Goyle, 1931). Leptospiral etiology of Andaman Haemorrhagic Fever (AHF) was unraveled in Diglipur in North Andaman (Sehgal *et al.*, 1995).

1.3. *Leptospira*, the spirochetal organism

1.3.1. Taxonomy

Leptospira belongs to:

Division - **Gracillicutes**

Class - **Scotobacteria**

Order - **Spirochaetales**

Family - **Leptospiraceae**

Genus - ***Leptospira***

The family Leptospiraceae consists of three genera viz., *Leptospira*, *Leptonema* and *Turneria*.

1.3.2. Classification of *Leptospira*

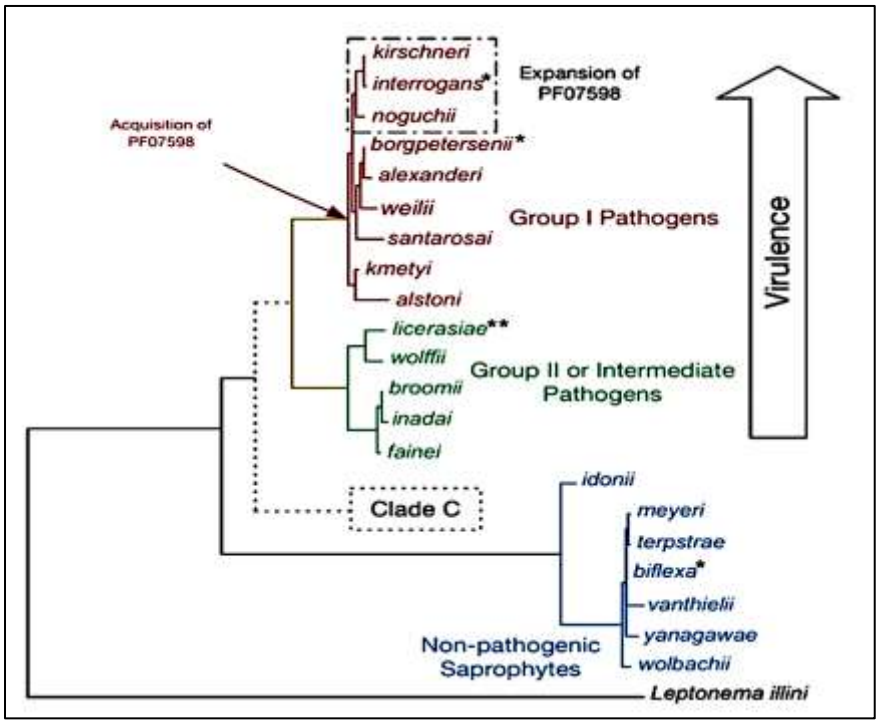
1.3.2.1. Serological classification

Based on antigenic relatedness, the genus *Leptospira* is classified into the pathogenic members belonging to *Leptospira interrogans* and the saprophytic members belonging to *Leptospira biflexa* (Levett, 2001). Saprophytic members can be morphologically distinguished from pathogens by forming spherical shapes in the presence of 1 M NaCl. They also growth at 13°C and in the presence of 8-azaguanine, unlike the pathogens. Each of these two species were further classified into serovars, based on the antigen-relatedness tested by the Cross-Agglutination Absorption Test (CAAT) (Terpstra, 1992). Currently, there are 300 pathogenic serovars and 60 non-pathogenic serovars (Picardeau, 2017), with antigenically-related serovars further sub-grouped into

serogroups for convenience in serologic testing (Levett, 2001), often used for epidemiological surveys.

1.3.2.2. Genotypic classification

The genotypic classification is based on the degree of genetic relatedness and today there are 21 species of the genus *Leptospira* known. Fig.1.2 shows the phylogenetic relatedness of these species, which based on 16S rRNA phylogeny groups them into pathogenic, intermediate and non-pathogenic *Leptospira* respectively (Lehmann *et al.*, 2014). In the Group I pathogens, *L. interrogans*, *L. kirschneri* and *L. noguchii* are evolutionarily-related and associated with fatal human disease (Brenner *et al.*, 1999).



(Lehmann *et al.*, 2014)

Figure 1.2. Classification of *Leptospira*. The figure shows phylogenetic tree based on 16S rRNA sequences from 21 leptospiral species. Recently identified genus *Leptonema illini* is placed distantly in the tree. The complete genomes are available for six strains belonging to three species showed by *. Three strains have high quality draft genome represented by **.

Group II pathogens can cause mild and self-resolving illness (Lehmann *et al.*, 2014). Genotyping of *Leptospira* spp. has also been done by multi-locus variable-number

tandem-repeat (Salaun *et al.*, 2006), multi-locus sequence typing (Ahmed *et al.*, 2006) and MALDI–TOF analysis (Rettinger *et al.*, 2012). With technological advancement, molecular techniques like Next-Generation Sequencing (NGS) will overtake phenotypic methods for identification and characterization of bacterial isolates (Richter & Rossello-Mora, 2009), therefor providing insights into different aspects such as classification, evolution, virulence and others (Thibeaux *et al.*, 2018).

1.3.3. Biology of *Leptospira*

Leptospira are Gram - negative spirochaetal bacteria that are thin and long (diameter of 0.15 µm and 10 - 20 µm long). They possess a characteristic spiral shape and exhibit corkscrew movement that helps to visualize them by dark-field microscopy. This motility enables them to enter the mammalian host through the skin and connective tissues (Picardeau, 2017). As leptospires are extremely thin, they cannot be visualized by the conventionally used staining techniques but can be detected by silver staining (Faine, 1999). Their corkscrew movement is due to the presence of two endoflagella at each end of the body (Fig. 1.3) extending from the cytoplasmic membrane into the periplasmic space (Levett, 2001).

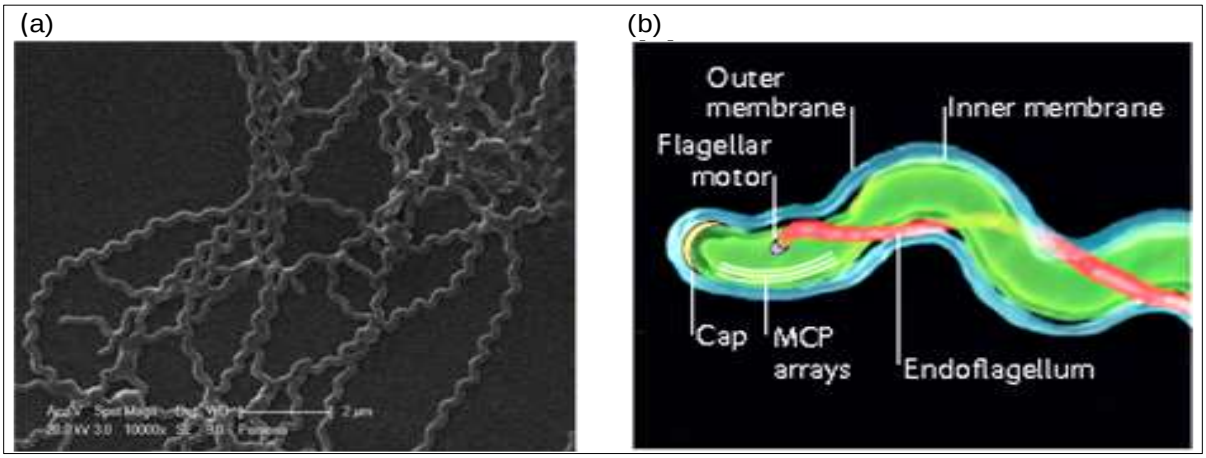


Figure 1.3. Leptospiral cell morphology and structural organization. Scanning Electron Micrograph (Philips FEI, XL30, USA) of *L. interrogans* serovar Pomona done at University of Hyderabad shows the characteristic spiral shape. In the second panel (Picardeau, 2017), the outer and inner membranes, and the endoflagellum along with the motor propelling it can be seen. MCP refers to methyl-accepting chemotaxis proteins seen near the flagellar motor at polar end.

1.3.4. Growth and Metabolism

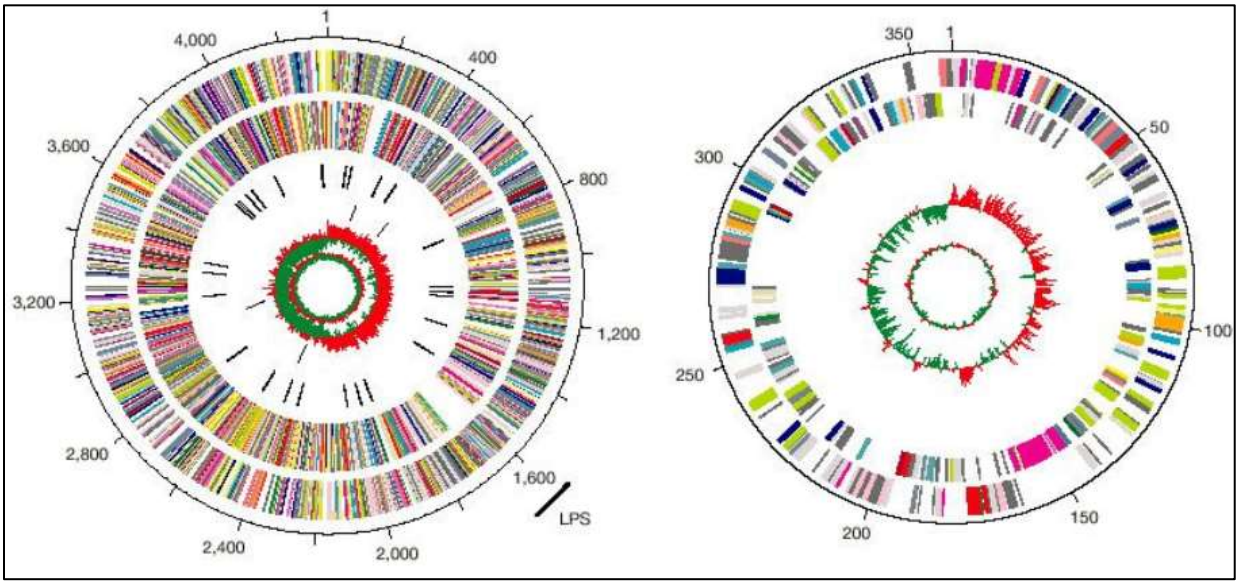
Leptospires are obligate aerobes and are relatively slow-growing bacteria with a doubling time of 6 – 8 hours. These organisms cannot utilize glucose and use lipids as carbon and energy source (Baseman & Cox, 1969). Tween 80 is commonly used as a source of long-chain fatty acids (Henneberry & Cox, 1970) and due to the inherent toxicity of free fatty acids, albumin is included in the medium. The growth medium must be supplemented with Vitamins B1 and B12, both of which are present in high amounts in rabbit serum (Ellis & Michno, 1976). The commonly used medium is the Ellinghausen and McCullough Modified Johnson and Harris (EMJH) medium (Ellinghausen & McCullough, 1965a; b), in which the organisms are maintained at 30°C. The liquid EMJH medium is used for isolating the organisms and performing all experimental studies, with the semi-solid media prepared by dissolving 0.2% agar in liquid EMJH medium is used for long-term maintenance of these organisms, usually for at least two months. In this medium, they grow below the surface, where the organisms can be seen as a discrete turbid zone, called as Dinger's ring (Levett, 2001). Korthof's medium, another preferred medium for cultivation of *Leptospira* directly from blood and urine samples, is prepared from a peptic digest of animal tissues, with added hemoglobin and inactivated serum. The semi-solid Korthof's medium, with 0.1% agar has been used to grow leptospires from infected rat kidney and urine (Saito *et al.*, 2015). Fletcher's liquid medium, prepared by adding rabbit serum to Fletcher's base medium maintains the cell viability for a long period without the need for sub-culturing (WHO, 2003).

A recently reported semi-solid medium is the *Leptospira* Vanaporn Wuthiekanun (LVW) agar medium containing Noble Agar base and 10% normal rabbit serum. Growth in this medium requires an initial incubation at 30°C in 5% CO_2 for 2 days prior to continuous culture at 30°C. This medium is cheaper and easy to prepare and facilitates optimal growth of the organisms (Wuthiekanun *et al.*, 2013). It is also useful for long term year-long maintenance without the need for sub-culturing (Wuthiekanun *et al.*, 2014). Culturing of leptospires from clinical specimens is often done upon inclusion of 5-fluorouracil and / or nalidixic acid to kill other contaminating bacteria, as these compounds do not inhibit the growth of leptospires (Adler & de la Pena Moctezuma, 2010).

1.3.5. Leptospiral genome

1.3.5.1. Whole genome sequencing

Currently, whole genome sequencing of nine leptospiral serovars are available. They include *L. interrogans* serovars Lai (Ren *et al.*, 2003), Copenhageni (Nascimento *et al.*, 2004), two strains of *L. borgpetersenii* serovar Hardjo L550 (Bulach *et al.*, 2006), two strains of *L. biflexa* serovar Patoc (Picardeau *et al.*, 2008), two strains of an intermediate spp. *L. licerasiae* (Ricaldi *et al.*, 2012) and *L. santarosai* serovar Shermani (Chou *et al.*, 2012). In general, the leptospiral genome is larger than that of the other spirochetes. It is 4.3 Mb in serovar Lai while it is only 1 Mb in Borrelia burgdorferi and Treponema pallidum. Almost all of them contains two chromosomes, with the non-pathogenic serovar Patoc containing an additional 74 kb circular replicon (Picardeau et al., 2008). In serovar Lai, the larger chromosome (CI) is 4.3 Mb, while the other (CII) is only 359 Kb in length. The genome has 36% GC content and contains 4,360 protein coding genes in CI (Fig. 1.4) and 367 genes in CII (Ren *et al.,* 2003).



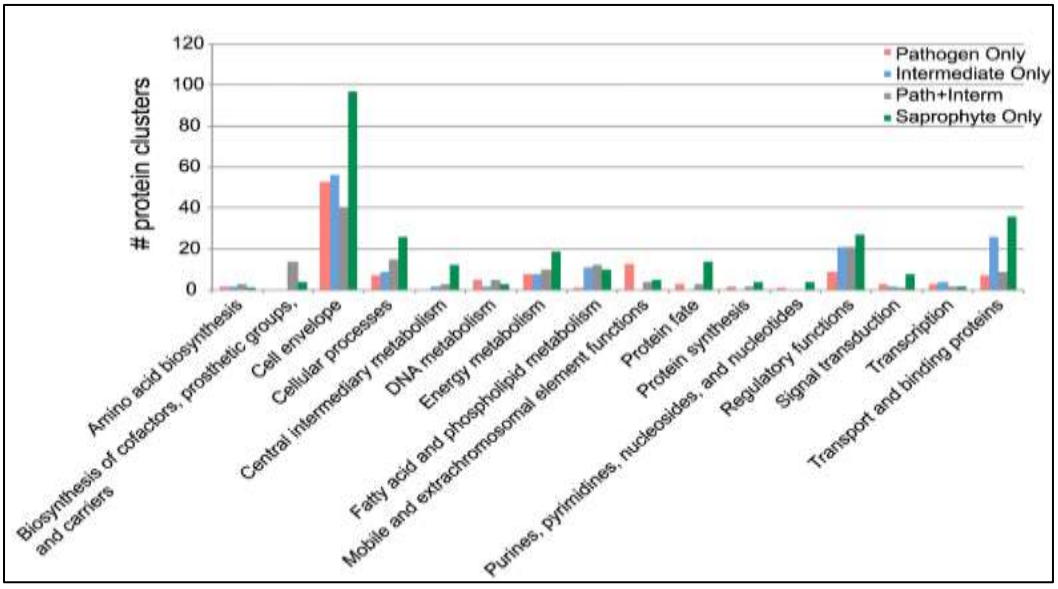
(Ren *et al.*, 2003)

Figure 1.4. Diagrammatic representation of protein coding sequences (CDSs) in the two circular chromosomes I and II of *L. interrogans* serovar Lai genome. Panels (a) and (b) show the larger chromosome CI and smaller chromosome CII. Kilobases is represented in outer scale. Coding genes are shown in different colours according to their functions.

1.3.5.2. Comparative genomics:

There is 95% identity in the genomes of the serovars Lai (strain 56601) and Copenhageni (strain Fiocruz L1-13) belonging to *L. interrogans*. The total protein coding genes in Copenhageni is 3728 (3454 and 274 in CI and CII) with a larger number in serovar Lai (4727 distributed as 4360 and 367 on CI and CII respectively). While they are almost similar, regions of chromosomal inversion are seen and there is variation in the number and distribution of insertion sequence elements (Nascimento *et al.*, 2004).

The genome of *L. borgpetersenii* serovar is 3.9 Mb, that is ~ 16% (~700 Kb) smaller than that of serovar Lai, which shows large number (627) of unique genes encoding proteins necessary for survival in the non-living environments. This enables the serovar Lai to survive outside the mammalian host for considerable periods before entering a mammalian host. This is not possible in the serovars belonging to *L. borgpetersenii*, indicating only host to host transmission (Bulach *et al.*, 2006). A recent study reported the use of protein clusters (Fig. 1.5) that can be used



(Fouts *et al.*, 2016)

Figure 1.5. Protein clusters unique to the genome of leptospiral spp. The figure shows genes involved in metabolic pathways unique to pathogenic, intermediate, and saprophytic groups. The shared genes between pathogens and intermediates play a significant role in biosynthesis of cofactors and fatty acid metabolism. 10 out of 16 groups was dominated by saprophyte-specific genes, and involved in signal transduction, energy metabolism, gene regulation, cell envelope and transport functions. These can be seen by different colors specific to species.

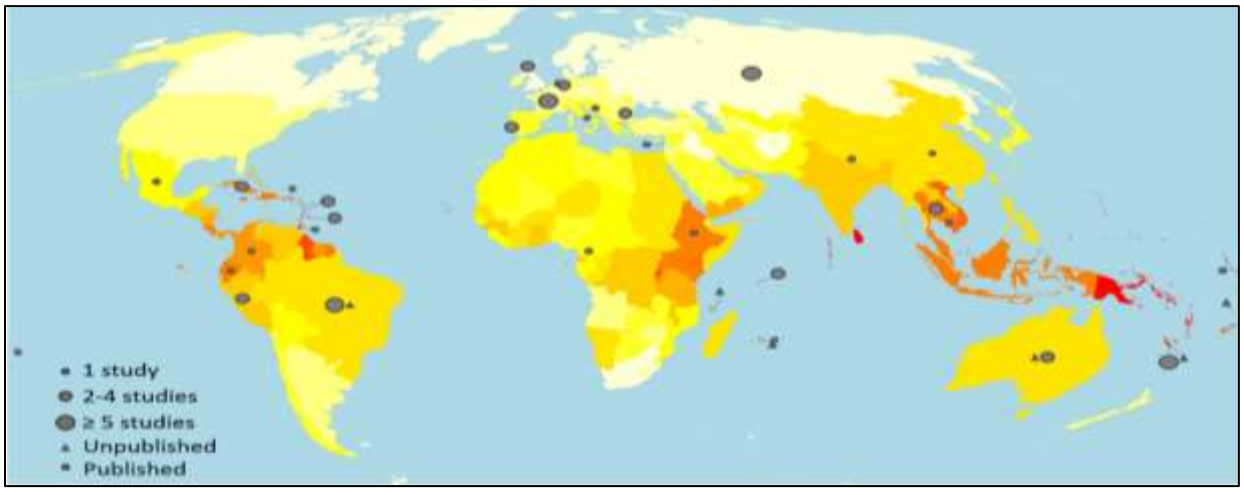
to categorize *Leptospira* into saprophytic, intermediate and pathogenic groups (Fouts *et al.*, 2016). There were larger number of genes in the pathogens, with the least in the saprophytes. There were several genes common to the pathogens and intermediate group, with much less similarity seen between pathogens and saprophytes. It was inferred that the members of the intermediate spp. were more related to the pathogens.

Comparative genomics has made possible the identification of proteins like sphingomyelinases, hemin-binding protein HbpA (Asuthkar *et al.*, 2007), LipL32, LipL36, LipL41 and LipL45 (Cullen *et al.*, 2004), Lig proteins (Choy *et al.*, 2007), LfhA, a putative factor H binding protein (Verma *et al.*, 2006) as pathogen-specific proteins. The corresponding orthologs were absent in *L. biflexa*, implicating their relevance in the survival of the pathogens within the mammalian host.

1.4. The disease leptospirosis

1.4.1. Epidemiology

Accurate statistical data on the incidence of leptospirosis is not available. The World Health Organization (WHO) established a reference center called 'Leptospirosis Epidemiology Reference Group (LERG) to estimate the morbidity and mortality of this



(Costa *et al.*, 2015)

Figure 1.6. Predictable annual morbidity of leptospirosis worldwide. The disease incidence per 100,000 population was 0–3 (white), 7–10 (yellow), 20–25 (orange) and more than 100 (red).

disease globally (Stein *et al.*, 2007). Other surveillance agencies include the Pan American Health Organization (Pereira *et al.*, 2018) and the European Centre for Disease Prevention and Control (ECDC), the latter reporting an incidence of 0.1 case per 100,000 population during 2015 (ECDP., 2018).

A recent survey reported incidence of 1.03 million cases of leptospirosis annually with 58,900 deaths (Jimenez *et al.*, 2018). Fig. 1.6 showing the highest estimates of leptospirosis in global burden disease regions of South and Southeast Asia, Oceania, Caribbean, Andean, Central, and Tropical Latin America, and East Sub-Saharan Africa (Costa *et al.*, 2015). Floods along the coastline, heavy rainfall, poor sanitation, inadequate drainage and lack of awareness are the major causes of leptospirosis in these regions.

1.4.1.1. Global scenario

Leptospirosis is a major public health issue in several islands in the Western Indian Ocean that experience tropical climate. Human leptospirosis has been extensively studied in Reunion Island, Mayotte, and Seychelles (Desvars *et al.*, 2013). Seychelles, with its sub-equatorial climate and 80% of humidity throughout the year reported the highest incidence with 101 cases of leptospirosis per 100,000 inhabitants (Biscornet *et al.*, 2017). Among East - Asian countries, high incidence was seen in China (1-10 cases per 100,000 population) with high mortality due to pulmonary haemorrhage (Hu *et al.*, 2014). There were fewer reports (< 1 per 100,000 population) from Hong Kong, Japan, South Korea and Taiwan (Victoriano *et al.*, 2009). Many cases of leptospirosis are reported in South Asian regions, with the identification of several different causative serovars (Naotunna *et al.*, 2016). The first confirmed case of human leptospirosis in the Maldives was reported in November 2000. Since then, the disease has been under national surveillance (Cosson *et al.*, 2014). In Nepal, a study reported that 21% patients with febrile illness showed IgM-specific anti-leptospiral antibodies (Regmi *et al.*, 2017). In Thailand, leptospirosis is the second most frequent cause of fever (Tangkanakul *et al.*, 2005). In New Zealand, cattle is main source of infection in dairy farm (Benschop *et al.*, 2017). Highest incidence 163 per 100,000 populations were reported in meat processing workers followed by livestock farm workers (92 / 100,000) and forestry-related workers (24 / 100,000) with major

infecting serovars identified as Hardjo, Pomona and Ballum (Thornley *et al.*, 2002). In U.S.A., the highest incidence was reported at Hawaii with 128 cases / 100,000 population (Katz *et al.*, 2002). The disease is endemic in the Caribbean islands with more than 500 cases (Pappas *et al.*, 2008).

1.4.1.2. Indian Scenario

The first incidence of leptospirosis in India was reported in Andaman Island in 1929 (Taylor & Goyle, 1931). Today there are several reports of leptospirosis, mainly from coastal regions such Mumbai, Madras, Kerala and Orissa (Ratnam *et al.*, 1983; Muthusethupathi & Shivakumar, 1987; Venkataraman *et al.*, 1991; Bharadwaj *et al.*, 2002; Mathur *et al.*, 2009; Shekatkar *et al.*, 2010). A retrospective study in a tertiary hospital setting in Hyderabad identified cases of leptospirosis with the serovar Lai identified as the major infecting serovar (Velineni *et al.*, 2007). The involvement of the eye was reported in Madurai, where an epidemic outbreak of uveitis with systemic leptospirosis was seen (Priya *et al.*, 2008). A subsequent study done to evaluate HbpA as a diagnostic antigen identified several cases of uveitis in Madurai (Sivakolundu *et al.*, 2012). The incidence of the disease is high in Kerala (Pappachan *et al.*, 2004; Sugathan & Varghese, 2005; Kuriakose *et al.*, 2008). In the state of Orissa, after cyclone during 1999, an increasing numbers of leptospirosis associated with febrile illness and haemorrhagic manifestation was reported (Mohanty, 1945; Jena *et al.*, 2004). Leptospirosis has been reported in Delhi, with co-infection of viral hepatitis E, malaria and dengue fever (Chaudhry *et al.*, 2013).

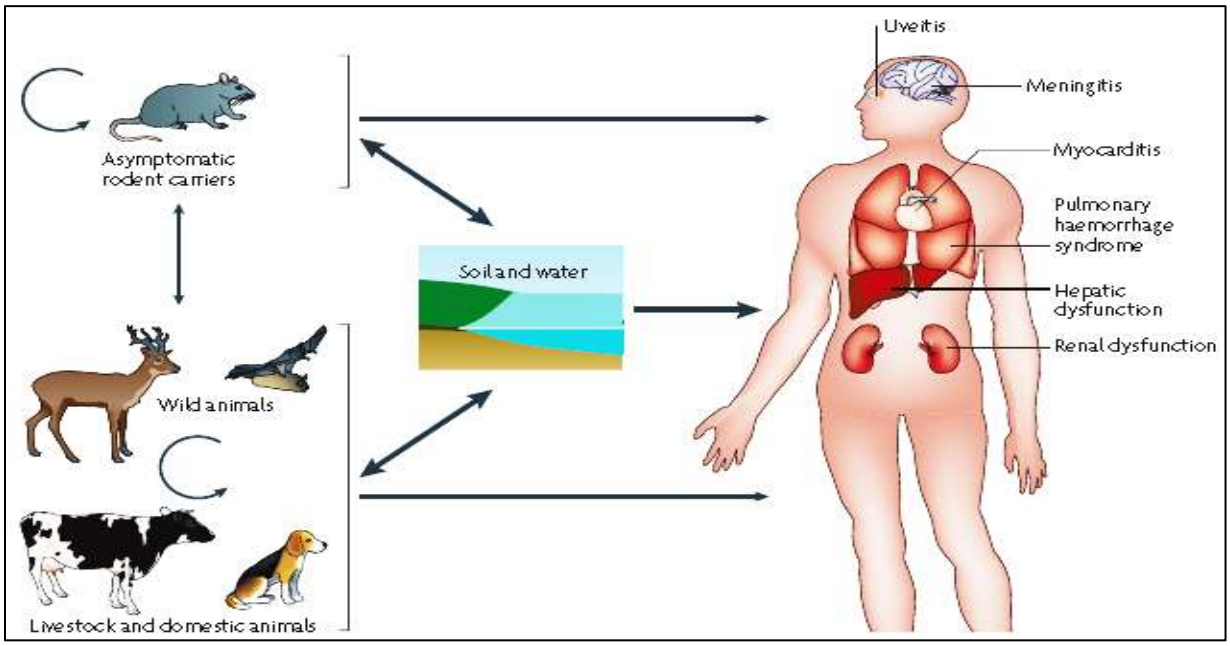
Leptospiral etiology was made for the Andaman Haemorrhagic Fever after five years of investigations *(*Sehgal *et al.*, 1995*).* The incidence of pulmonary involvement in leptospirosis increased from 9.4 % in 1929 to 52 % in 1997, that made the authors to raise the question whether these symptoms were new or were overlooked in earlier studies (Vijayachari *et al.*, 2015).

1.4.2. Transmission

Humans acquire the infection either by direct contact with infected animals, or indirectly from the immediate environment contaminated with leptospires shed *via* the urine of infected animals (Fig.1.7) (Haake & Levett, 2015). The risk of infection depends on the

ability of *Leptospira* to survive, persist, and infect new hosts (Barragan *et al.*, 2017). Cell aggregation and biofilm formation may contribute to the survival of leptospires outside their hosts (Ko *et al.*, 2009).

Livestock are the major source of infection for farmers, veterinarians and workers handling meat, while wildlife and rodents are the primary sources for hunters, pastoralists and urban dwellers (Mwachui *et al.*, 2015; Lau *et al.*, 2016). Leptospirosis prevalence is high in tropical regions as survival is higher in moist and humid climate. Factors favouring transmission include moist and humid climate, walking barefoot, close contact with animals and poor conditions of sanitation with improper sewage drainage and mixing with ground water (Dias *et al.*, 2007).



(Ko *et al.,* 2009)

Figure 1.7. Transmission of *Leptospira*. Leptospirosis is transmitted to humans by direct contact with urine of infected animals or indirect exposure to environmental surface water or soil that is contaminated with the urine of infected animals. Leptospires penetrate abraded skin or mucous membranes and enter in bloodstream and disseminate throughout the body tissue and cause severe multi-organ failure.

1.4.3. Clinical manifestations

In humans, there is a wide spectrum of symptoms, ranging from mild flu - like symptoms such as fever, headache, chills and myalgia to the severe, often fatal form called Weil's

syndrome. Upon entering into the human host, leptospires circulate in the blood (septicemic phase) for about a week and then migrate to tissues, preferably to the liver, lungs and kidneys upon the appearance of anti-leptospiral antibodies. The disease is often self-limiting due to strong humoral immune response, resulting in the generation of antibodies that kills the pathogens (immune phase). In the septicemic phase, one or more of the following can be seen in addition to fever and myalgia, conjunctival suffusion, abdominal pain, nausea, vomiting, diarrhoea, cough and pharyngitis (Bharti *et al.*, 2003). In Weil's disease, the affected organs often include the lungs, liver and kidneys. There is frequently haemorrhagic manifestations, reflected by jaundice, pulmonary haemorrhage, acute kidney injury (AKI) (Liao *et al.*, 2015). The organisms reside in the renal tubules and cause proximal tubule dysfunction, oliguria and hypokalemia (De Brito *et al.*, 2018). Chronic renal injuries are rare in humans but are seen in animals (Nally *et al.*, 2018). Leptospirosis causes uveitis that results due to the inflammatory response due to the invasion by the organism. Uveitis in patients following leptospirosis are well reported (Sivakolundu *et al.*, 2012; Sivakumar *et al.*, 2016; Diwo *et al.*, 2017). A significant concentration of serovar-specific LPS was observed in the aqueous humour of patients, suggesting an endotoxin-mediated process and also antibodies against the leptospiral lipoproteins LruA and LruB were detected in sera from patients who had leptospiral uveitis (Verma *et al.*, 2008).

In animals, the disease is often chronic. Problems such as abortions and still birth are reported (Lilenbaum & Martins, 2014; Libonati *et al.*, 2018). Frequent manifestation of leptospirosis pulmonary haemorrhage syndrome has been seen in infected dogs with a high morbidity and mortality (Gendron *et al.*, 2014). Infections in goats and sheep leads to infertility, abortion, stillbirth and weak lambs / goat kids. These aspects showed high rate of morbidity and lethality in livestocks which directly reduces the economic losses (Petrakovsky *et al.*, 2014).

1.4.4. Laboratory diagnosis

The difficulty in the definitive diagnosis of leptospirosis clinically warrants the need for good laboratory tests. The conventionally used staining techniques of clinical samples cannot be applied to these organisms. They cannot be visualized by staining with routinely used Gram stains as they are too thin to be seen in smear preparations. Dark

field microscopy (DFM) can identify them due to their characteristic corkscrew movement but cannot be used on clinical samples as artifacts like fibres can be mistaken for the organism. Culture is not easy to achieve and the preferred tests are based on the detection of antibodies upon infection, with the microscopic agglutination test (MAT) proving to be the 'gold standard'.

1.4.4.1. Culture

Culturing of *Leptospira* from blood or urine samples prior to initiation of antibiotic treatment can be done, often without much success. One or two drops of blood collected from the patient is immediately inoculated into 5 – 10 mL semi-solid or liquid medium with 5-fluorouracil that inhibits the growth of other bacteria (Faine., 1994; Levett, 2001). Organisms from human urine can be cultured from the beginning of the second week of symptomatic illness, but should be treated immediately as the organisms cannot survive for long due to the acidic nature of the urine (Haake & Levett, 2015; Soupe-Gilbert *et al.*, 2017).

1.4.4.2. Microscopic Agglutination Test (MAT)

MAT, first described by Martin and Pettit at Pasteur Institute, Paris (Martin & Pettit, 1918) is based on the agglutination of live organisms by antibodies against leptospiral lipopolysaccharides in the serum of infected patients / animals. The test is serogroup specific and can identify the infecting serovar with high specificity (Bharti *et al.*, 2003). In this test, the reference panel must include all the representative serovars and dark-field microscopy is used to detect the agglutination.

MAT detects both immunoglobulin M (IgM) and immunoglobulin G (IgG) class of agglutinating antibodies. Since this requires live organisms and technical skill for interpretation of the result, MAT cannot be used in routine laboratories. However, it is still used as a reliable method for screening purposes (Haake & Levett, 2015). In endemic areas of leptospirosis, where the population can present with basal level of antibodies, a single titer of 400 or more in a symptomatic patient is generally accepted, but titers as high as 1600 or more have been recommended (Levett, 2001). A paired sample is often preferred in which a rise in titre in a second sample collected 48 – 72 hours later is considered positive for the disease.

1.4.4.3. Polymerase Chain Reaction (PCR)

While PCR is a quick method for detected of bacterial DNA, the technique suffers from false positivity and thus cannot be used on its own as a diagnostic tool. However, there are several reports on using this test on blood samples collected within the first week after infection, based on the presumption that the organisms remain in circulation during this period, before the serum anti-leptospiral antibodies begin to appear. Pathogen specific leptospiral DNA can be amplified by using two sets of primes (G1 / G2 and B64-I / B64-II). G1 / G2 primers amplifies 285 bp from *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai* and *L. meyeri* and the B64-I / B64-II primers amplifies a 563 bp from *L. kirschneri.* Thus these sets of two primers can detect all pathogenic species in infected samples (Gravekamp *et al.*, 1993). PCR of a 631 bp product (Hookey, 1992) or a 330 bp product (Kumar *et al.*, 2001) targeting 16S rDNA from pathogenic serovars have been successfully used. PCR of the *hbpA* gene was used to identify all serovars of *L. interrogans* from clinical isolates collected globally (Sridhar *et al.*, 2008). PCR with pathogenic *flaB*-specific primers (*fla*B, G1-G2, B64I-II, and A-B) used to differentiate pathogenic and saprophytic leptospiral strains (Natarajaseenivasan *et al.*, 2010). Recent study overcome the PCR problem in differentiating the infecting serovars by using a set of primers (*ligB*Rpet and *ligB*Fpet) from pathogenic species specific for adhesion *ligB* gene (Martinez *et al.*, 2018). Several other PCR based assays have been developed which targets genes encoding *lipl32* (Branger *et al.,* 2005) and *ompL1* (Reitstetter, 2006). Immunocapture polymerase chain reaction (IC-PCR) are specific to *Leptospira* and is a promising tool for early diagnosis of leptospirosis, providing additional information about the infecting serovar or serogroup (Balassiano *et al.*, 2012).

1.4.4.4. Enzyme-linked immunosorbent assay (ELISA)

In general, ELISA is a simple and easy-to-perform test for the demonstration of serum antibodies against pathogenic organisms. Though it was reported long back for leptospirosis (Adler *et al.*, 1980), it was not useful due to the large number of cross-reacting antigens with the saprophytic members and due to the endemicity of the disease in several geographical locations. Earlier study reported ELISA based on antigens from whole cell sonicate, formalin – extract of a culture of leptospires (Terpstra *et al.*, 1985)

and also whole cell sonicates has been used to demonstrate anti-leptospiral antibodies in sera (McBride *et al.*, 2007). Variations in antigens preparation resulted in poor reproducibility of the results, therefore necessitating the identification of a specific antigen that can identify all infecting serovars. Pathogen specific recombinant protein-based serological tests offers great advantages as it does not require the maintenance of live organisms. Some of the candidate antigens identified were LipL32 (Vedhagiri *et al.*, 2013; Yaakob *et al.*, 2017), Omp1 (Subathra *et al.*, 2013), LipL41 (Chen *et al.*, 2013), LigA (Kanagavel *et al.*, 2014), LigB (Deneke *et al.*, 2014), Lsa63 (Alizadeh *et al.*, 2014), HbpA (Sivakolundu *et al.*, 2012).

1.4.4.5. Commercial kits

There are several commercially available kits in the market today but are not cost-effective. Table 1.1 lists the different kits available, some of which are 'point-of-care' devices and are thus easier to use for screening purposes. PanBio IgM ELISA is based on whole cell

Table 1.1. List of commercial diagnostic kits for leptospirosis

Tests	Company details
PanBio IgM *Leptospira*	PanBio Pty Ltd. (Brisbane, Australia)
Leptospirosis IHA test	MRL Diagnostics (Cypress, California, USA)
IgM Dip-S-Tick assay	Integrated Diagnostics (Baltimore, USA)
Biolisa IgM ELISA	BIOS GmbH (Müenchen, Germany)
Biognost IgM indirect fluorescent antibody (IFA) assay	BIOS GmbH Labordiagnostik (Gräfelfing, Germany)
IgM-ELISA (Serion – Virion)	Institut Virion, Serion GmbH (Würzburg, Germany)
Lepto Dipstick IgM test	Organon-Teknika Ltd. (Amsterdam, The Netherlands)
LEPTO IgM MICROLISA ELISA kit	J.Mitra & Co. Pvt. Ltd (India)
Leptocheck dipstick assay	Zephyr Biomedicals (India)
Leptocheck-WB	Zephyr Biomedicals (India)
LEPTO lateral flow	KIT (Amsterdam, The Netherlands)

Leptospira using non-pathogenic *L. biflexa* serovar Patoc as an antigen as it detects broadly cross-reactive antibodies. PanBio was reported to show high sensitivity and

specificity in two different studies (Zochowski *et al.*, 2001; Levett & Branch, 2002), with lower values in others (Blacksell *et al.*, 2006). Lepto Dipstick demonstrated 78.7% sensitivity and 88.3% specificity in acute phases from Andaman and Nicobar Islands (Sehgal *et al.*, 1999). Lateral flow assay showed 65.9% sensitivity and 93.3 % specificity in acute cases form mixed endemic diseases tested at district hospital in India (Smits *et al.*, 2001). These assays are rapid, easily be performed as an individual test, either with serum or whole blood samples. This requires only a single dilution with no requirement of special equipment and give consistent results. The bands can be stable for more than 12 months. Immuno-chromatographic test would be a suitable confirmatory test for screening of patients with acute leptospirosis.

1.4.5. Control measures

Control measures include timely diagnosis, effective treatment of the disease and development of vaccines for humans and animals. Basic preventive measures like as maintenance of proper hygiene, protective clothing, footwear and prevention of contact from contaminated environment can be adopted (Al-orry *et al.*, 2016). Rodent control is an important measure to be implemented (Fentahun & Alemayehu, 2012).

1.4.5.1. Chemotherapy

100 mg of Doxycycline is recommended twice a day for 7 days in anicteric leptospirosis (Levett, 2001). Benzyl penicillin are advisable by injection till five days comprising five million units per day (Faine., 1994).

1.4.5.2. Vaccines

Killed whole leptospiral cells cultured in serum-containing medium, referred as bacterins vaccine have been used since 1916. In developed countries, commercial *Leptospira* vaccines are used for animals (Levett, 2001). Bacterin preparations suffer from the disadvantage of exaggerated immune response due to the presence of the highly immunogenic LPS in it. It is reported that there are human vaccines containing killed leptospires for use in China, Cuba, Japan and France (Dellagostin *et al.*, 2017). While recombinant vaccines may be better, the success of recombinant vaccines has not been encouraging. The first evaluated recombinant subunit vaccine was *E. coli* membrane

fractions containing recombinant LipL41 and OmpL1 (Haake *et al.*, 1999). Others include LipL32 (Cullen *et al.*, 2005), LigA (Silva *et al.*, 2007) and LigB (Conrad *et al.*, 2017).

1.5. Pathogenesis

The mechanism of leptospiral pathogenesis is not well understood so far. A number of virulence factors are reported to modulate the severity and progression of the disease. Leptospirosis has been well studied in hamsters, guinea pigs and gerbils, as they are highly susceptible to this disease and develop severe infections. Rats and mice are the two main reservoirs and can only be infected in the laboratory with high doses of leptospires (Picardeau, 2017).

1.5.1. Attachment and invasion

The first step of pathogenesis is the attachment and internalization of pathogen to host cell. Skin abrasion, mucous membranes of conjunctivae and oral cavity are the best known potential entry sites (Haake & Levett, 2015). The direct attachment of *Leptospira interrogans* to the extra cellular matrix (ECM) proteins indicates the existence of multiple adhesion molecules (Evangelista *et al.*, 2014). They can also binds to endothelial cells, monocytes, macrophages, kidney epithelial cells and fibroblast cell line cultures *in-vitro* (Thomas & Higbie, 1990; Liu *et al.*, 2007). Virulent leptospires adhere and invade host cells more efficiently than the non-virulent strains (Fraga *et al.*, 2011).

1.5.2. Surface proteins and lipopolysaccharides

The outer membrane of leptospires contains LPS and several outer membrane surface proteins which play an important role in pathogenesis. The first identified leptospiral surface adhesion protein was a 36-kDa fibronectin binding protein isolated from the outer sheath of pathogenic leptospires (Merien *et al.*, 2000). Lsa24 is another leptospiral surface adhesion protein binds strongly to laminin in a specific, dose-dependent, and saturable fashion (Barbosa *et al.*, 2006; Vieira *et al.*, 2014). *Leptospira* immunoglobulin-like proteins (Lig) bind to fibronectin in *in-vitro* condition (Lin & Chang, 2008). rLig proteins also significantly binds to elastin and tropoelastin (Lin *et al.*, 2009). Leptospiral endostatin - like (Len) proteins such LfhH or LenA binds to complement factor H, fibrinogen and fibronectin (Barbosa *et al.*, 2006). Recently identified LcpA surface exposed protein binds

to human complement regulator C4BP. Lsa63, surface protein binds to laminin and collagen IV (Vieira *et al.*, 2010).

1.5.3. Immune response

Innate immune responses are the first line of defense by the mammalian host upon entry of an infecting pathogen. Upon invasion, leptospires initially appear and remain in blood circulation for about week (septicemic phase). This is followed by the acquired immune phase, characterized by antibody production (IgM and IgG) and excretion of leptospires in the urine (leptospiuric phase). The organisms are often killed and are cleared from the body by the immune response of the host. But, they may colonise in the convoluted tubules of the kidneys and other organs, resulting in complication seen in the severe form of the disease.

1.6. Hemolysins and sphingomyelinases

1.6.1. Introduction

Hemolysins are toxins produced by Gram - positive and Gram – negative bacteria (Goebel *et al.*, 1988). Bacteria secrete hemolysins in-order to acquire iron from heme or hemoglobin by lysing erythrocytes or by acting on cells. They damage host cell membrane either by pore formation or by enzymatic activities such as sphingomyelinase and phospholipase. Hemolysins are implicated as significant virulence factors as they contribute to inflammatory response during infection (Grimminger *et al.*, 1997).

1.6.2. Multiple sphingomyelinase genes

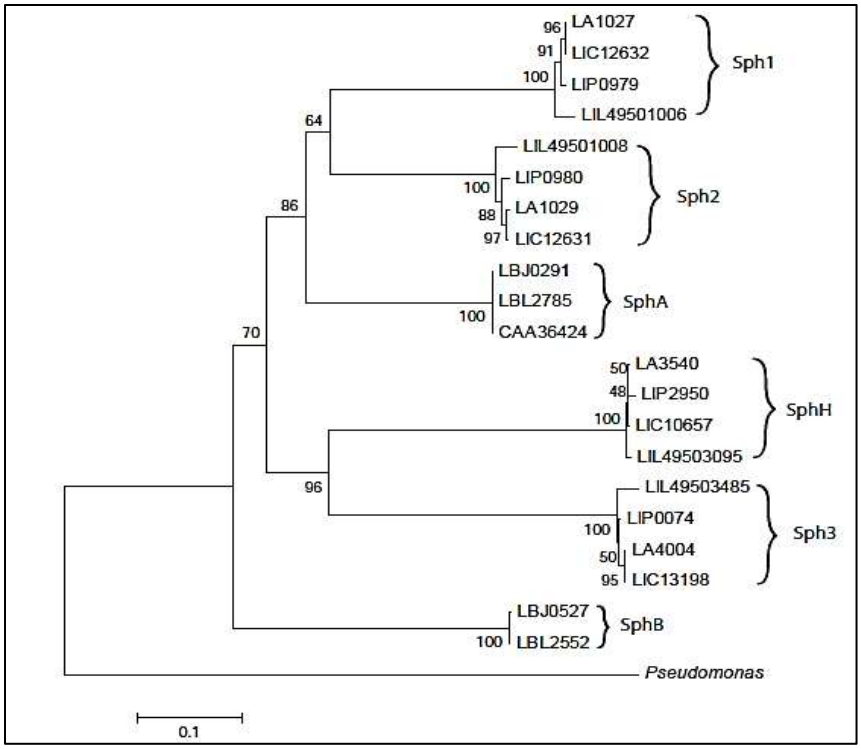
Genome sequencing studies revealed that pathogenic *Leptospira* possessed multiple hemolysin genes. The genome of *L. interrogans Lai* shows nine hemolysin genes, encoding four sphingomyelinases, one pore-forming hemolysin and four non-sphingomyelinases hemolysins (Ren *et al.*, 2003). They are thought to cause cell lysis and tissue damage due to sphingomyelinase, phospholipase, or pore - forming activities (Bernheimer & Bey, 1986; Lee *et al.*, 2002). The role of the five non-sphingomyelinase hemolysins in *L. biflexa* (LEPBIa0082, LEPBIa0717, LEPBIa2015, LEPBIa2375 & LEPBIa2477) are yet to be determined (Picardeau *et al.*, 2008).

Sphingomyelinases are enzyme that catalyse the hydrolysis of sphingomyelin into ceramide and phosphorylcholine. Based on its activities on optimum pH, sphingomyelinases are classified as acidic, neutral or alkaline. Most of the neutral sphingomyelinases of bacteria and mammals possess conserved catalytic core residues with overall sequence relatedness (Clarke *et al.*, 2011). Mammalian members of the neutral sphingomyelinase family are membrane-associated whereas the bacterial members are secreted. Bacterial sphingomyelinase are classified as SMaseC and SMaseD depending upon cleavage sites. SMaseC, with an active site containing His150, His285 and Mg^{+2} binding sites hydrolyses ester bond between ceramide and phosphorylcholine. SMaseD hydrolyzes the phosphodiester bond between ceramide-1-phosphate and choline. This protein has His44, His78 at its active sites and also requires Mg^{2+}. Sphingomyelinase from *Bacillus*, *Listeria, Staphylococcus*, *Pseudomonas*, *Leptospira*, *Helicobacter* are SMaseC types. They are smaller with their molecular mass ranging from 27 to 39 kDa. The sphingomyelinases expressed by *Leptospira* and *Pseudomonas* are bigger, possibly due to the presence of additional sequences at the C-terminal ends. SMase C are best known to bind and lyse RBCs from different species and also act on host cell membrane (Flores-Diaz *et al.*, 2016). Structural elucidation of the sphingomyelinases produced by *Bacillus cereus*, *Staphylococcus aureus* and *Listeria ivanovii* was made possible due to crystallization of the respective proteins.

1.6.3. Reports on leptospiral sphingomyelianses and hemolytic activity

Historically, the hemolytic activity associated with *Leptospira* was first reported in 1956 (Alexander *et al.*, 1956; Russell, 1956) and sphingomyelinase activity was first detected in *Leptospira* cultures in the 1960's (Kasarov & Addamiano, 1969), though the cloning of a sphingomyelinase gene was not reported until 1989 (del Real *et al.*, 1989). The gene *sphA* encoded a protein with both hemolytic and sphingomyelinase activities (del Real *et al.,* 1989*;* Segers *et al.,* 1992). 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.*, 1992). SphH of serovar Lai has been well studied (Lee *et al.*, 2000) and its pore-forming activity was demonstrated (Lee *et al.*, 2000; Lee *et al.*, 2002).

Genome sequencing uncovered the multiple sphingomyelinase genes, with the first report on *L. interrogans* serovar Lai identifying five sphingomyelinase genes *sph1*, *sph2*, *sph3*, *sph4* and *sphH* (Ren *et al.*, 2003), three (*sphA, sphB, sph4)* in *L. borgpetersenii* (Bulach *et al.*, 2006), seven in *L. santarosai* (Chou *et al.*, 2014) and none



(Narayanavari *et al.*, 2012b)

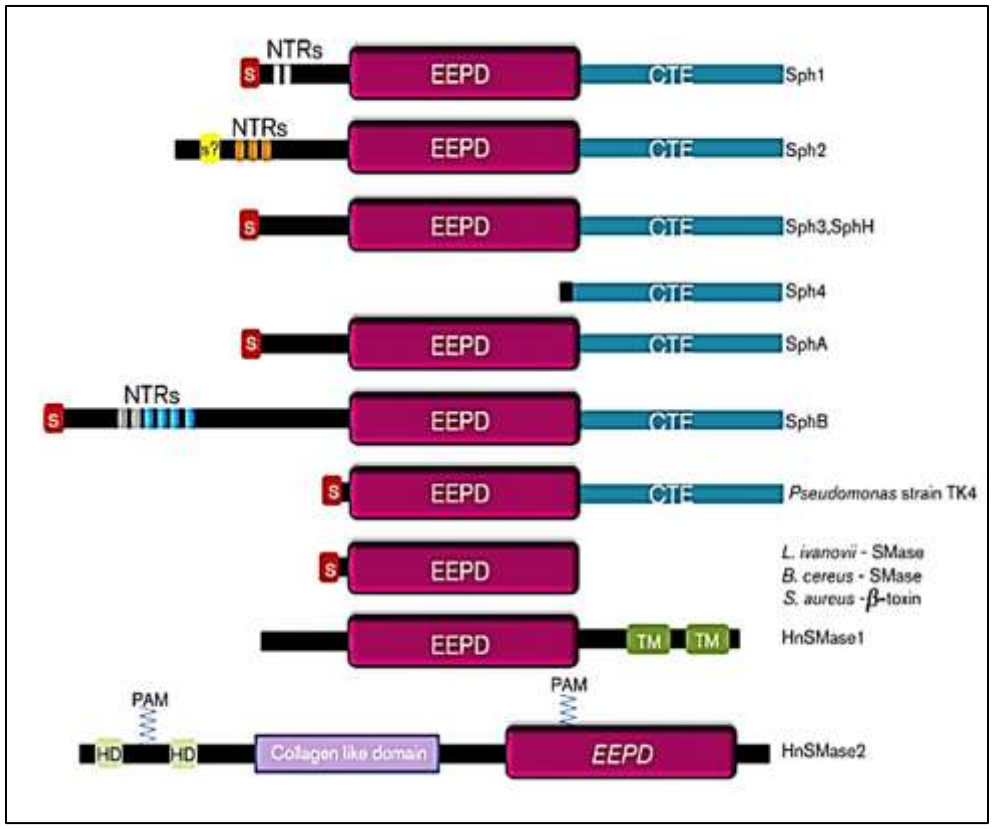
Figure.1.8. Evolutionary relatedness of leptospiral sphingomyelinase-like proteins. Figure showing phylogenetic tree of sphingomyelinases from *L. interrogans* and *L. borpetersenii.* The tree was built from multiple sequence alignments of the amino acid sequences of leptospiral sphingomyelinases. The tree was rooted with the Pseudomonas species TK4 sphingomyelinase. Figure shows sphingomyelinases from *L. Interrogans* serovar Lai (Sph1: LA1027, Sph2: LA1029, Sph3: LA4004, SphH: LA3540), serovar Copenhageni (Sph1: LIC12632, Sph2: LIC12631, Sph3: LIC13198, SphH: LIC10657), serovar Pomona (Sph1: LIP0979, Sph2: LIP0980, Sph3: LIP0774, SphH: LIP2950) and serovar Manilae (Sph1: LiL49501006, Sph2: LiL49501008, Sph3: LiL49503485, SphH: LiL49503095). Two *L. borgpetersenii* serovar Harjobovis strain JB197 (SphA: LBJ 0291, SphB: LBJ 0527) and *L. borgpetersenii* serovar Harjobovis strain JB197 strain L550 (SphA: LBL 2785 SphB: LBL 2552) was included in the study.

in the non-pathogen *Leptospira biflexa* (Picardeau *et al.*, 2008). The evolutionary relatedness among sphingomyelinases from *L. interrogans* serovars Lai, Copenhageni,

Pomona, Manilae and *L. borgpetersenii* serovars Hardjobovis strains JB197 and L550 grouped them into six clusters, though the sphingomyelinases from *L. interrogans* and *L. borgpetersenii* form separate clusters. Sph1 and Sph2 encoding genes of *L. interrogans* are adjacent to each other in the chromosome (Fig.1.8). A single copy of SphA is present in same genome position in *L. borgpetersenii* (Narayanavari *et al.*, 2012b).

1.6.4. Structural elucidation of leptospiral sphingomyelianses

Earlier studies in our lab included homology modeling of the sphingomyelinases of serovar Lai. Multiple sequence alignment of Sph1, Sph2, Sph3 and SphH showed that they possess the exo-endo phosphatase domain (EEPD) that is absent in Sph4 (Fig. 1.9).



(Narayanavari *et al.*, 2012b)

Figure 1.9. Schematic representation of leptospiral sphingomyelinase-like proteins. Panel represents occurrence of exo-endo phosphatase in sphingomyelinases. In *Leptospira* and in other bacterial species, NTRs represents N-terminal repetitive sequences; S is signal peptide; EEPD represents exo-endo phosphatase domain; CTE abbreviate for C-terminal extension; TM represents transmembrane domain; HD is hydrophobic domain and PAM represent palmitate.

(Narayanavari *et al.*, 2012b). Using the crystal structures of the sphingomyelinases of *B. cereus*, *Listeria ivanovii* and *S. aureus*, homology modeling was done. Leptospiral sphingomyelinases showed an extended C-terminal region, not seen in other bacteria. Another distinguishing feature was the absence of the hydrophobic β-hairpin seen in the other bacterial sphingomyelinases. It was shown that the presence of several hydrophobic amino acids, extending out from the surface of the protein molecule facilitates its attachment to the sphingomyelin on the eukaryotic cell membrane (Narayanavari *et al*., 2012b).

The leptospiral sphingomyelinase-like proteins and Pseudomonas strain TK4 sphingomyelinase possess 186 aa C-terminal extension that is missing in the other bacterial sphingomyelinases (Sueyoshi *et al.*, 2002; Narayanavari *et al.*, 2012b). Deletion of this in *Pseudomonas* completely abolished the haemolytic activity without affecting the sphingomyelinase activity, indicating that the C-terminal extension is indispensable for haemolytic activity (Sueyoshi *et al.*, 2002). This observation suggests that the function of the C-terminal extension is to interact with the target host membrane to position the enzymic domain near the sphingomyelin substrate (Sueyoshi *et al.*, 2002).

1.6.5. Sph2 is a true sphingomyelinase

Our earlier studies showed Sph2 to be a true sphingomyelinase. This was established by analyzing the active site amino acid residues and experimental demonstration of the hemolytic and enzymatic activity of Sph2. The hemolytic activity was demonstrated using sheep erythrocytes and the enzymatic activity was performed on sphingomyelin as the substrate, with thin layer chromatography used to identify the products ceramide and phosphorylcholine (Narayanavari *et al.*, 2012a).

Our recent study confirmed the functional role of Sph2. A mutant strain, defective in Sph2 expression was generated by transposon mutagenesis. This strain showed a marked reduction in both the hemolytic and sphingomyelinase activities that were partially restored upon complementation with the full length *sph2* gene. The Sph2 was shown to be secreted in its active form. As Sph2 lacks the N-terminal signal peptide, the mechanism for secretion and processing of Sph2 remains to be elucidated. Since pathogenic *Leptospira* encodes for TolC, HlyB and HlyD orthologs, it is highly likely that

Sph2 and other sphingomyelinase use the Type I secretion pathway for exporting these toxins secretory mechanism (Narayanavari *et al.*, 2015).

1.6.6. Role of external factors in the expression of sphingomyelinase

1.6.6.1. Effect of sodium chloride

Much remains to be understood about the regulation of expression of *sph2*. There was poor expression of *sph2* gene in *L. interrogans* serovar Lai, Copenhageni, and Manilae grown in the conventional EMJH medium, with serovar Pomona showing higher levels. Upon addition of sodium chloride and raising the osmolarity to physiological concentration, there was up-regulation of Sph2 in all the serovars, with marked increase in the serovar Pomona (Narayanavari *et al.*, 2015). Combined addition of sodium chloride and rat serum increases the level dramatically.

1.6.6.2. Effect of iron limitation

We reported for the first time the influence of iron limitation on the expression of sphingomyelinase in serovar Lai. It was seen that under these conditions, a 42 kDa sphingomyelinases was released in outer membrane vesicles (Velineni *et al.*, 2009). While our study demonstrated the 42 kDa as sphingomyelinase, it was not identified as cleavage product of any of the annotated molecules. A later study (Narayanavari, 2012), identified SphH to be markedly elevated upon iron limitation. It is to mentioned that this up-regulation was seen only upon raising the temperature to 37°C and not at 30°C. HbpA, the iron-regulated hemin-binding protein iron (Sritharan, 2005; Asuthkar *et al.,* 2007). This was also expressed under these conditions of iron and temperature. It is therefore not surprising that both HbpA and sphingomyelinases are expressed in the mammalian host that is known to limit iron availability and raise the temperature upon bacterial infection.

1.6.7. *In-vivo* expressed proteins and its clinical significance in leptospirosis

LipL32 is pathogen-specific and is expressed during acute lethal infections. This an outer membrane protein, localized on the cell surface and highly immunodominant (Haake *et al.*, 2000). It was referred to as hemolysis-associated protein-1 (HAP-1) (Hauk *et al.*, 2009) but its association with hemolytic activity remains to be understood. Another protein

of interest is HbpA, mentioned above. The diagnostic potential of this protein was reported from our lab (Sridhar *et al.*, 2008; Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012; Sarguna *et al.*, 2015), not only for screening human cases but also bovine samples. Targeted reduced expression of LigA and and LigB proteins potentially reduce the virulence in hamsters suggest these proteins involve in pathogenesis (Pappas & Picardeau, 2015). The expression of LipL36 was seen downregulated in infected hamster kidney and liver tissues (Haake *et al.*, 1998; Barnett *et al.*, 1999). However Omp1 and LipL41 were found to be upregulated in pathogenic leptospires upon colonizing the proximal convoluted renal tubules of hamster (Barnett *et al.*, 1999). Increased expression of outer membrane proteins such as Loa22, LipL32, Lip21 was seen in recovered leptospires from infected guinea pig tissues and a chronically infected rat tissue. This study demonstrated that host environment modulated leptospiral OMP expression (Nally *et al.*, 2007).

OBJECTIVES OF THE STUDY

I. Studies on the sphingomyelinases of *L. interrogans* serovar Pomona

- **a.** Comparative analysis of the sphingomyelinases produced by the pathogenic *Leptospira interrogans* serovars Lai and Pomona and the non-pathogenic serovar Andamana
- **b.** Infection of Vero cells with *L. interrogans* serovar Pomona: correlation of sphingomyelinase expression with the cytotoxic effect on Vero cells

II. Clinical significance and diagnostic potential of sphingomyelinases and the hemin-binding protein HbpA in diagnosis of leptospirosis

- **a.** Diagnostic potential of sphingomyelinase and HbpA in the sero-diagnosis of leptospirosis
- **b.** Differential diagnosis of leptospirosis from dengue fever: demonstration of pathogen-specific sphingomyelinase, HbpA, LipL32 and LipL41 proteins in the urine of patients with leptospirosis

CHAPTER – II

MATERIALS AND METHODS

2.1. Source of materials

2.1.1. Chemicals and reagents

Bacterial grade media components were purchased from HiMedia (Mumbai, India). Special chemicals and routine analytical reagents used for the study are listed in Appendix I.A1.

2.1.2. Bacterial strains

Escherichia coli strains DH5α, BL21 (DE3) and BL21 (DE3) pLysS were purchased from Novagen (USA). Table 2.1 shows the list of leptospiral strains used in the study.

Table 2.1. List of reference strains of *Leptospira* spp.

Species	Serovar	Strain	Source
L. interrogans	Australis	Ballico	Regional Medical Research Center, ICMR, Port Blair, Andaman and Nicobar Islands. (WHO Collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis, India)
	Bankinang	Bankinang I	
	Canicola	Hond Uttrecht IV	
	Hardjo	Hardjoprajitno	
	Hebdomadis	Hebdomadis	
	Pomona	Pomona	
L. weillii	Celledoni	Celledoni	
L. borgpetersenii	Tarassovi	Perepelicin	
L. biflexa	Andamana	CH11	
	Patoc	Patoc	
L. interrogans	Lai	56601	Dr. Rudy Hartskeel, KIT Biomedical Research, The Netherlands.
L. borgpetersenii	Hardjobovis	L550	Prof. Ben Adler, Monash University, Australia.

2.1.3. Plasmids

The commercial plasmids used in this study are listed in Table 2.2A and the lab collection of recombinant plasmids are listed in Table 2.2B.

Table 2.2. List of commercial and recombinant plasmids used in the study

A. Commercial plasmids			
No.	**Plasmid**	**Company**	**Features**
i)	pET28a (+)	Novagen, USA	N-terminal T7-Tag sequence, optional C-terminal His-Tag sequence, Kanamycin resistance encoding gene
ii)	pGEX-4T1	GE Healthcare, UK	N-terminal GST, Thrombin cleavage site, Ampicillin resistance encoding gene.

B. Recombinant Plasmids			
No.	**Plasmid**	**Gene of interest**	**Features**
i)	pMS508	*hbpA*$_{720}$	720 bp fragment (1410 – 2130 bp of *hbpA*) cloned into pET28a (+); rHbpA$_{720}$ is 240 aa long, containing residues 470 – 710.
ii)	pMS502	*sph3*$_{632}$	632 bp fragment (366 – 998 bp of *sph3*) cloned into pET28a (+); the encoded protein, rSphCD is 210 aa long, containing residues 123 - 332.
iii)	pMS511	*sph4*$_{420}$	420 bp fragment (298 – 698 bp of *sph4*) cloned into pET28a (+); rSph4 is 140 aa long, containing residues 99 – 239.
iv)	pMS515	*lipL41*	Full length *lipL41* cloned into pGEX-4T1

2.1.4. Mammalian cell line

Vero cells (Green African Monkey Kidney Epithelium Cell line) was from ATCC.

2.1.5. Antibodies

Antibodies against LipL32, Lip41, Fla1 and $Sph2_{163}$ (163 aa containing aa 27–190 of the 607aa long Sph2) was a kind gift from Prof. David Haake, UCLA. The laboratory collection included antibodies against $rSphCD_{210}$, rSph4 and rHbpA (the antibody was raised against the 55 kDa fragment containing aa 229–710 of the 710 aa long HbpA). All these antibodies were raised in rabbits.

2.2. Preparation of leptospiral growth medium

2.2.1. Ellinghausen - McCullough - Johnson and Harris (EMJH) enrichment medium

0.23 g of EMJH base was dissolved and made up to 90 mL with double-distilled water and autoclaved at 15 lbs / sq. inch for 20 min. After cooling, 10 mL of EMJH enrichment media was added under aseptic conditions. The EMJH semi-solid medium was prepared by the addition of 0.15 g of Noble agar to EMJH basal medium before autoclaving, with the enrichment medium added upon cooling to 50ºC.

2.2.2. EMJH-BSA (bovine serum albumin) medium

The following protocol was used for the preparation of 100 mL of EMJH-BSA medium. Dissolved 0.23 g of EMJH base in 75 mL of double distilled water and autoclaved at 15 lbs / sq. inch for 20 min. Dissolved 2 g of BSA in 22 mL of distilled water with continuous stirring (4% final concentration), with care taken to avoid foaming. The reagents listed in Table 2.3 were added to BSA, followed by EMJH basal medium. The solution was mixed well, sterilized by filtration through 0.22 µm pore size GVWP filters (Millipore Corporation, MA, USA) and stored at 4ºC.

Table 2.3. Salts and vitamins for the preparation of EMJH-BSA medium

No.	Reagent	Stock solution	Stock solution for 100 mL of medium
1	$CaCl_2.2H_2O$ + $MgCl_2.6H_2O$ (SRL)	1 g of each / 100 mL DDW	150 µL
2	$ZnSO_4.7H_2O$ (Qualigens)	400 mg / 100 mL DDW	100 µL

3	$CuSO_4.5H_2O$ (SRL)	300 mg / 100 mL DDW	10 µL
4	Vitamin B12* (Merck)	20 mg / 100 mL DDW	100 µL
5	Tween 80** (Merck)	1ml / 10 mL DDW	100 µL
6	Glycerol (Qualigens)	1ml / 10 mL DDW	1.25 mL
7	Sodium Pyruvate** (SD Fine Chemicals)	11 mg / 1 mL DDW	363 µL

* Except Vitamin B12, autoclaved all the solutions and stored as aliquots at - 20ºC

** Tween 80 and Sodium Pyruvate were prepared fresh before use

2.2.3. Growth of *Leptospira*

Leptospira cultures were maintained in semi-solid EMJH enrichment medium in screw capped test tubes at 30°C. Growth was monitored regularly by observing under the dark-field microscope (Nikon Eclipse E600, Japan). The cultures were regularly sub-cultured after an interval of 25-30 days**.**

The organisms were grown in liquid EMJH-enrichment medium for preparing genomic DNA. The following published procedure (Matsunaga *et al.*, 2005) was followed when the organisms were subjected to physiological osmolarity conditions. The leptospires were grown in 100 mL liquid EMJH-enrichment medium till log phase, added 2.4 mL of sterile 5 M NaCl (29.2 g of NaCl in 100 mL water) to get final concentration of 120 mM NaCl and incubated for 4 h at 30°C as per published protocol.

The organisms were grown in liquid EMJH-BSA medium for preparing whole cell sonicates used for screening for antibodies from patients' serum.

2.3. Preparation and analysis of leptospiral whole cell sonicates

2.3.1. Preparation of leptospiral cell-free whole cell sonicates (WCS)

The organisms, grown in liquid EMJH medium as described above were harvested by centrifuging at 13,000 rpm for 20 minutes. The cells were washed twice with 0.1 M phosphate buffered saline pH 7.2 (Appendix I.A2), sonicated for 5 min (20 second pulses with 30 second intervals at 40 Hz in a Vibra Cell sonicator, USA) and centrifuged at 13,000 rpm for 20 minutes. The supernatant was carefully removed and used as cell free whole

cell sonicate. The total protein was estimated by the commercial BCA kit (Sigma Aldrich, St. Louis, MO, USA).

2.3.2. Separation of proteins by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were fractionated by SDS-PAGE as per published protocol (Laemmli, 1970).

Reagents

- 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 double-distilled water and the volume was made up to 100 mL.
- Resolving gel buffer (1.5 M Tris-HCl pH 8.8 with 0.4% SDS)**:** 36.3 g of Tris base and 0.8 g of SDS were dissolved in 150 mL of double-distilled water, pH was adjusted to 8.8 with 0.1 N HCl and the volume was made up to 200 mL.
- Stacking gel buffer (Tris-HCl, 0.5 M pH 6.8 with 0.4% SDS)**:** 12.0 g of Tris base and 0.8 g of SDS were dissolved in 150 mL of double-distilled water, pH was adjusted to 6.8 with 0.1 HCl and volume was made up to 200 mL.
- Ammonium persulfate (APS) 10%**:** 0.1 g of APS was dissolved in 1 mL of double-distilled water. This was prepared fresh before use.
- Tetramethyl ethylene diamine (TEMED)**:** This was used as obtained commercially.
- Sample buffer (2X): Added 0.62 mL of 2 M Tris-HCl pH 6.8, 0.4 g SDS (4%), 2 mL of Glycerol (20%), 1 mL of β-mercaptoethanol (10%), 0.2 mg bromophenol blue (0.02%) and made up to 10 mL with double-distilled water.
- Running buffer**:** Dissolved 24.75 g of Tris base and 108 g of glycine in 900 mL of double-distilled water, added 7.5 g of SDS and volume was made up to 1.5 L.
- Coomassie blue stain**:** 0.25 g Coomassie Brilliant Blue - R250 was dissolved in 1 mL of methanol and volume was made up to 100 mL using methanol **:** glacial acetic acid **:** double-distilled water (5:2:5).
- Destaining solution**:** This was prepared by mixing methanol, glacial acetic acid, double-distilled water in the ratio of 1**:**1**:**8.

Procedure

The resolving gel (5-20% gradient / 10% gel) was prepared as indicated in Table 2.4 and allowed to polymerize for 40 min. The stacking gel (Table 2.5) was poured over it after

positioning the comb. Protein samples were mixed with 2X sample buffer (1:1 v/v), boiled for 10 min, centrifuged at 13,000 rpm for 10 min to remove any insoluble material and the clear supernatant was loaded onto the gel (gradient gel; Hoefer electrophoresis unit, Amersham Pharmacia Biotech AB, CA, USA, 10% gel; Broviga electrophoresis unit, Balaji Scientific Services, Chennai, India). Gradient gel electrophoresis was carried out initially at 100 V and upon entry of the marker dye into the resolving gel it was run at a constant current of 25 mA per plate till the bromophenol blue reached the bottom of gel. Gradient gels were subjected to electrophoresis for an additional 30 min for optimal resolution of the protein bands. Electrophoresis of 10% gel was carried out at an initial voltage of 70 V till the bromophenol blue dye entered the resolving gel and then at 100 V. The gel was stained with Coomassie Brilliant Blue for 30 min followed by destaining overnight.

Table 2.4. Recipe for the preparation of resolving gel

	Volume (mL)		
Reagents	**Gradient gel**		**10%**
	5%	**20%**	
Acrylamide: bisacrylamide	2.75	10.6	10.6
Resolving gel buffer	4.0	4.0	8.0
Double distilled water	9.3	1.4	13.4
Ammonium per sulphate	0.08	0.08	0.16
TEMED	0.008	0.008	0.008

Table 2.5. Recipe for the preparation of stacking gel

Reagents	**Volume (mL)**
Acrylamide: bisacrylamide	1.5
Stacking gel buffer	2.5
Double distilled water	6.0
Ammonium per sulphate	0.03
TEMED	0.01

2.3.3. Western blot analysis (Towbin *et al.*, 1979)

Reagents

- 10X Transblot buffer (250 mM Tris and 1.3 M glycine)**:** 15.17 g of Tris base and 48.8 g of glycine were dissolved in 300 mL of double-distilled water and made up the volume 400 mL.
- Working buffer (1X Transblot buffer)**:** This was prepared by mixing 200 mL of 10X stock with 400 mL of methanol and 1.4 L of double-distilled water.
- 1X Tris-buffered saline (TBS, pH 7.4)**:** Dissolved 6.05 g of Tris base and 8.7 g of NaCl in 800 mL of double-distilled water, pH was adjusted to 7.4 with 0.1 HCl and volume made up to 1 L with double-distilled water.
- TBS / T**:** TBS containing 0.05% Tween 20.
- Ponceau S stain**:** Ponceau S, trichloroacetic acid and sulfosalicylic acid were mixed in 2:30:30 (w/v) ratio and the final volume was made up to 100 mL with double-distilled water. Working solution was prepared by adding 1 mL of stock solution to 9 mL of double-distilled water.

Procedure

Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membrane at 30 V overnight or 60 V for 2½ h using 1X transblot buffer in transblot transfer apparatus (Balaji Scientific Services, Chennai, India). The transferred proteins on the membrane was visualized with Ponceau S stain and the molecular weight marker bands were marked with a pencil. The membrane was blocked for 2 h using 5% non-fat milk solution (NFM) dissolved in TBS. The membrane was washed twice with TBS / T and incubated overnight with primary antibody in TBS (the dilution for the various antibodies are indicated in the Results). The membrane was washed twice with TBS / T and incubated with goat anti-rabbit IgG-ALP conjugate (1:5000 dilution in TBS) at room temperature for 2½ h. The blot was washed thoroughly with TBS / T and developed with the ready-to-use substrate 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium solution (BCIP / NBT).

2.3.4. Biological activity of Sphingomyelinases (Narayanavari *et al.*, 2012a)

2.3.4.1. Hemolytic assay

Reagents

- Alsever's solution**:** detailed in Appendix I.A3.
- Preparation of 10% RBC**:** mentioned in Appendix I.A4.
- 0.5 M $MgCl_2.6H_2O$**:** Dissolved 1 g of $MgCl_2.6H_2O$ was dissolved in 8 mL of double-distilled water and made up to 10 mL.
- 0.9% saline**:** 0.9 g of NaCl was dissolved in double-distilled water and made up to a final volume of 10 mL.

Procedure

The assay was performed in a 96 well round - bottomed microtitre plate. The reaction mixtures were added in a final volume of 200 µL and performed in duplicate. The samples included a) 250 µg leptospiral whole cell sonicate, b) 100 µL of spent growth medium (from 1 mL culture containing 2 x 10^8 leptospires) and c) 0.8 µg of commercial sphingomyelinase C from *Staphylococcus aureus* (Sigma Aldrich, St. Louis, MO, USA), of which were made up to a final volume of 156 µL with 0.9% saline; the latter served as test control (TC). The positive (PC) and negative controls (NC) included 156 µL of double distilled water and 0.9% saline respectively. To all the wells with the respective samples mentioned above, added 4 µL of 10 mM $MgCl_2.6H_2O$ and 40 µL of 10% suspension of washed sheep erythrocytes, mixed well and incubated for 2½ h at 37°C. The plates were then incubated at 4°C for 30 min and centrifuged (Kubota, Tokyo, Japan) at 2,500 rpm for 10 min. 100 µL of the supernatant was transferred to another flat-bottomed microtitre plate and the $OD_{570\ nm}$ was measured in a Microplate Reader (BioRad, Microplate reader Model 680XR, USA) using 0.9% saline as blank.

Hemolytic activity in the respective test sample was calculated using the formula

$$\% \text{ hemolysis} = [(OD_{Test} - OD_{NC}) / OD_{TC}] \times 100,$$

where NC and TC are the negative and test control.

2.3.4.2. Assay of sphingomyelinase activity

Reagents

- Ether: methanol (9:1 v/v)
- Hexane: ethyl acetate (3:7 v/v)
- Sphingomyelin (2 mg / mL)**:** 2 mg was dissolved in 1 mL of ether: methanol mixture
- 0.5 M $MgCl_2.6H_2O$
- Ceramide stock (1 µg / µL)**:** Dissolved 25 µg of ceramide in 25 µL of ether.
- Sphingomyelinase C from *S. aureus*
- TLC Silica gel 60 plates

Procedure

This was performed as established earlier (Narayanavari *et al.*, 2012a). The reaction mixture, in a total volume of 1 mL of ether:methanol (9:1 v / v) contained 2 mg of the substrate sphingomyelin (bovine brain; Sigma Chemical Co., USA), 150 – 250 µg of leptospiral whole cell sonicate and 25 mM $MgCl_2$ (50 µL of 0.5 M $MgCl_2.6H_2O$), the latter two samples prepared in 0.9% saline. An identical reaction set up without the leptospiral protein served as negative control. Additional control included the positive substrate control, namely sphingomyelinase C from *Staphylococcus aureus* (Sigma Aldrich, St. Louis, MO, USA) that was set up as for the test sample. The mixture was vigorously shaken for 4 h at 37°C, the two phases separated and 10 µL of the organic phase was subjected to thin layer chromatography on a Silica gel – 60 glass plate (E. Merck AG, Darmstadt, Federal Republic of Germany), using hexane : ethyl acetate (3:7 v / v) as the mobile phase. The spots were visualized with 5% phosphomolybdic acid in ethanol. Commercial ceramide was used as product control.

2.4. Molecular biology techniques

2.4.1. Growth of *E. coli* strains

All the *E. coli* strains were grown in Luria Bertani (LB) medium. The liquid medium was prepared was follows: dissolved 1 g of tryptone, 0.5 g of yeast extract and 1 g of NaCl in 75 mL double-distilled water, adjusted pH to 7.0 with 1 N NaOH and made up the volume

to 100 mL. LB agar plates were prepared by adding 1.5 g agar to 100 mL of liquid LB media. All media were sterilized by autoclaving at 15 lbs / sq. inch for 20 min. The agar plates were prepared by pouring about 20 mL media into 90 mm petri plates, allowed to solidify and stored at 4°C.

When required, LB agar antibiotic plates were prepared by the addition of the appropriate antibiotic (20 µg / mL chloramphenicol / 30 µg / mL kanamycin / 100 µg / mL ampicillin) to the media that was cooled to 50°C.

2.4.2. Purification of genomic DNA from *L. interrogans* serovar Lai

Reagents

- 1 M Tris-HCl**:** Dissolved 121.1 g of Tris base in 800 mL of double-distilled water, followed by addition of 42 mL of concentrated HCl. The pH was adjusted to 8.0 with 1 N HCl and made up to 1 L with double-distilled water and autoclaved at 15 lbs / sq. inch for 20 min.
- 0.5 M EDTA**:** Dissolved 186.1 g of disodium EDTA in 800 mL of double-distilled water, dissolved sodium hydroxide pellets with stirring on a magnetic stirrer till the pH was 8.0 (~20 g of NaOH pellets). The volume was made up to 1 L with double-distilled water and autoclaved at 15 lbs / sq. inch for 20 min.
- TE buffer, pH 8.0 (10 mM Tris-HCl and 1 mM EDTA)**:** Pipetted 1 mL of 1 M Tris-HCl buffer and 0.2 mL of 0.5 M EDTA and made up to 100 mL with double-distilled water.
- Lysozyme (10 mg / mL stock)**:** Dissolved 10 mg of lysozyme in 1 mL of double-distilled water and stored at - 20°C.
- Proteinase K (10 mg / mL stock)**:** Dissolved 10 mg of Proteinase K in 1 mL of double-distilled water and stored at - 20°C.
- 10% SDS**:** Dissolved 1 g of SDS in 10 mL of double-distilled water.
- 5 M NaCl**:** Dissolved 29.2 g of NaCl in 60 mL of double-distilled water and made up to 100 mL, autoclaved at 15 lbs / sq. inch for 20 min.
- Chloroform: Isoamylalcohol (24:1)**:** Added 24 volumes of chloroform with 1 volume of isoamyl alcohol, mixed and stored at room temperature.
- Phase lock tubes (Eppendorf, Germany).

Procedure

Leptospiral genomic DNA was isolated using published protocol (Narayanavari *et al.*, 2012a). 10 mL culture of serovar Lai was harvested by centrifugation at 10,000 rpm for 15 min and the organisms were washed twice with TE buffer. The pellet was re-suspended in 400 µL of TE buffer and incubated with 50 µL of lysozyme for 1 h at 37°C, followed by the addition of 6 µL proteinase K and 70 µL of 10% SDS and incubation 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, that was previously centrifuged for 1 min. Added 750 µL of chloroform: isoamyl alcohol mixture, mixed gently and centrifuged at 13,000 rpm for 3 min. The supernatant was carefully transferred into a new tubes, added 450 µL of isopropanol and kept at room temperature for 30 min to precipitate the DNA. The sample was centrifuged at 12,000 rpm for 15 min and the DNA pellet was washed with 1 mL of 70% ice-cold ethanol. The pellet was dried at room temperature and dissolved in 20 µL of TE buffer. The concentration of the DNA was determined in Nanodrop (Thermo Scientific, USA) and checked by agarose gel electrophoresis for shear.

2.4.3. Mini-prep isolation of plasmid DNA (Birnboim & Doly, 1979)

Reagents

- Solution I: Added 0.9 g of glucose, 2.5 mL of 1 M Tris-HCl pH 8.0 and 0.37 g of EDTA in 70 mL of double-distilled water, adjusted pH to 8.0 with 0.1 N HCl and made up to 100 mL. Stored at 4°C.
- Solution II: Dissolved 0.8 g of NaOH and 1.5 g of SDS in 70 mL of double-distilled water and made up to 100 mL. This was prepared fresh before use.
- Solution III: Added 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of double-distilled water. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Stored at 4°C.

Procedure

Pelleted 1.5 mL of a culture of recombinant *E. coli* by centrifugation for 10 min at 13,000 rpm at 4°C. Added 100 µL of Solution I to the cell pellet, re-suspended them by vortexing and incubated on ice for 5 min. Added 200 µL of freshly prepared Solution II to the cell

pellet and incubated on ice for 5 min. Added 150 µL of ice-cold Solution III, mixed by gently inverting the tube five times and incubated in ice for 5 min. The tube was centrifuged at 13,000 rpm for 10 min at 4°C, transferred the supernatant to a new tube, added DNase-free RNase to a final concentration of 10 µg / mL and incubated at 37°C for 30 min. Added equal volume of phenol: chloroform (1:1 v / v) and centrifuged at 13,000 rpm for 10 min. The aqueous phase was transferred to a new tube and pelleted the plasmid DNA by adding two volumes of isopropanol and incubating at room temperature for 30 min. The plasmid DNA, pelleted by centrifuging at 13,000 rpm for 15 min was washed with 70% ethanol, air dried and dissolved in 20 µL of TE buffer. The concentration of the DNA was determined in Nanodrop (Thermo Scientific, USA).

2.4.4. Agarose gel electrophoresis

Reagents

- 50 X Tris-acetate buffer (TAE)**:** Dissolved 24.2 g of Tris base in 60 mL distilled water, added 5.71 mL of glacial acetic acid, 10 mL of 0.5 M EDTA and made up to 100 mL with double-distilled water.
- The working solution was prepared by adding 20 mL of the stock to 980 mL of distilled water.
- Ethidium bromide**:** 1 mg / mL of ethidium bromide in double-distilled water.
- Agarose: Sigma Aldrich, St. Louis, MO, USA.
- Molecular marker: 1 kb DNA ladder (MBI Fermentas, USA).
- 6X loading dye (MBI Fermentas, USA).

Procedure

0.8 and 1% agarose gels were prepared for the separation of high molecular weight (genomic) and plasmid / PCR products respectively. 0.5 gm of agarose (1% gel) was added to 50 mL of TAE buffer, heated to dissolve the agarose, added 5 µL of ethidium bromide and poured in the trough with the comb in position.

The samples were prepared by adding 6X loading buffer (1 µL of dye for 5 µL sample) and loaded on the gel. 1 µL of 1 kb DNA ladder was loaded as the molecular marker. The samples were subjected to electrophoresis in a horizontal agarose gel unit (Balaji Scientific Services, India) at 100 V till the bromophenol blue dye was about 1 inch

from the bottom of the gel. The unit was switched off, the agarose gel removed and viewed in the UV trans-illuminator (UVP GelDoc, Cambridge, UK).

2.4.5. Cloning of *hbpA* (Sambrook *et al.*, 1989)

2.4.5.1. PCR amplification of *hbpA*

720 bp *hbpA* fragment *(*1410 - 2130 bp) was amplified from the genomic DNA of *L. interrogans* serovar Lai strain 56601 using gene specific primers (For – *Nde*I - 5'-GCG GCCGCATATGGGTTTTCGTCCTCCGAGTTTT-3'; Rev – *Sal*I - 5'-ATAGGAGTCGACA AACTGGGCCGAGAATCCACC-3'). The reaction mixture included 2 µL of 10x Dream Taq buffer, 2 µL of 10 mM dNTP mix, 1 µL of 10 pmol each of forward and reverse primers, 100 ng of leptospiral genomic DNA and 0.5 unit of Dream Taq DNA Polymerase (Fermentas, Thermo Scientific, Pittsburgh PA, USA) in a total volume of 20 µL. PCR was performed in Master cycler (Eppendorf, CA, USA) using the following program: initial denaturation for 3 min at 95°C followed by 30 cycles of amplification for 1 min at 95°C, 30 sec at 60°C, 1 min at 72°C and final extension for 5 min at 72°C. An identical reaction mixture without template DNA was included as negative control. The PCR products were separated by electrophoresis in 1% agarose gel.

2.4.5.2. Restriction digestion of vector and *hbpA*

1 µg of pET28a (+) plasmid DNA and 1 µg of *hbpA* amplicon were separately subjected to double digestion with *Nde*I and *Sal*I. The reaction mixture contained 2 µL of 10X buffer O (Fermentas, Thermo Scientific, Pittsburgh PA, USA), 10 units of each enzyme in a total volume of 20 µL. The tubes were incubated at 37°C overnight. After heat inactivation at 65°C for 10 min, they were purified using PCR reaction clean-up kit (Invitrogen, USA) as per manufacturer's instructions.

2.4.5.3. Ligation and preparation of recombinant DNA

Ligation was performed in the molar ratio of 1:3 (100 ng of vector DNA and 300 ng of insert DNA) taken in 20 µL of ligation mixture containing 2 µL of 10X ligation buffer and 1 µL of T4 DNA ligase (5 units / µL). The ligation mixture was incubated at 4°C overnight. The reaction mixture was incubated at 65°C for 10 min to inactivate the enzyme.

2.4.5.4. Transformation into *E. coli* DH5α

2.4.5.4.1. Preparation of ultra-competent cells

Reagents

- Inoue transformation buffer**:** Dissolved 10.88 g of $MnCl_2.4H_2O$ (55 mM), 2.20 g of $CaCl_2.2H_2O$ (15 mM), 18.65 g of KCl (250 mM) and 20 mL of 0.5 M PIPES (10 mM) in 700 mL of double-distilled water. The pH was adjusted to 6.7 with 0.1 N HCl and the volume was made up to 1 L.

Procedure

Ultra-competent cells were prepared as per standard procedure (Sambrook & Russell, 2006). A single colony was inoculated in 5 mL LB broth and incubated in an orbital shaker overnight at 37°C. 1 mL of this was inoculated into 100 mL of LB broth and allowed to grow at 18°C overnight on shaker. The culture was harvested at $OD_{600\ nm}$ of 0.4 - 0.5 by centrifuging at 5000 rpm for 10 min at 4°C. The cell pellet was suspended in 30 mL of ice-cold Inoue transformation buffer, centrifuged at 5000 rpm for 10 min at 4°C and re-suspended in 8 mL of ice cold Inoue transformation buffer. 0.6 mL of DMSO was added to the cell suspension, incubated in ice for 10 min, aliquoted 100 µL of the cell suspension in sterile cryovials, frozen in liquid nitrogen and stored at -80°C.

2.4.5.4.2. Transformation

One vial was thawed and maintained on ice. 5 µL of ligation mixture was added and incubated for 30 min. The cells were subjected to heat shock at 42°C for 90 s and chilled on ice for 2 min, added 800 µL of LB broth and incubated at 37°C for 45 min. The cells were plated on kanamycin (30 µg / mL) LB agar plates and incubated at 37°C overnight. The colonies obtained were then subjected to screening.

2.4.6. Screening and selection of a recombinant *hbpA* clone

Six colonies were picked randomly and inoculated separately into 5 mL of liquid LB medium supplemented with 30 µg / mL of kanamycin and incubated overnight at 37°C with shaking. Plasmid DNA was isolated from all the six clones and 1 µg of the respective plasmid DNA was subjected to restriction digestion with *Nde*I and *Sal*I (details as explained above). The digested samples were separated by agarose gel electrophoresis

and the release of the cloned fragment in these samples were recorded as positive. All further work were performed with one positive sample. The plasmid was subjected to sequence analysis (Xcelris Labs Ltd., Ahmedabad, India) and the generated sequence of the cloned insert was analysed with ChromasPro (Version 2.4, Technelysium Pty. Ltd., Australia). The recombinant *hbpA* containing plasmid was labelled as pMS508.

2.4.7. Expression of rHbpA$_{34}$

Expression of rHbpA$_{34}$ was performed by transforming pMS508 into the expression host *E. coli* BL21 (DE3) pLysS. Upon screening and confirmation of a positive clone, the recombinant clone was plated on a kanamycin agar plate and a single colony was inoculated into 5 mL of kanamycin-supplemented LB medium and incubated at 37°C in an orbital shaker. 2 mL of this culture was inoculated in 200 mL LB broth containing kanamycin and chloramphenicol and incubated at 37°C in an orbital shaker. When the growth was in mid-log phase and reached an $OD_{600\ nm}$ of 0.6, 10 mL of the cell suspension was pipetted and labeled as induced control. 1 mM IPTG was added to the remaining culture and incubated for 2½ h at 37°C.The organisms from both uninduced and induced cultures were pelleted by centrifugation at 10,000 rpm for 15 min at 4°C and subjected to sonication at 20 second pulses with 30 second intervals at 40 Hz in a Vibra Cell sonicator, USA for 5 minutes. The sonicate was centrifuged at 10,000 rpm for 20 min at 4°C, the supernatant was transferred into a new tube and the pellet solubilized with 10 mM Tris-HCl containing 0.6% SDS, pH 8. Protein concentration in the supernatant and the pellet was determined by the commercial BCA kit.

2.4.8. Identification of rHbpA$_{34}$ by SDS-PAGE and immunoblotting

30 µg of the protein in the supernatant and pellet were subjected to SDS-PAGE and transferred to nitrocellulose as described earlier. Identical blots were developed with commercial anti-His antibody-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Dallas, Texas, USA) and with anti-HbpA antibodies (laboratory collection; raised against a 55 kDa fragment at the C-terminal end of HbpA). The former was developed as per manufacturer's instructions and developed with the substrate diaminobenzidine (DAB) and H_2O_2 (10 mg DAB in 15 mL TBS with 0.08% H_2O_2). Anti-HbpA antibody was used at 1,000 dilution and goat anti-rabbit IgG-ALP conjugate (1:5000) was used as secondary antibody. Incubation with the primary antibody was

performed overnight at room temperature and the secondary antibody-conjugate was incubated for 2½ h at room temperature. The colour was developed with the ready-to-use 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium solution (BCIP / NBT; Sigma Aldrich, St. Louis, MO, USA).

2.4.9. Expression of rSph3, rSph4 and rLipL41 proteins

The recombinant clones for the three recombinant proteins rSph3, rSph4 and rLipL41 are available in the lab. These clones carry the recombinant plasmids pMS502, pMS511 and pMS515 respectively. Both pMS502 and pMS511 were generated by cloning the respective genes into pET28a (+); they were however transformed into the expression host *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) respectively. The clone with pMS515 contained the full length *lipL41* gene in pGEX-4T1 vector in the expression vector *E. coli* BL21 (DE3). The following were the antibiotics used for growing the three clones: kanamycin (30 µg / mL) and chloramphenicol (20 µg / mL) for the rSph3 clone, kanamycin (30 µg / mL) for the rSph4 clone and ampicillin (100 µg / mL) for rLipL41 clone.

2.4.10. Purification of the recombinant proteins

2.4.10.1. Affinity chromatographic purification of rHbpA$_{34}$, rSph3 and rSph4 using AKTA Prime Plus Purifier System (GE, Healthcare)

Reagents

- Equilibration buffer**:** 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, 25 mM Imidazole, pH 8.0.
- Wash buffer**:** 5 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, 50 mM Imidazole, pH 8.0.
- Elution buffer**:** 5 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, 500 mM Imidazole, pH 8.0.

Procedure

The respective clones, grown 200 mL cultures with the appropriate antibiotic was processed as detailed above. As all the three recombinant proteins were obtained as inclusion bodies, the insoluble pellet containing the recombinant protein was processed

as follows. The pellet was solubilised in 20 mL of equilibration buffer by incubation in a gel rocker for 1 h at room temperature and the solubilized protein was obtained in the supernatant upon centrifugation at 10,000 rpm for 10 min at 4°C. The recombinant protein was then subjected to affinity chromatography using the AKTA Prime Plus system.

5 mL of Nickel Sepharose resin was washed with equilibration buffer, packed and maintained at 4°C overnight. The column was fixed to the instrument, washed with equilibration buffer and the column was allowed to run at a flow rate of 1 mL / min. The washes were monitored by reading at 280 nm till a stable baseline with negligible reading was observed. The solubilised protein sample, in a volume of 20 mL was filtered using 0.22 µm pore size GVWP filters (Millipore Corporation, MA, USA) and loaded onto the column and allowed to pass through the column at the same flow rate of 1 mL / min. Wash buffer was added to remove unbound proteins and the column was washed with approximately 10 column volumes (~ 50 mL) of wash buffer. The protein in 10 mL of elution buffer was added and allowed to flow at the same flow rate. 1 mL fractions were collected using fractionation collector.

30 µL of each eluate was subjected to SDS-PAGE on a 10% polyacrylamide gel. The fractions showing a single band of the protein were pooled and concentrated by ultrafiltration using the 3 kDa Amicon ultrafitration tubes (Millipore). The excess urea and the imidazole were removed stepwise by adding 10 mM Tris-HCl pH 8 with decreasing concentrations of urea (4 M to 0 M). The final sample was concentrated to 1 mL and the total protein determined by the commercial BCA kit. 30 µg of the purified protein was subjected to SDS-PAGE and Western blotting with the respective antibodies.

2.4.10.2. Purification of rLipL41 by preparative gel electrophoresis

Reagents

- Gel elution buffer**:** Added 2.5 mL of 1 M Tris pH 8.8, 0.18 g of glycine, 1 g of SDS and 5 mL of glycerol in 70 mL of double-distilled water and volume was made up to 100 mL.

Procedure

Prior to this, the expression of the rLipL41 was studied and confirmed by Western blotting with anti-LipL41 antibodies. For purification, the recombinant clone was grown as

mentioned above. The insoluble recombinant protein was solubilized in the sample loading buffer and 1 mg of total protein was subjected to SDS-PAGE in 5 - 20% gradient gel as mentioned in Section 2.3.2. The band corresponding to rLipL41 was cut with a surgical blade, crushed by passing through a syringe and the protein eluted out by adding 3 mL of gel elution buffer and incubating at 37°C overnight with shaking. The soluble protein, obtained by centrifugation at 4,000 rpm for 10 min was transferred to a new tube and the residual protein in the gel slices was eluted upon addition of a small volume of gel elution buffer. The samples were pooled and the protein precipitated by adding five volumes of chilled acetone and incubating at - 20°C overnight. The protein pellet, obtained by centrifugation at 13,000 rpm for 20 min was washed with fresh acetone, dried and solubilized in a minimal volume (≤ 50 µL) of 10 mM Tris-HCl pH 7.8 with 0.6% SDS. It was centrifuged at 12,000 rpm for 5 min to remove any insoluble material and the soluble protein after determining protein concentration by BCA kit was stored at -80°C.

2.5. Infection of Vero cell line with pathogenic *L. interrogans* serovar Pomona

Infection of Vero cells was performed with the serovar Pomona since the objective of this study was to study sphingomyelinase expression by pathogenic leptospires under different environmental conditions, and *in-vitro* studies showed that serovar Pomona exhibited higher hemolytic activity that was specifically associated with the secreted sphingomyelinase. The experiments included a) infection of Vero cells with the leptospires; studying the associated changes in the Vero cells and demonstrating the sphingomyelinase expression by the pathogen and b) demonstrating the biological activity of the released sphingomyelinase on a new batch of Vero cells.

2.5.1. Maintenance and propagation of Vero cell line

Reagents

- Dulbecco's Modified Eagle Medium: Preparation of the DMEM (Sigma Aldrich, St. Louis, MO, USA) medium is detailed in Appendix I.A5. This was used as the incomplete medium. The complete medium was prepared fresh by adding 5 mL of heat-inactivated sterile FBS (10%) to 44.5 mL of DMEM medium followed by addition of 1% antibiotic-antimycotic solution (Penicillin, 100 units / mL, Streptomycin, 100 units / mL and Amphotericin B, 25 ng / mL).

- Fetal Bovine Serum: Heat inactivation (Appendix I.A6) of FBS (Invitrogen, CA, USA).
- Phosphate buffered saline (PBS)**:** 0.1M, pH 7.2 (Appendix I.A2).
- 0.25% EDTA Trypsin: commercially available (Invitrogen, CA, USA).
- Cryomix: DMEM with 20% each of FBS and DMSO.
- 5x Normal saline: Dissolved 4.5 g NaCl in double distilled water and made up to 100 mL and autoclaved.

  Working solution of normal saline was prepared by adding 1 part of above to four parts of sterile distilled water.
- Trypan Blue Stock (0.2%)**:** Trypan blue (Hi-Media; supplied as 0.5% solution). Added 0.4 mL of this to 0.6 mL of double-distilled water to get 0.2% stock solution. Working solution was prepared by mixing 4 parts of 0.2% Trypan Blue with 1 part of 5x normal saline.
- Hemocytometer.

The growth and maintenance of Vero cells were performed as per published procedures (Ammerman *et al.*, 2008).

2.5.1.1. Revival of frozen cells and propagation of the Vero cells

A frozen cryovial of Vero cells was quickly thawed and the contents dropped into 4 mL of DMEM medium in a 15 mL Falcon tube. It was centrifuged at 2000 rpm for 5 min and washed twice with incomplete DMEM medium and re-suspended in 1 mL of complete DMEM medium. The number of viable cells were determined as detailed below. 1 x 10^7 cells were seeded into a T-25 flask containing 4 mL of complete DMEM medium. The flask was first incubated in the upright position and then kept in the horizontal position in carbon dioxide incubator (5% CO_2). The cells were allowed to adhere and when the complete monolayer was formed, they were trypsinized as follows. The monolayer was washed twice with PBS (4 mL) and then incubated with 1 mL of 0.25% trypsin-EDTA for 1 min till the cells rounded up and loosened. The flask was gently tapped to release the cells which were collected in a 15 mL Falcon tube. The cells were washed with incomplete

DMEM and re-suspended in 2 mL DMEM complete medium and the viability was determined before seeding into new flasks.

2.5.1.2. Viability count

To 10 µL of cell suspension added 40 µL of DMEM medium and 10 µL of Trypan blue and mixed. A coverslip was placed on the haemocytometer and 10 µL of the cell suspension was loaded and allowed to flow under the cover slip. The number of colourless cells, representing the viable cells in each of the four large squares were counted; the cells that had taken up the blue dye were also counted to determine the total dead cells in the sample. The following calculation was used to determine the total number of viable cells.

No. of viable cells / mL = average no. of cells per square x dilution factor x 10^4

2.5.1.3. Cryopreservation of Vero cells

2 mL of the cryomix was added to 2 mL cell suspension dropwise, taking care to mix gently by swirling in order to prevent osmotic shock and minimize the heating effect due to DMSO addition. 1 mL aliquots were transferred into four labelled cryovials and cooled step-wise by maintaining them at for 4°C (2 h), -20°C (2 h), - 80°C (overnight) and finally transferring them to liquid nitrogen for long term storage.

2.5.2. Infection

Infection of the cell line with serovar Pomona was performed as per published protocol (Xue *et al.*, 2010). All infections were performed at an MOI of 1:100, either in 6 / 12 - well plates or in T-75 cell culture flasks (Corning Incorporated, USA). The cell line was propagated as described above and the leptospires were grown in EMJH liquid medium that was subjected to sodium chloride as described earlier in Section 2.2.2. The leptospires were washed with PBS and re-suspended in 1 mL DMEM medium before addition to the Vero cells.

2.5.2.1. Flow cytometry for assaying cell viability

Reagents

- 0.1 M PBS pH 7.2**:** Appendix I.A2.
- Anti-SphCD$_{210}$ antibodies: This was used as 1:200 dilution in 0.1 M PBS pH 7.2.
- FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA). This consist of three component as mentioned:
  - **a.** FITC Annexin V (component no. 51-65874X): 5 µL of this was added per test.
  - **b.** Propidium Iodide (PI) (component no. 51-66211E): 5 µL of this was added per test.
  - **c.** 10X Annexin V Binding Buffer (component no. 51-66121E): 0.1 M HEPES / NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$**.**
- Working solution of 1X Binding buffer: This was prepared by adding 1 mL of 10X Annexin V Binding Buffer to 9 mL of autoclaved double distilled water.

Procedure

Identical cultures of serovar Pomona grown in EMHJ-enrichment medium were subjected to the following conditions: untreated, induced with 120 mM NaCl (detailed in Section 2.2.3) and incubated with anti-SphCD$_{210}$ antibodies. The latter was performed upon washing the harvested leptospires with PBS and incubation with anti-SphCD$_{210}$ antibodies (1:200 dilution) for 1 h and washed with PBS prior to infecting Vero cells. Vero cells were seeded at 1 x 10^6 cells / well into 6-well cell culture plates. The experimental set-up included establishment of the cultures for performing tests in duplicate. The respective leptospiral cultures (1 x 10^8 cells) were added to the Vero cells, incubated for 4 h, trypsinization performed and harvested the Vero cells by centrifugation at 2500 rpm. Cells were washed twice with cold PBS and then re-suspended in 1 mL of the binding buffer. 100 µL (1 x 10^5 cells) was transferred to 5 mL FACS tube and added 5 µL each of FITC-conjugated Annexin V and Propidium iodide (PI). The cells were gently mixed, incubated for 15 min at room temperature in the dark and added 400 µL of the binding buffer. All the different samples were subjected to flow cytometry in BD LSRFortessa^{TM} flow cytometry (BD Biosciences, USA). The data was then analysed with FlowJo 10.4.2. software. Uninfected Vero cells, with and without addition of the two stains were used as controls.

2.5.2.2. Physical and morphological changes of Vero cells upon infection

2.5.2.2.1. Microscopic examination of infected cell lines

1 x 10^6 Vero cells were seeded on sterile 15 mm diameter round coverslips in 6-well cell culture plate (Corning Incorporated, USA). Duplicate sets were infected with 1 x 10^8 leptospires for 2 h, with two sets of uninfected Vero cells used as controls. The cell monolayers were washed twice with PBS to remove non-adherent leptospires. The coverslips from one set of infected cells were subjected to Modified Fontana silver staining (Rodríguez González *et al.*, 2013). This and the other two sets of Vero cells, including the control cells were examined in bright field using Olympus CX41 microscope (Olympus Corporation, Japan) fitted with Mp3 Micro Publisher 3.3 RTV (Q-imaging, BC, Canada) at 100x magnification and at 63x magnification (oil immersion) using Phase contrast microscopy (Carl Zeiss, Germany).

2.5.2.2.2. Scanning Electron Microscopy

Scanning electron microscopy of the infected Vero cells was performed as per published protocol (Barocchi *et al.*, 2002). 1 x 10^6 Vero cells was seeded on 12 mm diameter circular coverslips in a 12-well cell culture plate and infected with 1 x 10^8 leptospires for 30 min, 60 and 120 min respectively; uninfected cells were included as controls. The cell monolayers were washed twice with PBS and fixed with 2.5% glutaraldehyde overnight at 4°C. The cells were washed with graded series of ethanol (10 -100%), increasing the alcohol content by 10% at each step of washing performed for 10 min; the final wash with 100% ethanol was performed twice. The coverslip was then rapidly transferred to a critical-point drier and dried to critical point dehydration. The coverslip was mounted on specimen stubs and sputter coated with gold-palladium before observation under SEM (Philips FEI, XL30, USA).

2.5.2.3. Detection of sphingomyelinase expressed by the pathogen in infected Vero cells

2.5.2.3.1. Confocal Microscopy of infected Vero cells

Reagents

- DAPI (4′,6-diamidino-2-phenylindole): supplied as 1 mg / mL (Sigma Aldrich, St. Louis, MO, USA).

- Stock solution (10 µg / mL): added 10 µL of the above to 990 µL of PBS.
- Working solution (0.1 µg / mL): added 10 µL of stock to 990 µL of PBS.

Procedure

1 x 10^6 Vero cells was seeded on sterile 15 mm diameter round coverslips in 6-well cell culture plate (Corning Incorporated, USA) and infected with 1 x 10^8 leptospires for 2 h. The monolayers were washed twice with PBS and treated with 50 µg / mL of gentamicin for 30 min to kill extracellular leptospires. The monolayers were washed twice with PBS, fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) for 30 min and then permeabilized with 0.01% Triton X-100 for 10 min to allow antibody penetration into the cells. The cell monolayers were washed twice with PBS, blocked with 1% BSA for 1 h and incubated for 1 h with anti-sphingomyelinase antibodies (rabbit anti-$Sph2_{163}$ antibodies; 1:250 dilution). The cell monolayers were washed twice with PBS followed by incubation with goat anti-rabbit IgG antibody-FITC (Merck Life Science Pvt. Ltd. Darmstadt, Germany; 1:500 dilution) for 1 h. The monolayers were washed twice with PBS and incubated with 0.1 µg / mL DAPI for 10 min for staining the Vero cell nuclei. The coverslips were sealed with paraffin wax and observed at 63x magnification with oil immersion under Laser Confocal Microscope (Carl Zeiss, Germany). The FITC and DAPI fluorescence were viewed at 590 / 617 nm and 360 / 460 nm wavelengths (excitation / emission) respectively.

2.5.2.3.2. Analysis of the transcript and protein levels of sphingomyelinase expressed by the serovar Pomona upon infection

2.5.2.3.2.1. Infection procedure

1 x 10^7 Vero cells were seeded in a T-75 cell culture flask and incubated in 5% CO_2 at 37°C, 24 h. The cell monolayer was washed twice with PBS and added 1 x 10^9 leptospires which was resuspended in 1 mL DMEM medium and incubated 2 h in 5% CO_2 at 37°C. The cell monolayer was washed twice with PBS and trypsinized. The cells were collected in 15 mL Falcon tube and washed twice with PBS. The pellet was resuspended in 500 µL of 0.1% Triton X-100 for 30 min to lyse the Vero cells, followed by centrifugation

at 13,000 rpm for 20 min at 4°C to pellet the leptospires. An aliquot was taken for determining the recovered leptospires cell count as mentioned in Appendix I.A7.

2.5.2.3.2.2. Isolation of RNA from serovar Pomona

Four sets of serovar Pomona were used for isolation of total RNA from serovar Pomona. Sets 1 and 2 included organisms grown in axenic medium in the absence and presence of 120 mM sodium chloride. Vero cells were separately infected with sets 1 & 2 and the two lots of recovered organisms were denoted as sets 3 and 4 respectively. Added 1 mL of Trizol (Invitrogen, CA, USA) to the cells pellet, resuspended thoroughly by pipetting and incubated at room temperature for 5 min. Added 200 µL of chloroform, mixed gently by inverting the tube repeatedly, centrifuged at 12,000 rpm for 15 min at 4°C and the upper aqueous layer pipetted into a new tubes. Added 0.1 volume of 3M sodium acetate and 0.8 volume of isopropanol, mixed and incubated overnight in -20°C. The tube was centrifuged at 12,000 rpm for 15 min at 4°C, pellet was washed with 1 mL of 75% ethanol and air dried. Dissolved the pellet in 20 µL of RNA free DEPC water. The RNA samples were subjected to clean-up using the RNeasy Mini kit (Invitrogen, CA, USA) as manufacturer's instructions. DNA was digested by addition of 5 µL of 10X Turbo DNase buffer and 1 µL of Turbo DNase (Ambion, Texas, USA) followed by incubation in 37°C water bath for 2 h. The reaction was terminated with 2 µL of DNase inactivation reagent at 37°C for 2 min followed by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was transferred into a new tube and concentration was determined in NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, DE, USA) and 200 ng of RNA was analysed for the integrity of 16S and 23S rRNA by 2% agarose gel electrophoresis.

2.5.2.3.2.3. qRT-PCR

1 µg of the total RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, CA, USA) according to the manufacturer's instructions in a Master Cycler gradient thermal cycler (Eppendorf, USA) with priming at 25°C for 5 min followed by reverse transcription at 42°C for 30 min and enzyme inactivation at 85°C for 5 min. A suitable dilution of the cDNA was used as the template for the amplification of the *sph2* gene and 16S rRNA (internal control) using established primers (Narayanavari

et al., 2015). The reaction mixture, consisted of 4 µL of 1:20 dilution cDNA, 5 pmol of forward and reverse primer and 5 µL of 2X iQ5 SYBR Green supermix (Bio-Rad) in a total volume of 10 µL was subjected to amplification in the CFX96 Real-time PCR Detection System (Bio-Rad) using the following program: initial denaturation for 15 min (95°C) followed by 40 cycles of amplification (15 s at 95°C, 30 s at 58°C, 30 s at 72°C) and a final extension of 2 min at 72°C. The difference in gene expression, expressed as fold-change, was calculated using the $2^{-\Delta\Delta CT}$ method. The relative fold change was calculated by comparison with the EMJH control. The assay was performed using duplicate biological replicates.

2.5.2.4. Effect of rSphCD$_{210}$ on Vero cell

Study with rSphCD$_{210}$ protein was performed and analysed Confocal Microscopy (Section 2.5.2.3.1), Scanning Electron Microscopy (Section 2.5.2.2.2)**.**

2.6. Clinical studies

Two clinical studies were conducted. In the first study, a total of 190 serum samples from suspected cases of leptospirosis and controls were screened for antibodies by MAT, commercial PanBio ELISA kit and in-house ELISA for antibodies against sphingomyelinase, HbpA and LipL41. In addition, lateral flow device was prepared and tested for anti-HbpA antibodies. In the second study, testing of both serum and urine samples were performed in patients with leptospirosis and compared with dengue cases. The tests employed on these samples included MAT and ELISA.

2.6.1. Screening of serum samples for anti-leptospiral antibodies

2.6.1.1. Microscopic Agglutination Test (Sivakolundu *et al.*, 2012)

Cultures listed in Table 2.1 were grown to mid-log phase in liquid EMJH - enrichment medium, with care taken to ensure that there was no clumping of the organisms. In a 96 well microtiter plate, added 50 µL of PBS to all the wells. Row A served as negative control with no serum added in the wells A1-A12. In all the wells in row B (B1-12), 48 µL of PBS and 2 µL of test serum was added and mixed well. The serum in each of the wells was serially diluted from rows B to H as follows. 50 µL of sample from B1 was diluted serially

from rows B to H and discarding 50 µL from row H (dilution being 1: 50 in row B to 1:3200 in row H). This process was performed from wells 1-12 with a multi-channel pipette. The various live leptospiral serovars were prepared and examined by dark-field microscopy at 40x magnification (Nikon Eclipse E600, Japan) to ensure there was no agglutination. 50 µL of the specific serovar was added from rows A – H. Thus, 12 serovars were added in wells 1-12. After addition, the plate was gently moved to mix the contents for 15-20s and incubated for 3 h at 37°C.

A drop from each well was placed on a glass slide and check for agglutination under a dark field microscope. A serum sample was considered positive when 50% organisms were agglutinated or there was 50% reduction in the number of leptospires.

2.6.1.2. PanBio IgM ELISA

Patients' serum samples were screened for IgM-specific anti-leptospiral antibodies by commercial PanBio IgM ELISA kit (Inverness Medical Innovation Pty. Ltd., Australia). To the coated test wells in the strips provided, added 100 µL of serum (diluted 1:100) and incubated for 30 minutes at 37°C. After three washes, as in routine ELISA, 100 µL peroxidase conjugated anti-human IgM was added and incubated for 30 min at 37°C. After three washes to remove the unbound conjugate, 100 µL tetramethyl benzidine and hydrogen peroxide was added and incubated for 10 min. The reaction was stopped with 100 µL of 1 M phosphoric acid and the absorbance was read at 450 / 600 nm in an ELISA reader (Model 680XR; Bio-Rad, CA, USA).

2.6.1.3. Enzyme-linked immunosorbent assay (Sivakolundu *et al.,* 2012) for screening for the presence of anti-leptospiral antibodies

ELISA was performed for the detection of antibodies against rHbpA / rLipL41 / rSph / IgM-specific anti-leptospiral antibodies in the serum of patients suspected of leptospirosis. The respective antigen (250 ng) was used for the coating of the ELISA plate. Whole cell sonicate proteins (500 ng) were used for determining the IgM titres of serum antibodies against leptospiral proteins. In addition, ELISA was performed to determine the presence of urinary antibodies, the processing of which is detailed below.

Reagents

- Sodium carbonate – bicarbonate coating buffer (0.05 M, pH 9.2)**:** 159 mg of sodium carbonate and 293 mg of sodium bicarbonate were dissolved in 90 mL double-distilled water, adjusted pH to 9 with 0.1 N HCl and volume was made up to 100 mL with double-distilled water. The solution was autoclaved and stored at 4°C.
- Phosphate buffered saline (PBS; 0.1 M, pH 7.2)**:** Appendix I.A2.
- PBS / T**:** 0.1 M PBS containing 0.05% Tween 20.
- Blocking solution**:** 0.1 M PBS containing 5% BSA.
- Secondary antibodies.
- Substrate for alkaline phosphatase
  - Diethanolamine buffer: Added 24.2 mL of diethanolamine (Sigma Aldrich, St. Louis, MO, USA) and 0.5 mM $MgCl_2$ in 200 mL double-distilled water and adjusted pH to 9.8 with 0.1 N HCl and made up the volume to 250 mL with double-distilled water.
  - Dissolved 1 tablet (5 mg) of p- nitrophenylphosphate (pNPP; Sigma Aldrich, St. Louis, MO, USA) in 5 mL of diethanolamine buffer to get 1 mg / mL solution.
- Substrate for Peroxidase**:**
  - 0.05 M phosphate-citrate buffer (pH 5): Added 5.11 g of citric acid and 7.3 g of disodium hydrogen phosphate in 900 mL of double-distilled water, adjusted pH to 5 with 0.1 N HCl and the volume was made up to 1 L.
  - 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution: Dissolved 1 mg TMB tablet in 10 mL of 0.05 M phosphate citrate buffer and added 3 µL of hydrogen peroxide prior to use.
- Stop solution:
  - For Peroxidase**:** Added 6.8 mL of H_2SO_4 (1.25 M) and volume was made up to 100 mL with double-distilled water.
  - For alkaline phosphatase**:** 4 g of NaOH (1N) was dissolved in 80 mL of double-distilled water and volume was made up to 100 mL.

Procedure

Flat-bottomed 96-well polystyrene microtitre plates (Corning Inc., Corning, New York, USA) were coated with 250 ng of the respective recombinant antigen in 100 µL of coating buffer and incubated 3 h at 37°C in a humid box, followed by overnight incubation at 4°C. The plates were washed twice with PBS/T and incubated with blocking solution for 1 h at 37°C. 100 µL of human serum (1:200 dilution) was added and incubated for 1 h at 37°C, the plates were again washed twice with PBS/T and 100 µL of goat anti-human IgG (Fc specific)-peroxidase conjugate conjugate / goat anti-human IgM (µ-chain specific)-peroxidase conjugate (1:5000 dilution: Sigma Aldrich, St. Louis, MO, USA) was added to the wells and incubated for 1 h at 37°C. The colour was developed in the dark with 100 µL of freshly prepared TMB / pNPP substrate solution and the reaction was stopped using 100 µL of the appropriate stop solution after 20 min. The absorbance at OD_{450} nm was read with an ELISA reader (Model 680XR, BioRad, USA) for peroxidase-catalysed reactions and at OD_{405} nm for alkaline phosphatase. 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.

2.6.1.3.1. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (Version 5.01, GraphPad Software Inc., San Diego, CA). The results were analysed by non-parametric Mann Whitney test and were considered significant when $p<0.05$. The cut-off values for in-house ELISA were calculated by adding two times standard deviation to the mean value of normal endemic controls. Sensitivity, specificity and predictive values of the test were determined using MAT as the gold standard.

- Sensitivity = [a / (a + c)] × 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.
- Specificity = [d / (b + d)] × 100; where d = number of serum samples negative by ELISA and MAT; b = number of serum samples negative by MAT but positive by ELISA.
- Positive predictive value = a / (a + b) × 100.

- Negative predictive value = d / (c + d) × 100.

2.6.1.4. Development of a lateral flow device using $HbpA_{34}$

1 µg / µL of $rHbpA_{34}$ was coated on the lateral flow device by company Genomix Molecular Diagnostics Pvt. Ltd, (Hyderabad, India). 5 µL of undiluted serum, followed by 200 µL sample buffer (50 mM phosphate buffer containing 0.1% BSA, 0.1% Triton X-100 and 0.03% SDS) was added to sample well. The development of the test and control bands was visualized within 10 minutes upon addition. A total of 20 human serum samples were screened by this method.

2.6.2. Screening of urine samples for leptospiral antigens

2.6.2.1. Processing of urine samples

5 mL of urine was precipitated with 5 mL of saturated ammonium sulphate solution. After incubating for 20 min at 37°C, it was centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, the pellet was dissolved in 200 µL distilled water and dialysed against phosphate buffered saline (0.1 M, pH 7.2, 0.9% NaCl). The protein concentration was estimated with the BCA kit (Sigma Aldrich, St. Louis, MO, USA).

2.6.2.2. Detection of urinary leptospiral antigens

2.6.2.2.1. ELISA

400 ng of urinary proteins was used as antigen, polyclonal mono-specific rabbit antibodies raised against LipL32, LipL41, Fla1, HbpA, $SphCD_{210}$, $Sph2_{163}$ and Sph4 were added at 1:1000 dilution and goat anti-rabbit IgG (Fc specific)-alkaline phosphatase conjugate (Sigma Aldrich, St. Louis, MO, USA) was used at 1:5000 dilution. The colour was developed with 100 µL of p-nitrophenyl phosphate (Sigma Aldrich, St. Louis, MO, USA) for 20 min and the OD_{405nm} was read in an ELISA reader (Model 680XR, BioRad, USA).

2.6.2.2.2. Western blotting analysis

50 µg urinary proteins were subjected to SDS-PAGE (5 -20% gradient) and immunoblot analysis using standard procedures. Four identical blots of each urine sample was incubated separately with antibodies (diluted 1:1000) against LipL32, $SphCD_{210}$ and $Sph2_{163}$ and HbpA. Goat anti-rabbit IgG - alkaline phosphatase conjugate (Sigma Aldrich,

St. Louis, MO, USA) was used at 1:5000 dilution and the blot was developed with NBT-BCIP (Sigma Aldrich, St. Louis, MO, USA).

2.6.2.3. Identification of serum / urine reactive leptospiral antigens

This was performed by first performing Western blotting analysis of leptospiral whole cell sonicates using antibodies from serum and urine of leptospirosis patients, followed by the identification of the strongly reactive proteins using MALDI-TOF-MS/MS analysis**.** 60 µg of the four leptospiral whole cell sonicates (Lai, Pomona, Hardjobovis and Andamana) were subjected to SDS-PAGE and immunoblotting with serum and urine, used at a dilution of 1:200, followed by the addition 1:5,000 dilution of anti-human IgG-alkaline phosphatase conjugate (Sigma Aldrich, St. Louis, MO, USA). The reacting bands were detected by addition of the substrate NBT / BCIP solution (Sigma Aldrich, St. Louis, MO, USA).

Six leptospiral proteins detected with serum and urinary antibodies were purified by SDS-PAGE in 5-20% gradient gel and confirmed by immunoblotting. The approximate molecular mass of these proteins was determined using UVP Vision Works LS Image Acquisition and Analysis Software. They were subjected to MALDI-TOF-MS/MS analysis (Sandor Life Science Pvt. Ltd, Hyderabad, India) and identified using Mascot; protein score > 40 was considered to be of significant matching.

CHAPTER – III

RESULTS

The findings in the study are grouped into two parts; first, the demonstration of sphingomyelinase by pathogenic *L. interrogans* serovar Pomona upon infecting Vero cells (3A) and second, the clinical significance and diagnostic potential of this protein and the hemin-binding protein HbpA (3B). The latter, also a pathogen-specific protein was reported and evaluated as a diagnostic marker in our earlier studies, as detailed in Chapter I. Here, a smaller fragment has been chosen as the antigen for screening by ELISA and tested for its effectiveness in the lateral flow device.

3A.1. Sphingomyelinase expression by *L. interrogans* serovar Pomona upon infecting Vero cells

Sphingomyelinases are expressed in low levels by leptospires cultured in laboratory media, but an increase in the osmolarity to physiological levels (120 mM sodium chloride) augments its expression (Matsunaga *et al.*, 2005). We compared the sphingomyelinases produced by the pathogenic *L. interrogans* serovars Pomona and Lai under these conditions and further, demonstrated the absence of these virulence proteins in the non-pathogenic serovar Andamana. The infection studies were performed with serovar Pomona due to the higher hemolytic activity seen with this serovar. We report here not only an increase in the transcript and protein levels of sphingomyelinase but also a processed form of the protein that was secreted into the medium. Since sphingomyelinases are known to lyse host cells, the viability and cell surface changes of the Vero cells upon infection were studied.

3A.1.1. Expression of sphingomyelinase(s) by the pathogenic serovars Pomona and Lai grown in EMJH-enrichment medium

3A.1.1.1. Expression profile as analysed by SDS-PAGE and immunoblot analysis

Sphingomyelinase (*sph*) genes vary in number and sequence among the pathogenic *Leptospira* spp. (Ren *et al.*, 2003; Bulach *et al.*, 2006; Chou *et al.*, 2012) and are absent in the non-pathogens (Picardeau *et al.*, 2008). In the serovar Lai, there are five *sph* genes encoding Sph1, Sph2, Sph3, SphH and Sph4, of which Sph2 was shown to be a true sphingomyelinase (Narayanavari *et al.*, 2012b). With the exception of Sph4, four of them possess the exo-endophosphatase domain. In the absence of information on the conditions that trigger the expression of one or more of these sphingomyelinases and

their post-translational modifications / cleavage to yield a functional molecule, the common exo-endophosphatase domain was earlier cloned and expressed as rSphCD$_{210}$. Antibodies against this recombinant protein and rSph2$_{163}$ (163 aa fragment carrying the N-terminal region of Sph2; given by Dr. Haake, UCLA, USA) were used to identify sphingomyelinase(s) in this study.

It is well established that addition of sodium chloride to a final concentration of 120 mM, equivalent to the osmolarity of serum induced the expression of sphingomyelinases (Matsunaga *et al.*, 2005). Hence, the three serovars, including the two pathogens were grown under these conditions and their whole cell sonicates were analysed for sphingomyelinase expression. Fig. 3A.1 clearly shows the sphingomyelinase bands in the two pathogenic serovars Lai and Pomona, with none detected in the lane corresponding to serovar Andamana.

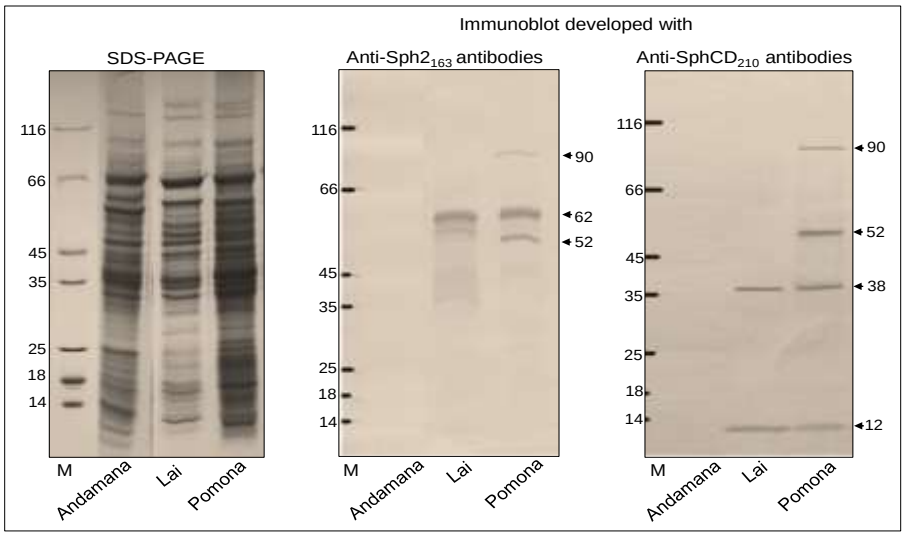


Figure 3A.1. SDS-PAGE and immunoblot analysis of the whole cell sonicates of the serovars Lai, Pomona and Andamana grown in EMJH-enrichment medium. All the three serovars were grown to log phase in EMJH-enrichment medium and supplemented with 120 mM final concentration of sodium chloride. 60 µg of cell-free whole cell sonicates of these serovars were electrophoretically fractionated by SDS-PAGE on 5-20% polyacrylamide gel (Panel a), transferred onto nitrocellulose membrane and probed with anti-Sph2$_{163}$ (Panel b) and anti-SphCD$_{210}$ antibodies (Panel c) diluted 1:1000 respectively. M represents the molecular weight markers.

The immunoblots showed the differing banding pattern with the antibody used for the analysis. In the serovar Lai, two bands of approximate molecular mass of 59 and 54 kDa were recognized by anti-Sph2$_{163}$ antibodies that were not recognized by anti-rSphCD$_{210}$ antibodies (Fig. 3A.1). The latter reacted with two low molecular weight bands of 38 and

12 kDa respectively. Three sphingomyelinases were seen in the immunoblots of serovar Pomona developed with anti-$Sph2_{163}$ antibodies, of which the 62 and 52 kDa bands were prominent with the 90 kDa band showing faint reactivity. Interestingly, two of these polypeptides (90 and 52 kDa) also reacted with the antibody against the common domain (anti-$rSphCD_{210}$ antibodies). The serovar Pomona, like serovar Lai, expressed the 38 and 12 kDa sphingomyelinases recognized by anti-$rSphCD_{210}$ antibodies.

3A.1.1.2. Hemolytic assay All the three leptospiral serovars were grown to a cell density of 2 x 10^8 cells / mL. 100 µL of the spent growth medium and 250 µg of whole cell sonicate from these identically grown cultures were used for the hemolytic assay. A marked increase from 0.96% to 48.7% was noted in the hemolytic activity of the spent growth medium of the serovar Pomona upon induction with salt.

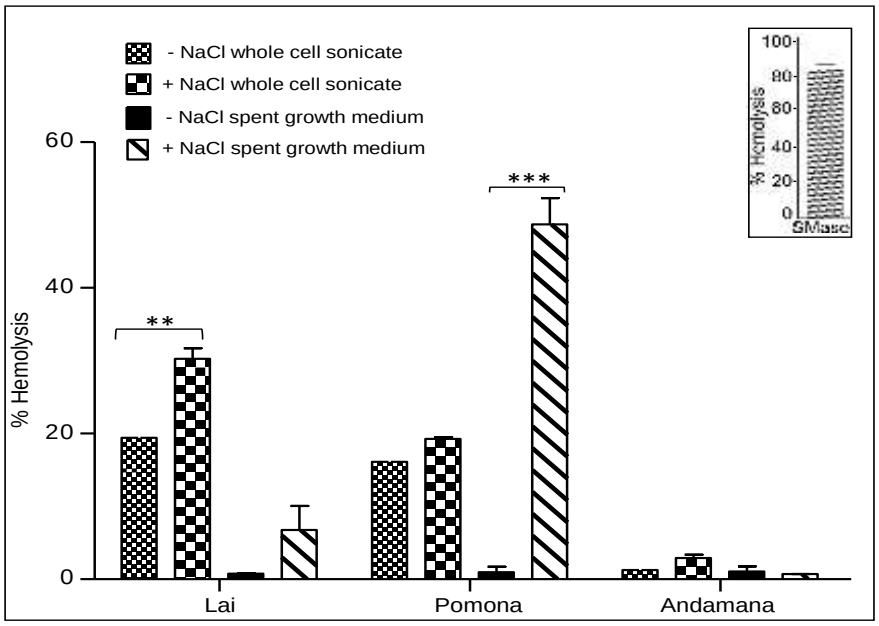


Figure 3A.2. Hemolytic assay. The figure shows the hemolytic activity of the whole cell sonicates and spent growth media of the serovars Lai, Pomona and Andamana performed in microtiter plates. The organisms were grown in the absence (- NaCl) and presence (+ NaCl) of 120 mM sodium chloride. RBCs in normal saline and distilled water were taken as negative and positive controls respectively, with the latter representing 100% lysis. The marginal lysis seen with RBCs in normal saline was subtracted from the absorbance of all the test samples and the % hemolysis was calculated with reference to the absorbance of the positive control. The inset shows the hemolysis seen with commercial Sphingomyelinase C (SMase) from *Staphylococcus aureus* (Sigma Aldrich, St. Louis, MO, USA). The error bars represent the standard deviation calculated from two identical experiments performed in duplicates. The results were analysed by Unpaired t test and considered as statistically significant when $p < 0.05$.

This dramatic increase was not seen in the pathogenic serovar Lai that showed only a 9-fold rise in the hemolytic activity. Though not appreciable, differences in the hemolytic activity were observed with the whole cell sonicates; the hemolytic activity of serovar Lai was relatively higher than that of serovar Pomona upon addition of salt to cultures.

Mention must be made of the fact that 120 mM final concentration of sodium chloride is equivalent to physiological osmolarity and does not contribute to the hemolysis. The inability of the serovar Andamana to lyse RBCs is an anticipated finding due to the absence of the sphingomyelinases and its inclusion served as an additional control (Fig. 3A.2).

3A.1.2. Infection of Vero cells with *L. interrogans* serovar Pomona

The Vero cell line maintained as monolayers was infected with the serovar Pomona at an MOI of 100. Upon addition of the live organisms, the performance of the Vero cells in terms of viability and survival were first recorded. The leptospires recovered from the infected Vero cells as well as the growth medium were analysed for sphingomyelinase(s).

3A.1.2.1. Flow cytometry analysis of infected Vero cells for the measurement of cell death

Fig. 3A.3 shows the flow cytometry analysis of Vero cells infected with serovar Pomona under different conditions, as indicated in the figure. The various populations represent viable and non-apoptotic (Annexin V^- / PI^-), early and late apoptotic (Annexin V^+ / PI^-; Annexin V^+ / PI^+) cells and dead cells.

Most of the uninfected control Vero cells (99.9% of unstained and 84.2% stained) belonged to the viable and non-apoptotic population. Upon infection with untreated and salt-induced leptospires, this population reduced drastically to 12.7% and 0.44% respectively. Based on our hypothesis that sphingomyelinase contributed to the cell damage and death, leptospires pre-incubated with anti-$SphCD_{210}$ antibodies were used for infecting the Vero cells. There was increase in the viability of the organisms, with the viable cells population rising to 17.5%.

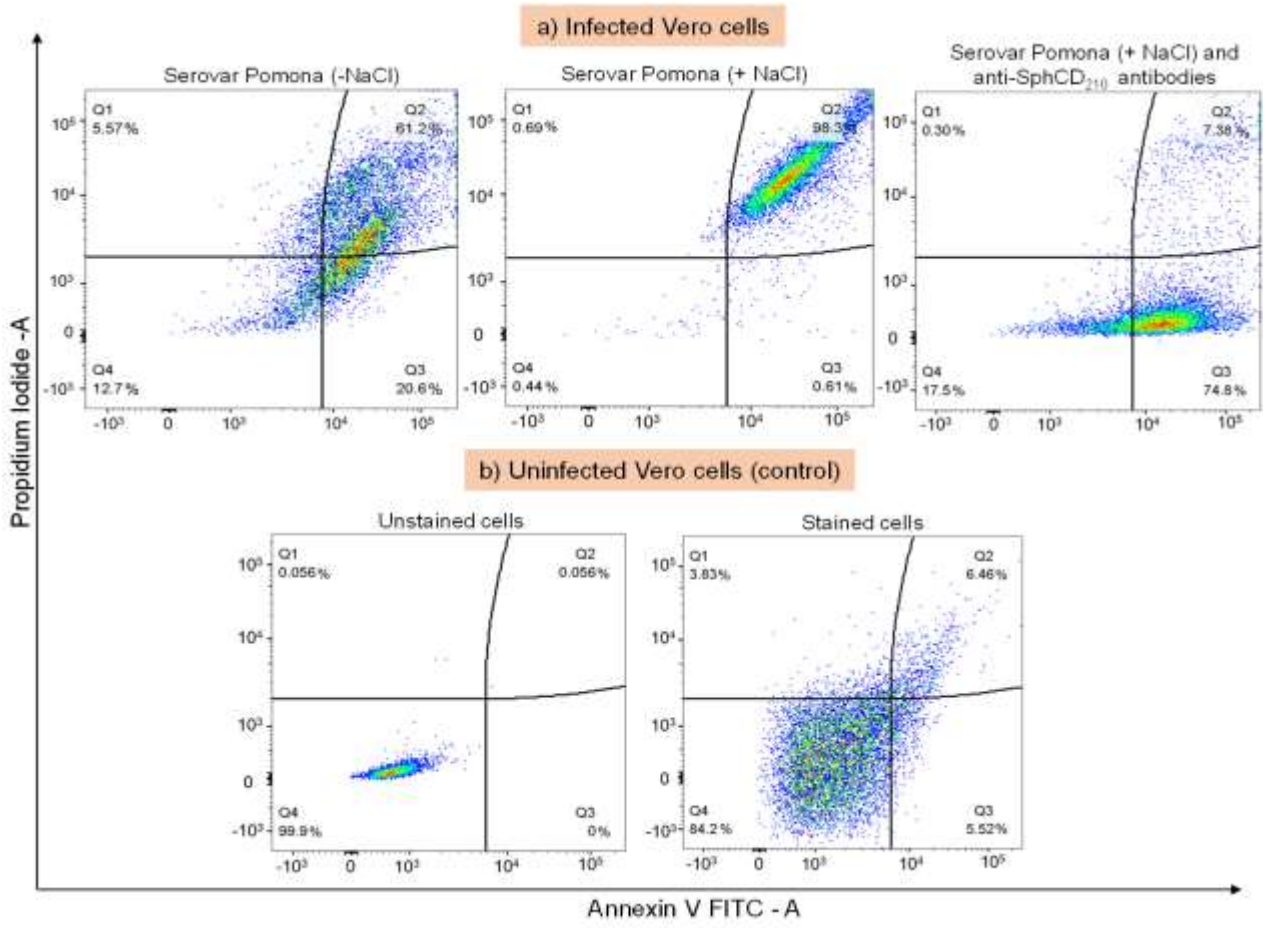


Figure 3A.3. Flow cytometry analysis of infected Vero cells. Vero cell monolayers were infected with serovar Pomona at an MOI of 100. The infected cells (Panel a) and the uninfected control cells (Panel b) were stained with Annexin V-FITC and propidium iodide (PI) and subjected to analysis by flow cytometry. The four quadrants, labelled Q1 to Q4 represent populations of cells that are viable (Q4 - Annexin V^- / PI^-), early apoptotic (Q3 - Annexin V^+ / PI^-), late apoptotic cells (Q2 - Annexin V^+ / PI^+) and necrotic (Q1 - Annexin V^- / PI^+) cells. The experiment was performed in duplicate and the data was analyzed using FlowJo 10.4.2. software. The data analysis was performed upon acquisition of at least 10,000 events.

The population of late apoptotic cells were high upon infection, with 98.39% population being Annexin V^+ / PI^+ positive (Q2) when sodium chloride - treated leptospires were used for infecting the Vero cells. Considerable cell death resulted upon addition of untreated leptospires, with 61.2% seen in this population group. In the antibody-treated group, the predominant population (74.8%) was early apoptotic cells, with 7.38% progressing to the late apoptotic (Q2; Annexin V^+ / PI^+) population. It is likely that the latter group would increase with time, due to other factors influencing the viability of the cells.

3A.1.2.2. Detachment of Vero cells from substratum upon addition of live leptospires

The Vero cells became rounded and detached from the substratum, within 120 minutes upon addition of live serovars of the two pathogenic strains Lai and Pomona (Fig. 3A.4). These changes were seen only when the infecting pathogenic serovars were induced with sodium chloride. Further, the non-pathogenic serovar Andamana did not show any effect on the Vero cells and merely adhered to the glass surface, as visualized by silver staining (Fig. 3A.4a).

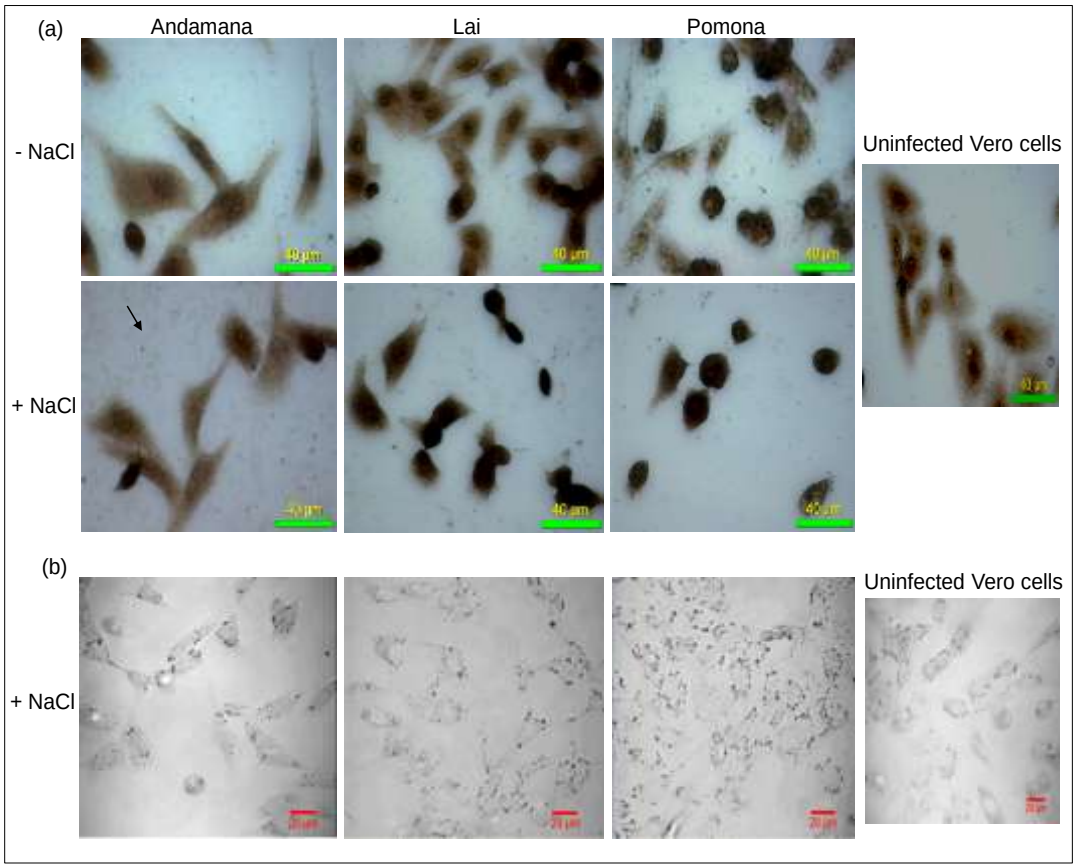


Figure 3A.4. Detachment of Vero cells upon infection with pathogenic leptospires. The leptospiral serovars Lai, Pomona and Andamana were added to Vero cell monolayers in three identical flasks. Panels (a) and (b) represent the images obtained by silver staining and phase contrast microscopy respectively. These images show the detachment of the Vero cells from the substratum upon infection by serovars Lai and Pomona treated with 120 mM sodium chloride (+ NaCl), with negligible effect seen in cultures without the salt treatment (- NaCl). The uninfected Vero cells served as negative control. The non-pathogenic serovar Andamana had no effect on Vero cells and merely adhered to the glass surface, as indicated by the arrow.

3A.1.2.3. Visualization of infected Vero cells using Scanning Electron Microscopy

Vero cells were infected with serovar Pomona and the infected cells were visualized by scanning electron microscopy (SEM) at increasing time intervals. The adherence of the bacteria to the mammalian cell surface was seen as clumps within 30 minutes upon infection. Damage to the cell surface was evident by 60 minutes and by 120 minutes, most of the organisms were internalized into the cell (Fig. 3A.5 b). There was considerable damage to the host cell surface as seen from the several perforations, a feature not seen in the uninfected Vero cells (Fig. 3A.5a).

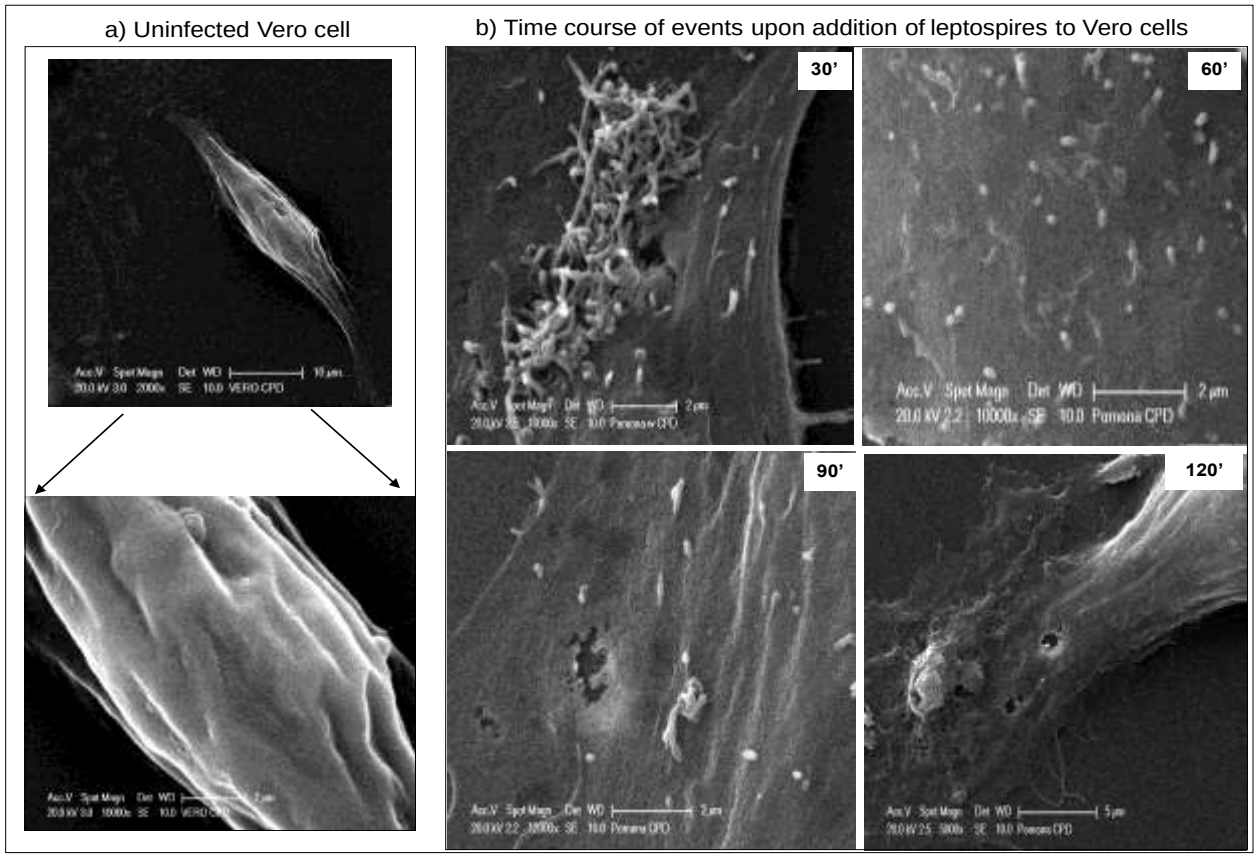


Figure 3A.5. Scanning electron microscopy of Vero cells upon leptospiral infection. Panel (a) represents the uninfected Vero cell used as control, in which the smooth cell surface can be clearly seen in the enlarged view. Leptospires, upon addition to the Vero cells (Panel b) adhered on the host cell surface and were seen as clumps within 30 minutes. Damage to the host cell surface was seen within 60 minutes and at 120 min, holes were seen on the Vero cells and almost all the leptospires adhering on the cell surface entered the cell.

3A.2. Analysis of leptospiral sphingomyelinase(s) upon infection

Infection of Vero cells by serovar Pomona was allowed for two hours, after which all the free leptospires in the medium were removed and the adherent cells, after suitable

washes, were lysed to release all leptospires. Sphingomyelinase(s) expressed by the pathogen was detected by a) confocal microscopy of infected cells with anti-sphingomyelinase antibodies, b) measurement of *sph2* transcript levels of the leptospires released from infected cells, c) immunoblotting of whole cell sonicate of the recovered leptospires and d) immunoblotting of the spent growth medium for detecting sphingomyelinase.

3A.2.1. Confocal microscopy: detection of sphingomyelinase in Vero cells infected with serovars Pomona and Lai

It is evident from the images that sphingomyelinase is produced by the two pathogenic serovars Pomona and Lai, as seen by the green fluorescence generated by reactivity with

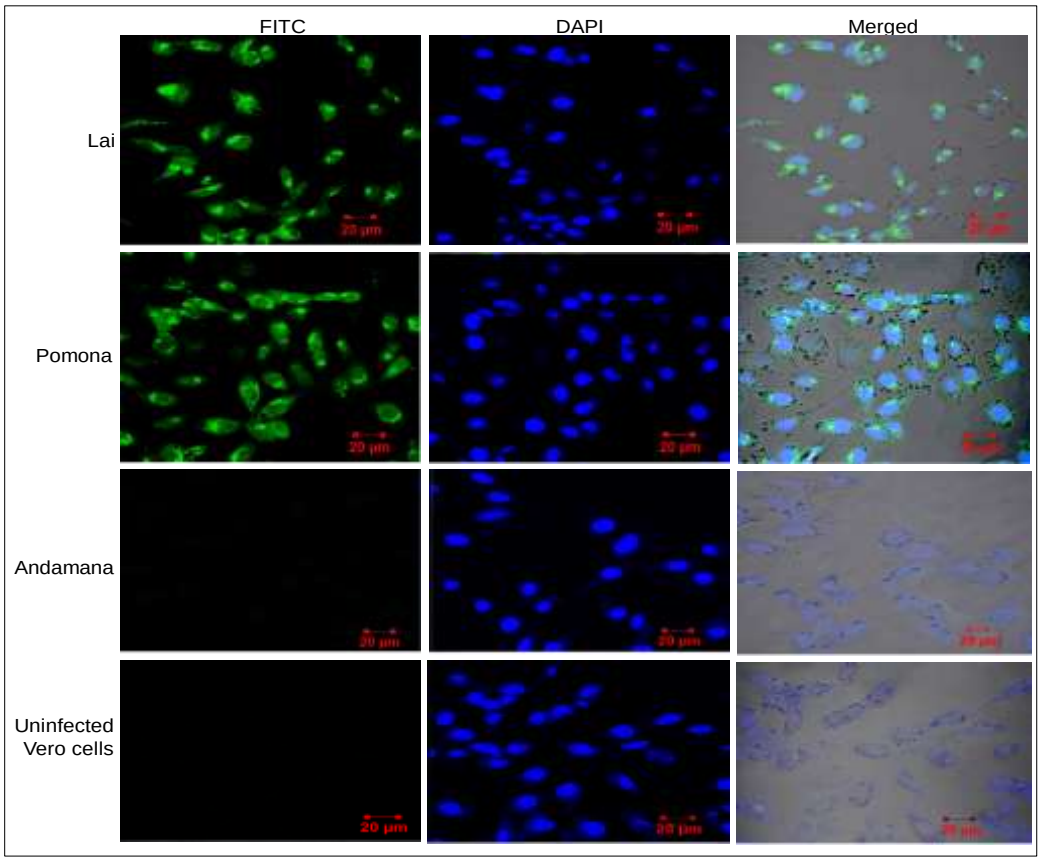


Figure 3A.6. Confocal microscopic demonstration of sphingomyelinase in Vero cells infected with pathogenic leptospires. Vero cells were infected with the salt-induced serovars Pomona, Lai and Andamana at a MOI of 1:100 for 2 h. The Vero cells were then subjected to confocal microscopy after incubating with anti-$Sph2_{163}$ antibodies for 1 h (1:250 dilution) followed by FITC-labelled anti-rabbit IgG antibodies for 1 h (1:500 dilution) of (green). DAPI (0.1 µg / mL) was used to stain the nuclei of the Vero cells. Scale bar, shown in red is 20 µm.

antibody against sphingomyelinase. The overlaid images highlighted the distribution of the sphingomyelinase-associated green fluorescence to the edges against the blue background of the stained nuclei. No fluorescence was seen in the uninfected control Vero cells and in cells infected with the non-pathogenic serovar Andamana (Fig. 3A.6).

3A.2.2. Elevated levels of *sph2* mRNA transcripts in serovar Pomona recovered from infected Vero cells

Infection of Vero cells was performed using serovar Pomona grown in the absence (set 1) and presence of 120 mM sodium chloride (set 2) in order to study the variation, if any on the levels of sphingomyelinase. Since Sph2 was considered as a true sphingomyelinase, *sph2* transcript levels were assayed in set 1, set 2 and in these two sets recovered from Vero cells upon infection (sets 3 and 4). The *sph2* transcript levels, assayed by qRT-PCR were first normalized with 16S rRNA transcripts. The relative fold changes of *sph2* mRNA of sets 2, 3 and 4 were represented in terms of set 1 (Fig. 3A.7).

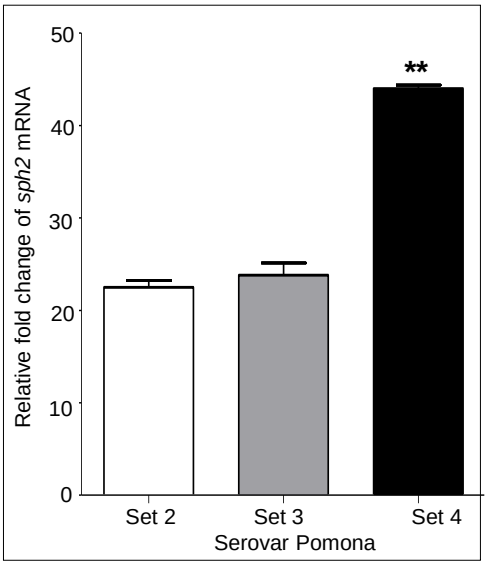


Figure 3A.7. qRT - PCR analysis of *sph2* mRNA. The transcript levels of *sph2* were measured in four sets of the serovar Pomona. Set 1 and 2 included the *in-vitro* cultured organisms that was untreated and treated with 120 mM NaCl. These two sets were used to infect Vero cells and the respective organisms recovered were referred to as sets 3 and 4. The *sph2* mRNA levels in all the four sets were normalized using 16S rRNA as control. The figure represents the fold changes in sets 2, 3 and 4 relative to set 1. The error bar represents the standard deviation of the values obtained from two different experiments, each performed in duplicate. The results were analysed by Unpaired t test and are considered as statistically significant when $p < 0.05$ (represented by **).

First, it was seen that there was a 23-fold increase in *sph2* in the *in-vitro* grown organisms upon induction with salt (set 2 *vs* set 1). As anticipated, when the salt-treated organisms were used for infecting Vero cells, there was a further increase, with a 44-fold rise seen in set 4 *vs* set 1. This clearly indicated the up-regulation of sphingomyelinase in the pathogen upon entry into the mammalian host. The host-induced expression was also seen in set 3 that was characterized by a 24-fold increase in *sph2* mRNA levels.

3A.2.3. Immunoblot analysis of leptospiral whole cell sonicate

Fig.3A.8 represents the two immunoblots developed with anti-Sph2$_{163}$ and anti-rSphCD$_{210}$ antibodies of the organisms recovered from infected Vero cells. The banding pattern was identical with both the antibodies. Interestingly, there was variation when compared to the pattern seen in organisms grown in axenic medium. An interesting observation was the 42 kDa band that was also seen in the spent growth medium (see below, Fig. 3A.9). There were two additional low molecular weight bands of 20 and 16 kDa respectively. Among the various bands seen under *in-vitro* conditions of growth, only the 90 kDa band was seen in these samples.

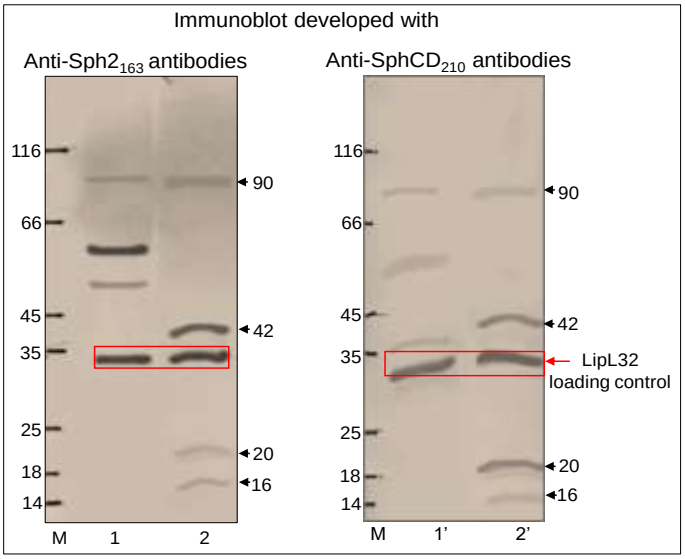


Figure 3A.8. Sphingomyelinase expression: immunoblot of serovar Pomona released from infected Vero cells. Vero cells were infected with serovar Pomona and after 2 hours, the culture medium was removed. The cell monolayers were washed with fresh medium and the cells were lysed to release the leptospires. 60 µg of the whole cell sonicate of the recovered organisms was subjected to immunoblotting with anti-Sph2$_{163}$ and anti-SphCD$_{210}$ antibodies (lanes 2 and 2'). In both the panels, serovar Pomona grown in axenic medium in the presence of sodium chloride was included (lanes 1 and 1') for comparison. The boxed region represents LipL32 used as loading control. M is the molecular weight markers.

3A.2.4. Secretion of sphingomyelinase by serovar Pomona: identification of a 42 kDa band in the growth medium

After incubation of Vero cells with salt-induced serovar Pomona for 2 h, the growth medium was collected and analyzed for sphingomyelinase. The presence of the protein was ascertained by: (i) Western blotting, (ii) assay for its biological activity by checking for haemolytic activity on RBC and (iii) assay for enzymatic activity on its substrate sphingomyelin.

3A.2.4.1. SDS-PAGE and Western blotting analysis

The growth medium of infected and uninfected (control) Vero cells were collected and concentrated by ultrafiltration using 10 kDa Amicon ultrafiltration tubes. Upon SDS-SDS-PAGE and Western blotting, a 42 kDa band from the infected sample reacted strongly with anti-SphCD$_{210}$ antibodies, which was clearly not seen in the control sample (Fig. 3A.9). This unequivocally identified the 42 kDa band as sphingomyelinase. It may be mentioned that a 42 kDa sphingomyelinase was identified in membrane vesicles of iron-limited serovar Lai in our earlier studies (Velineni *et al.*, 2009).

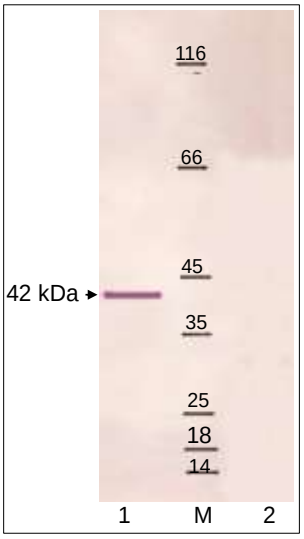


Figure 3A.9. Identification of a 42 kDa sphingomyelinase secreted upon infection of Vero cells. The growth medium of infected (lane 1) and uninfected (control; lane 2) Vero cells were concentrated by ultrafiltration and acetone precipitation, followed by solubilizing the protein pellet in solubilization buffer (10 mM Tris-HCl pH 7.2 containing 0.6% SDS). 60 µg of total protein from both samples were electrophoretically separated by SDS-PAGE on a 5-20% polyacrylamide gel and subjected to immunoblotting with anti-SphCD$_{210}$ antibodies (1:1000). M represents the molecular weight markers.

3A.2.4.2. Hemolytic activity of the culture medium of Vero cells upon infection with serovar Pomona

When sheep RBC was incubated with culture medium of infected Vero cells, 21.5% hemolysis was seen. Upon ultrafiltration, the concentrated 10-fold concentrated retentate showed 64.8% hemolysis, with no such lytic activity detected in the filtrate (Fig. 3A.10).

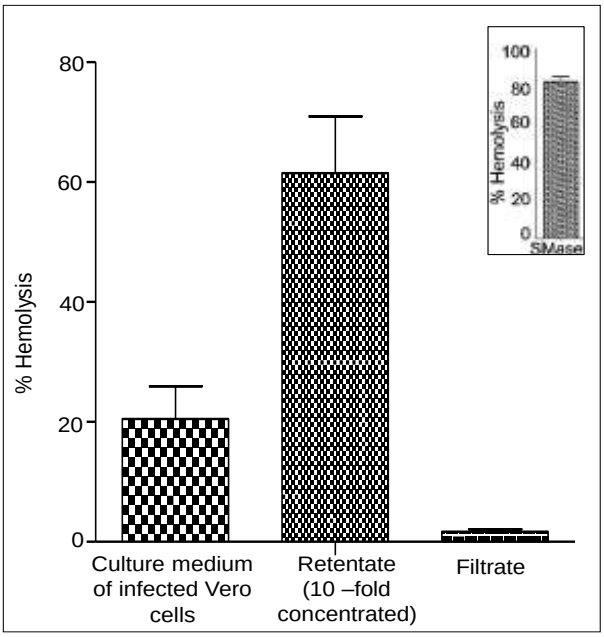


Fig. 3A.10. Hemolytic activity of the culture medium of infected Vero cells. Hemolytic assay was performed with the culture medium of infected Vero cells. Upon concentration of this medium using 10 kDa Amicon ultrafiltration tubes, both the retentate and the filtrate were tested for hemolytic activity. In all the three samples used for hemolytic assay, the total protein was 250 µg. The inset shows the hemolytic activity of Sphingomyelinase C (SMase) from *Staphylococcus aureus*. The error bars represent the standard deviation calculated from two identical experiments performed in duplicate.

3A.2.4.3. Enzymatic activity

The enzymatic activity of sphingomyelinase in the whole cell sonicate of serovar Pomona, recovered from infected Vero cells showed the release of ceramide and phosphorylcholine, as analysed by TLC. The mobility of the ceramide was identical to the commercial ceramide included in the analysis. The *in-vitro* cultured serovar Pomona was also included along with the serovars Lai and Andamana. In the two pathogenic serovars, the products were clearly seen indicating the presence of the sphingomyelinase. Nothing was seen in the lane corresponding to serovar Andamana as it does not express any sphingomyelinase (Fig. 3A.11).

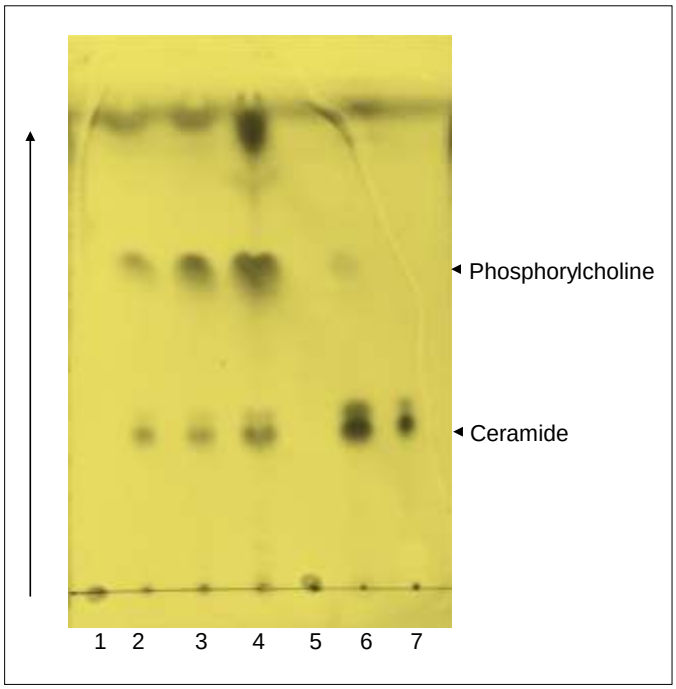


Figure 3A.11. Enzymatic activity of the leptospiral sphingomyelinase(s): identification of the reaction products by thin layer chromatography. The whole cell sonicates of serovar Pomona recovered from infected Vero cells (lane 4) and the three serovars Andamana, Lai and Pomona (lanes 1-3), grown in EMJH-sodium chloride medium were analysed for sphingomyelinase activity upon addition of the substrate sphingomyelin. After the enzymatic activity, the reaction mixture was subjected to chromatography, as detailed in Section 2.3.4.2. Pure sphingomyelin (lane 5) and ceramide (lane 7) were spotted separately to identify them in the test samples. The commercial sphingomyelinase (SMase) from *S. aureus* was included as positive control (lane 6). The arrow indicates the direction of separation of the products.

3A.3. Studies on sphingomyelinase secreted by serovar Pomona upon infection of Vero cells

In the above experiments, biologically active sphingomyelinase was demonstrated to be released into the culture medium of infected Vero cells. This was further substantiated by demonstrating cell damage upon addition of this culture medium to a fresh monolayer of Vero cells. The spent medium from infected Vero cells was filtered through 0.22 µm pore size GVWP filters (Millipore Corporation, MA, USA) and a volume equivalent to 100 ng total protein was added per mL of a fresh batch of Vero cells.

The sphingomyelinase in the spent growth medium was shown by confocal microscopy to bind the Vero cells and the cell damage was demonstrated by scanning electron microscopy.

3A.3.1. Confocal microscopy: binding of the secreted sphingomyelinase to uninfected Vero cells

A new batch of Vero cells was subjected to confocal microscopy before and after addition of the spent growth medium of infected Vero cells. Using anti-sphingomyelinase antibodies, the binding of the sphingomyelinase (in the spent growth medium of infected Vero cells) to the fresh batch of Vero cells was demonstrated. The green fluorescence intensified with time, against the background of the red propidium iodide staining of the cell nuclei (Fig. 3A.12).

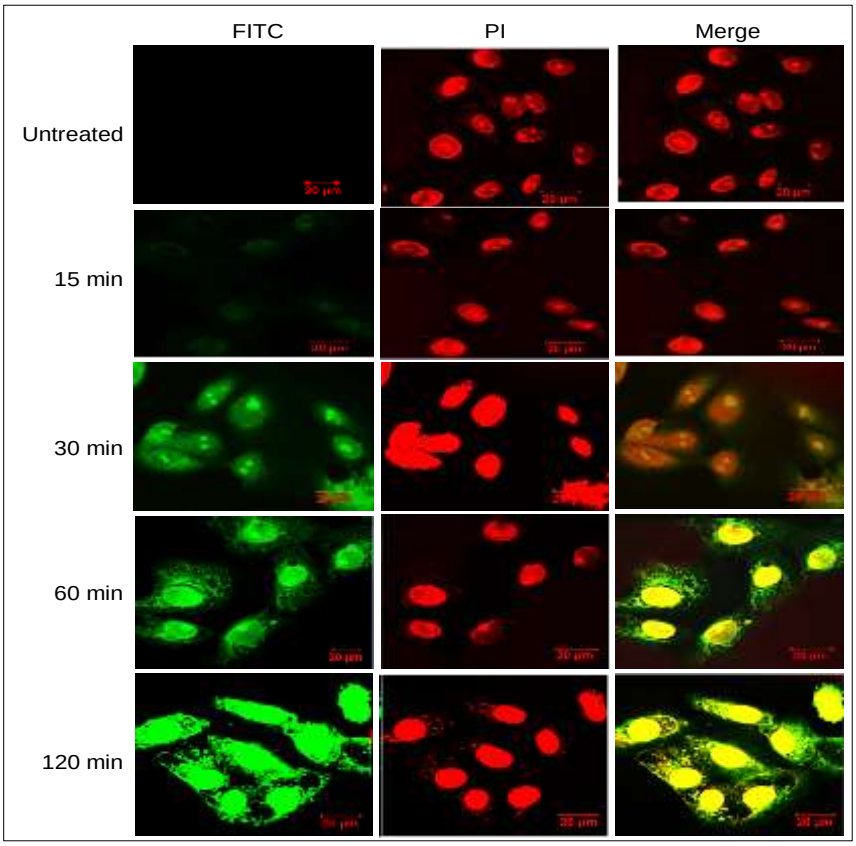


Figure 3A.12. Confocal microscopy demonstrating the binding of the sphingomyelinase in the spent growth medium to uninfected Vero cells. The total protein from the spent growth medium of Vero cells infected with live serovar Pomona was filter-sterilized. 100 ng of this was added per mL of medium was added to a new batch of uninfected Vero cells. Immuno-detection of the bound sphingomyelinase was studied at 15 min, 30 min, 60 min and 120 min upon addition. The following were used: anti-$Sph2_{163}$ antibodies (diluted 1:250), FITC-labelled anti-rabbit IgG antibodies (1:500 dilution) and propidium iodide (1 µg / mL). The latter was used to stain the nuclei by incubating the cells for 10 min. The coverslip was sealed with paraffin wax and observed under a Carl Zeiss laser confocal microscope with 590 / 617 or 535 / 617 nm wavelengths (excitation / emission) for detection of FITC / propidium iodide respectively. Scale bar 20 µm.

3A.3.2. Cytotoxic effect of the sphingomyelinase studied by phase contrast and scanning electron microscopy

Fig. 3A.13 shows the images obtained by phase contrast microscopy of Vero cells incubated with the sphingomyelinase-containing spent growth medium. Notable changes

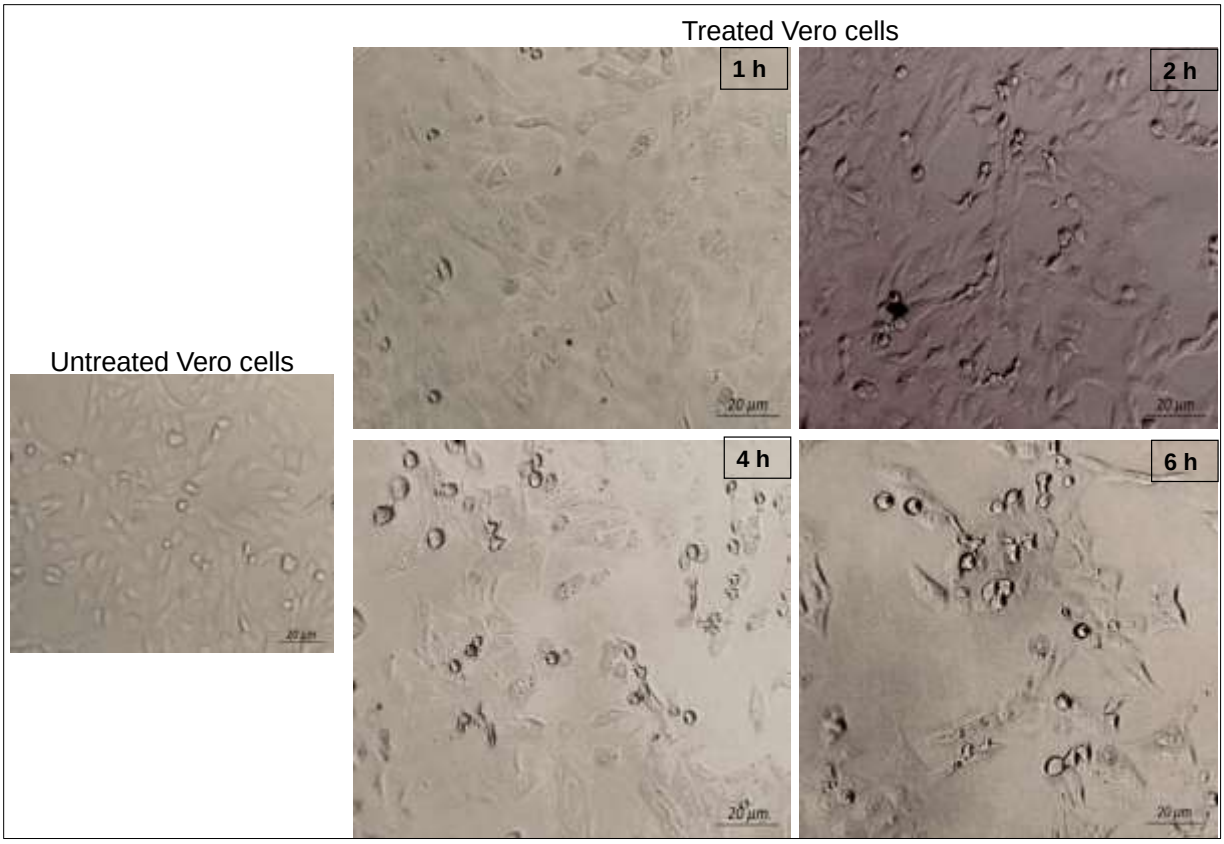


Figure 3A.13. Detachment of Vero cells upon addition of sphingomyelinase-containing spent growth medium. The cytotoxic effect of the shingomyelinase was evident on the fresh batch of Vero cells, with the cells becoming rounded and detaching from the substratum. The images were captured using the Inverted Microscope (Evos XL core, Life Technologies) at 20x magnification. Scale bar 20 µm.

in the morphology of Vero cells was observed from 2 h, with detachment and blebbing of cells observed with increasing time and at 6 h onwards, about 80% of cells were released from substratum and became rounded.

The pore inducing property of the sphingomyelinase was evident from the scanning electron micrographs presenting with significant alteration in the cell surface of Vero cells incubated with the spent growth medium containing sphingomyelinase (Fig. 3A.14).

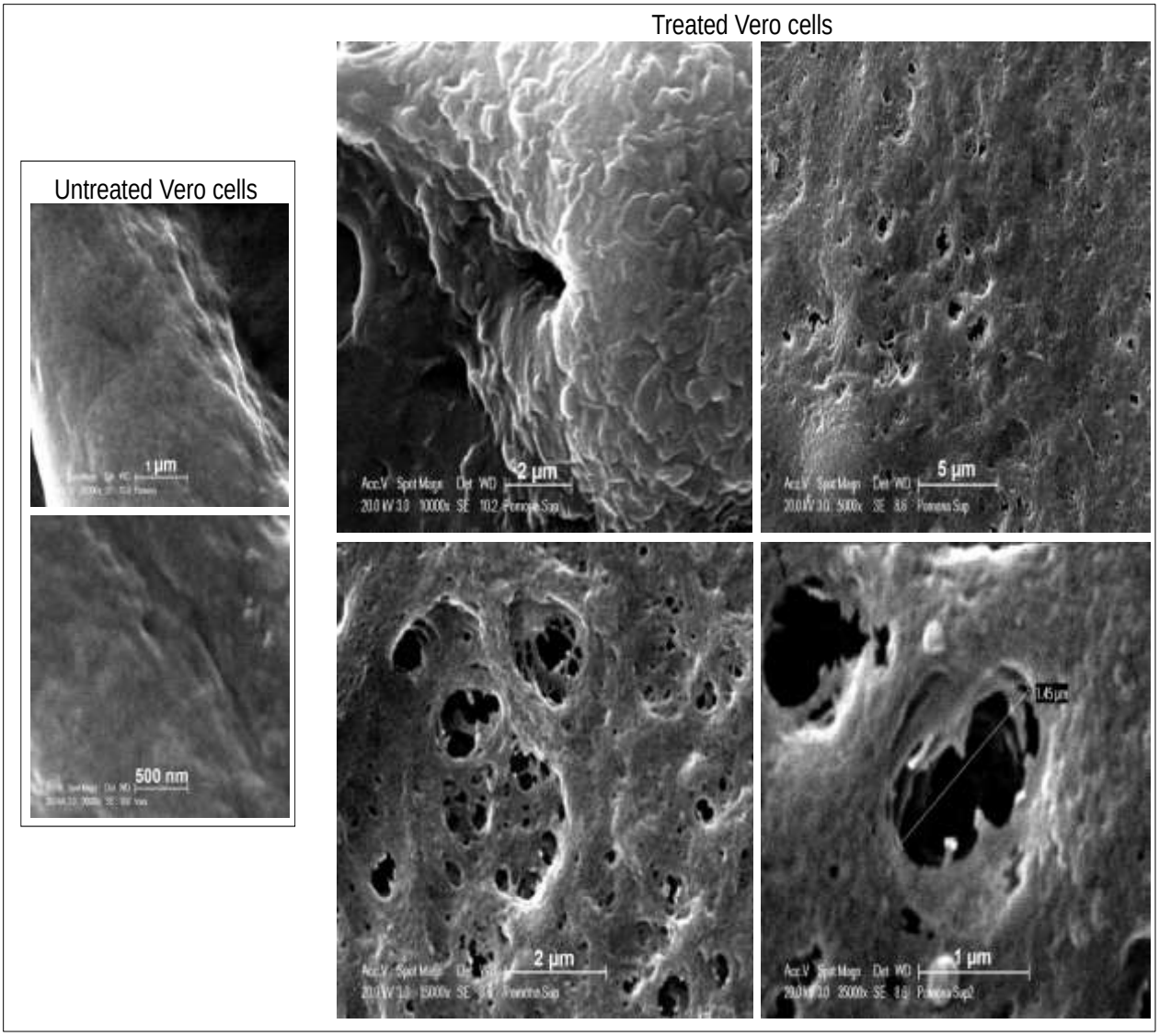


Figure 3A.14. Scanning electron micrographs of Vero cells subjected to treatment with sphingomyelinase-containing spent growth medium. The scanning electron micrographs clearly show pore formation on the Vero cells upon incubation with spent growth medium for 2 h. Image was taken using scanning electron microscope at magnification under 2500x to 50000x.

3A.4. Direct demonstration of the effect of purified sphingomyelinase on Vero cells

3A.4.1. Expression and purification of rSphCD$_{210}$ and rSph4

The recombinant clones expressing the two sphingomyelinases are available as laboratory collection. Though our main objective was to purify rSphCD$_{210}$ containing the exo-endophosphatase domain in order to demonstrate its cytotoxic effect on the Vero cells, rSph4 protein was included as a control, as it lacked the enzymatic domain. Fig.

3A.15. shows the IPTG-induced profile and the purified protein from SDS-PAGE and immunoblotting analysis.

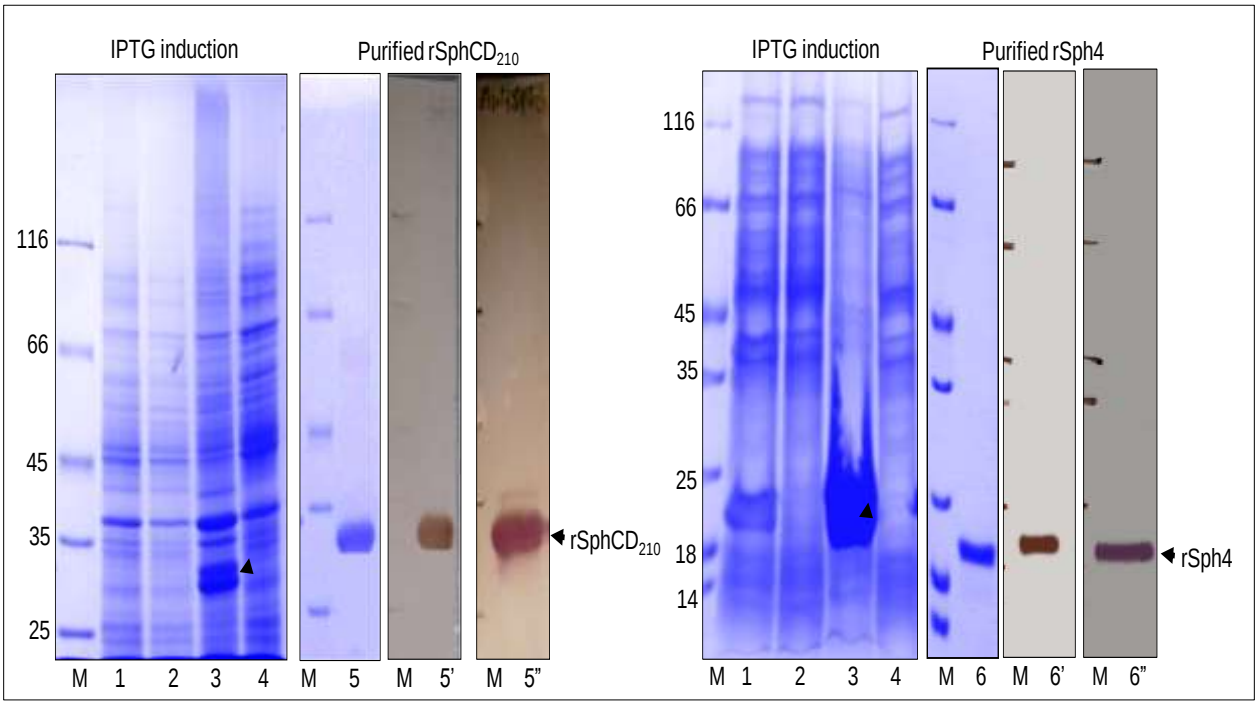


Figure. 3A.15. Purification of rSphCD$_{210}$ and rSph4. The figure shows the induction of the respective recombinant proteins by IPTG, with lanes 1 and 2 representing the pellet and supernatant of the un-induced clone and lanes 3 and 4 representing the pellet and supernatant upon induction with IPTG. Both the proteins were purified by affinity chromatography as detailed under Materials and Methods. The purified rSphCD$_{210}$ is shown in lanes 5 (SDS-PAGE), 5' and 5" (immunoblot developed with anti-His antibodies and anti-SphCD$_{210}$ antibodies respectively. The purified rSph4 is shown in the corresponding lanes 6, 6' and 6". M represents the molecular weight markers.

3A.4.2. Demonstration of the binding of rSphCD$_{210}$ to Vero cells by confocal microscopy

The purified rSphCD$_{210}$ bound to the cell surface of Vero cells, evident from the green fluorescence seen upon incubation with anti-rSphCD$_{210}$ antibodies. There was no signal in untreated Vero cells and in cells incubated with pre-immune serum. The nuclei in all the cells can be clearly seen from the red fluorescence of propidium iodide.

Changes in cell morphology due to addition of the sphingomyelinase is evident from the images (Fig.3A.16).

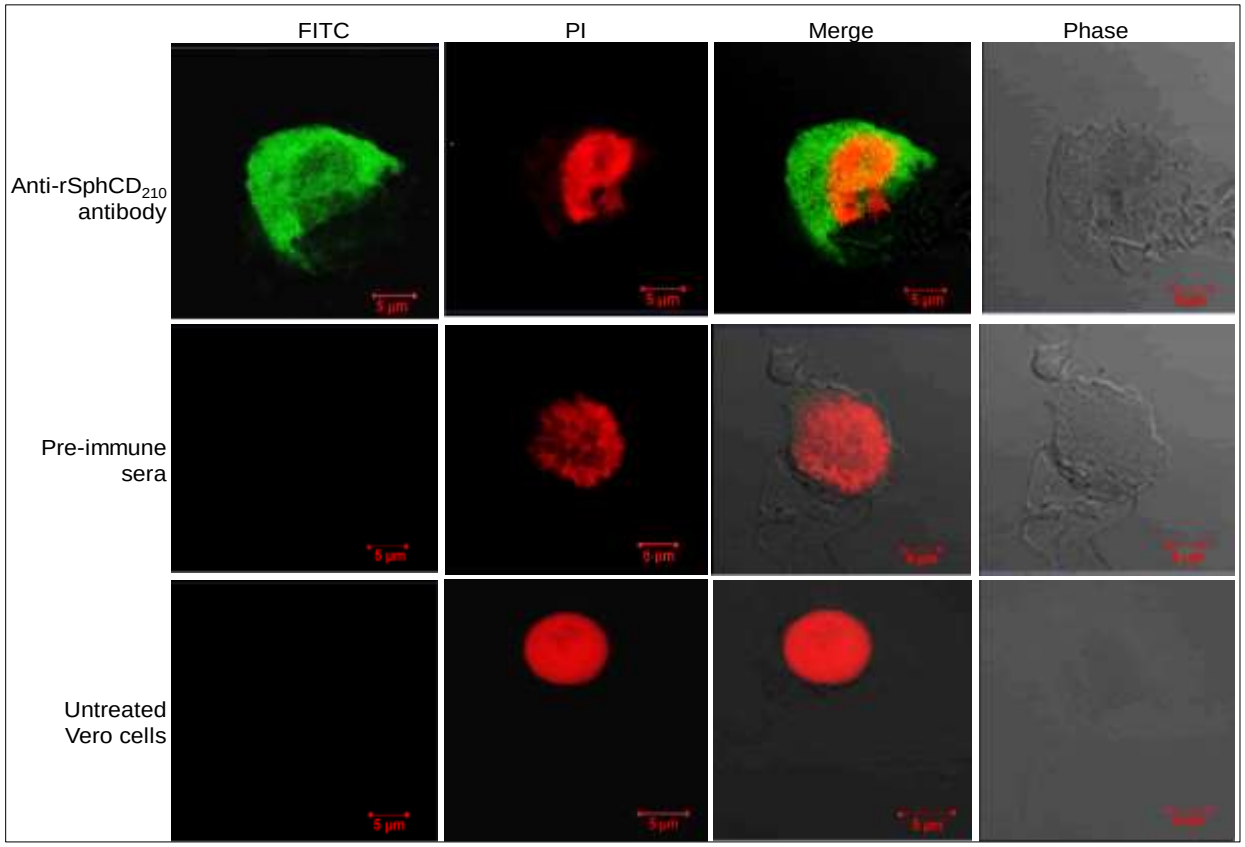


Figure 3A.16. Confocal microscopy of rSphCD$_{210}$ treated Vero cells. Identical preparation of Vero cells treated with rSphCD$_{210}$ were subjected to incubation with pre-immune serum and anti-SphCD$_{210}$ antibodies (1:250 dilution), followed by incubation with FITC-labelled goat anti-rabbit IgG (1:500). Nucleus were stained with 1 µg / mL propidium iodide for 10 min. The coverslip was sealed with paraffin wax and observed under a Carl Zeiss laser confocal microscope with 590 / 617 or 535 / 617 nm wavelengths (excitation/emission) for FITC or propidium iodide detection respectively at 63x magnification with oil immersion. Scale bar 5 µm.

3A.4.3. Cytotoxicity of rSphCD$_{210}$ on Vero cells

Time-dependent changes in the surface morphology of Vero cell upon addition of rSphCD$_{210}$ protein was examined (Fig.3A.17). Significant alteration in the morphology was observed after 30 min (Fig.3A.17a) that increased further, with formation of pores on cell surface that were clearly visible at 2 h (Fig.3A.17c and d). These changes were not seen in the untreated cells and in the cells treated with rSph4, lacking the enzymatic activity (Fig.3A.17e & f). Image was captured under 2500x to 20000x magnification. In the presence of the antibodies against rSphCD$_{210}$, pore formation was not seen

implicating sphingomyelinase as the factor effecting these surface changes and thereby causing cell damage (Fig. 3A.17g).

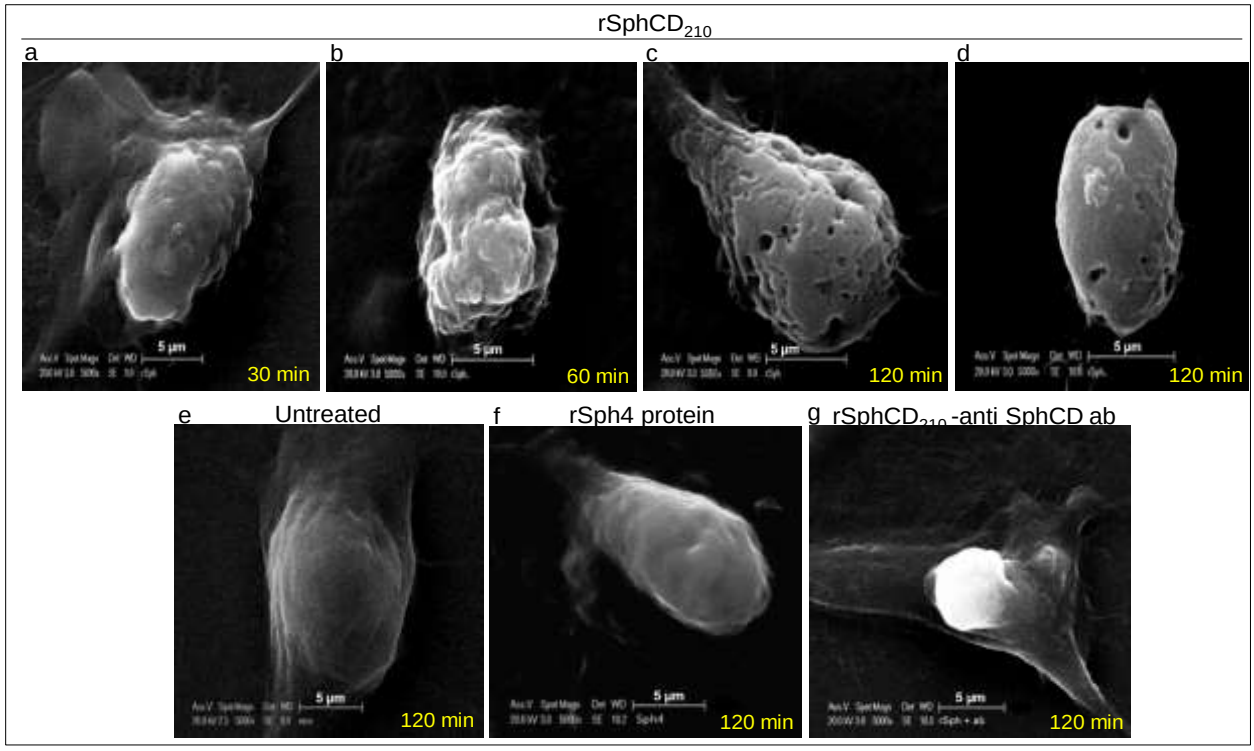


Figure 3A.17. rSphCD$_{210}$ - mediated pore formation on Vero cells. Panels 'a' to 'd' shows scanning electron micrographs of Vero cells added with 5 µg / mL of rSphCD$_{210}$ for 30 min, 60 min and 120 min respectively. Panel e represents untreated Vero cells. 5 µg / mL of rSph4 was added to Vero cells for 2 h shown in Panel f. 5 µg / mL of rSphCD$_{210}$ were pre-incubated with equal volume of anti-SphCD$_{210}$ antibody for 1 h at 37°C followed by addition to Vero cells for 2 h shown in Panel g. Images were captured under scanning electron microscope (Philips FEI, XL30, USA) at 5000x magnifications. Scale bar 5 µm.

3B. Diagnostic potential of sphingomyelinase and the hemin-binding protein HbpA in the screening for leptospirosis

Sphingomyelinases are virulence factors expressed by pathogenic *Leptospira.* Their biological effect on mammalian cells has been established in our previous chapter. Analysis of its expression *in-vivo* and understanding of its diagnostic and vaccine potential will help in the development of better control measures. In this study, the diagnostic implications of its presence in leptospirosis patients was studied. In addition, the iron - regulated hemin-binding protein HbpA, another pathogen - specific leptospiral protein (Asuthkar *et al.*, 2007), which was well-studied in our lab as a diagnostic marker for the

screening of leptospirosis (Sridhar *et al.*, 2008; Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012), was studied further. Earlier studies were performed with a 55 kDa fragment, encoding 482 aa of the C-terminal end whereas here, a smaller fragment of 34 kDa containing 240 aa of the C-terminal was selected based on B cell epitope mapping. Antibodies against $HbpA_{34}$ as well as sphingomyelinase were analysed in the first clinical study consisting of 190 study subjects. In the second study, consisting of a smaller group of 40 subjects, including patients with leptospirosis and dengue, leptospiral antigens were additionally demonstrated in the urine samples of these patients, highlighting the potential of the study in differential diagnosis of leptospirosis.

3B.1. Screening of antibodies against $HbpA_{34}$ and sphingomyelinase in the serum of leptospirosis patients – Clinical study I

3B.1.1. HbpA as a diagnostic antigen

3B.1.1.1. Identification of the antigenic determinant of HbpA

The region in the C-terminal end of HbpA was subjected to B-cell epitope mapping using a public domain software (http://bips.u-strasbg.fr/EMBOSS/). This was performed to specifically identify those regions within the outer loop, which are potential epitopes for humoral immune response. A 240 aa region, containing aa 470 – 710 was chosen (Fig.3B.1).

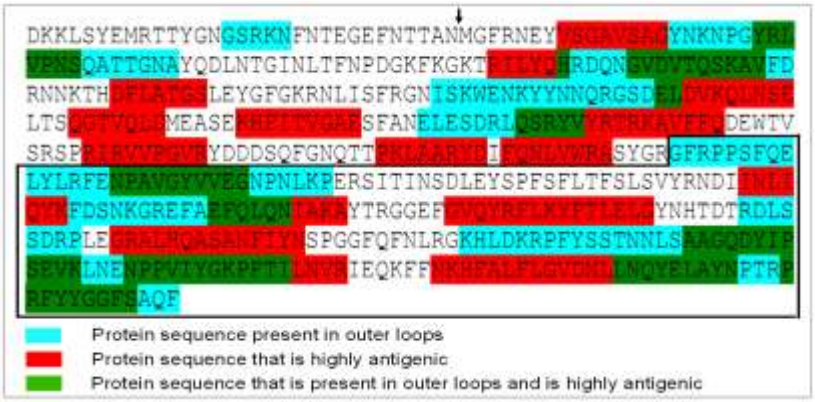


Figure 3B.1. B-cell epitope mapping of $HbpA_{34}$. The figure represents the region of HbpA with outer loop sequences. The region in blue represents five outer loops and the regions highlighted in red and green colour show nine potential B-cell epitopes. The six regions in blue and green colour, representing the B-cell epitopes present in the outer loop were considered for selection of the 240 aa fragment $HbpA_{34}$, shown as a boxed region. The arrow indicates the amino acid from which the entire stretch of the C-terminal region was chosen for cloning the 55 kDa fragment used earlier.

3B.1.1.2. Cloning and expression of rHbpA$_{34}$

The 720 bp *hbpA*$_{34}$ was cloned into pET28a (+) using conventional techniques and the recombinant plasmid pMS508 was generated (Fig. 3B.2). This was confirmed by demonstrating its presence upon double digestion and by sequence analysis (Appendix II, B1). The recombinant protein was expressed as an insoluble protein upon induction with 1 mM IPTG. The protein was purified to homogeneity by AKTA Prime Plus, as demonstrated by a single band seen in SDS-PAGE and in the immunoblots developed with anti-His and anti-HbpA$_{55}$ antibodies.

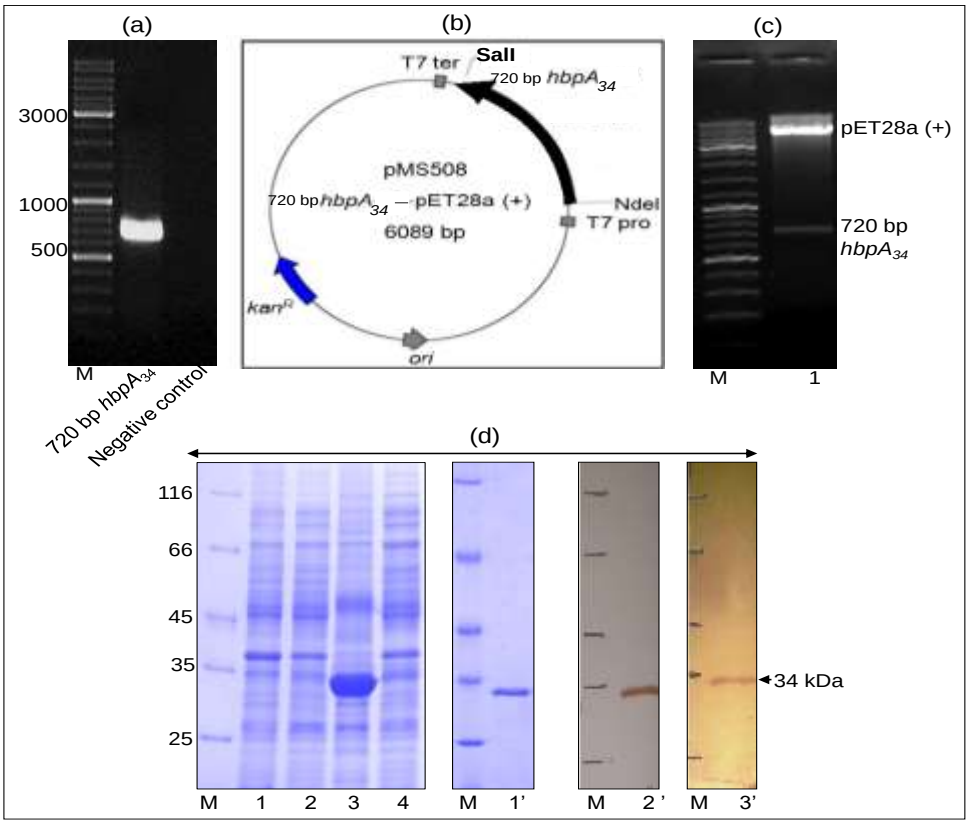


Figure 3B.2. Cloning, expression and purification of rHbpA$_{34}$. Panel (a) shows the 720 bp *hbpA* amplicon (lane 1) cloned into pET28a (+) vector to generate recombinant clone pMS508 (Panel b); lane 2 is negative control. Lane 1 in Panel (c) shows the release of the *hbpA* amplicon upon digestion of the recombinant plasmid with *Nde*I and *Sal*I enzymes. The expression of rHbpA$_{34}$ is represented in Panel (d), with lanes 1 & 3 loaded with uninduced and induced pellets and lanes 2 & 4 with uninduced supernatant and induced supernatant respectively. The rHbpA$_{34}$ was purified using AKTA Prime Plus and checked for purity. A single band (lane 1') can be seen in the Coomassie stained gel upon electrophoresis in a 10% gel upon SDS-PAGE. This band was recognized by anti-His (lane 2') and anti-HbpA$_{55}$ antibodies (lane 3'). M is the molecular weight marker.

3B.1.1.3. Performance of rHbpA$_{34}$ as an antigen in ELISA: comparison with MAT and PanBio ELISA in the screening for leptospirosis

A total of 190 serum samples were screened by HbpA$_{34}$-ELISA with MAT as gold standard and the commercially available PanBio ELISA kit. Among them, 130 samples were from patients suspected of leptospirosis, with the remaining 60 samples obtained from endemic normal subjects who served as controls. The patient group was further divided based on MAT positivity, with most of them testing MAT positive (Table 3B.1).

Table 3B.1. Grouping of study subjects based on MAT analysis

Study subjects			
Group	**Subjects (n = 190)**	**MAT**	**Criteria for inclusion**
I	108	Positive	Symptoms of leptospirosis
II	22	Negative	Symptoms of leptospirosis
III	60	Negative	Endemic controls

In the MAT-positive Group I, the predominant serogroup was Icterohaemorrhagiae (42%) with Autumnalis and Australis comprising 7.6% and 7% respectively. It is increasingly reported, including our own findings that MAT, though highly specific may fail to identify a positive sample due to omission of an infecting serovar in the reference panel of serovars (Surujballi & Mallory, 2004; Sivakolundu *et al.*, 2012). Since our interest was to develop an ELISA-based test for screening, we evaluated earlier the performance of HbpA$_{55}$, a 55 kDa fragment carrying the C-terminal region of HbpA. In order to increase the specificity, we cloned and expressed this 34 kDa HbpA fragment (rHbpA$_{34}$) and tested its potential in the screening of samples with leptospirosis. Fig. 3B.3 shows high titre of anti-HbpA antibodies in serum of Group I (n = 102) patients by ELISA which was statistically significant ($p < 0.001$) when compared to the endemic normals (Group III). Nine of the MAT negative cases in Group II tested positive for leptospirosis, who presented clinical symptoms of the disease. While the HbpA$_{34}$ ELISA could not identify 6 of the MAT positive samples in Group I.

Forty eight samples were not detected with the commercial PanBio ELISA (Table 3B.2). The statistical analysis and comparative evaluation of the various tests is discussed below

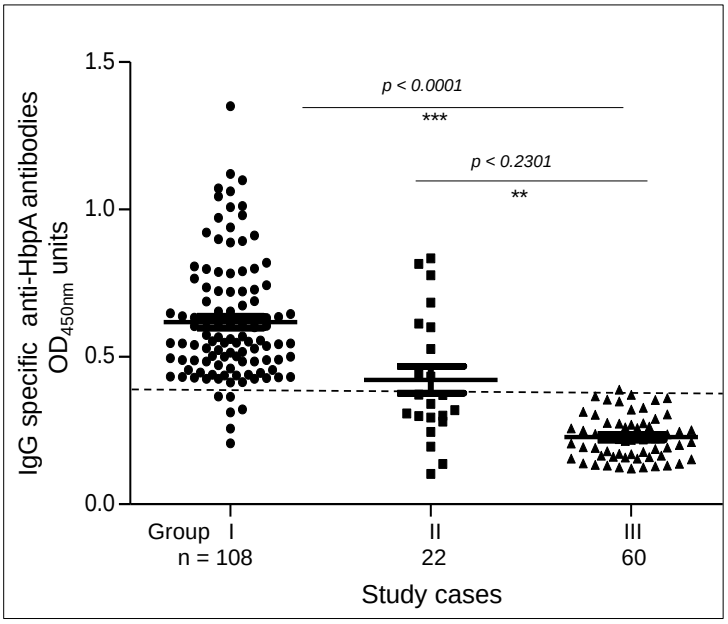


Figure 3B.3. ELISA based detection of anti-HbpA antibodies in patients with leptospirosis. The study subjects included the MAT positive (Group I) and negative (Group II) patients suspected of leptospirosis, with the endemic normals forming the Group III. The figure shows the levels of IgG-specific antibodies against rHbpA$_{34}$. The horizontal line indicates the mean absorbance value in each group. The dotted line represents the cut off value, calculated as detailed under Materials and Methods. Data are presented as mean ± SD of two independent experiments. The results were analyzed by non-parametric, Mann Whitney test and considered significant when $p < 0.0001$.

Table 3B.2. Evaluation of rHbpA$_{34}$-ELISA and PanBio IgM ELISA *vs* MAT

Tests	**MAT**			
rHbpA$_{34}$ ELISA		**(+)**	**(-)**	**Total**
	(+)	102	9	111
	(-)	6	73	79
	Total	108	82	**190**
		(+)	**(-)**	**Total**
PanBio ELISA	**(+)**	60	4	64
	(-)	48	78	126
	Total	108	82	**190**

3B.1.1.4. $HbpA_{34}$ – based lateral flow device for the screening of leptospirosis

Lateral flow device was prepared by coating $rHbpA_{34}$ as antigen (done at GENOMIX Molecular Diagnostics Pvt. Ltd, (Hyderabad, India), along with the control antigen (standardized by the company). Figure 3B.4 shows representative devices developed with patients' serum. Of the 20 samples tested, 12 were positive samples. Table 3B.3 shows the performance in comparison with MAT and $rHbpA_{34}$-ELISA.

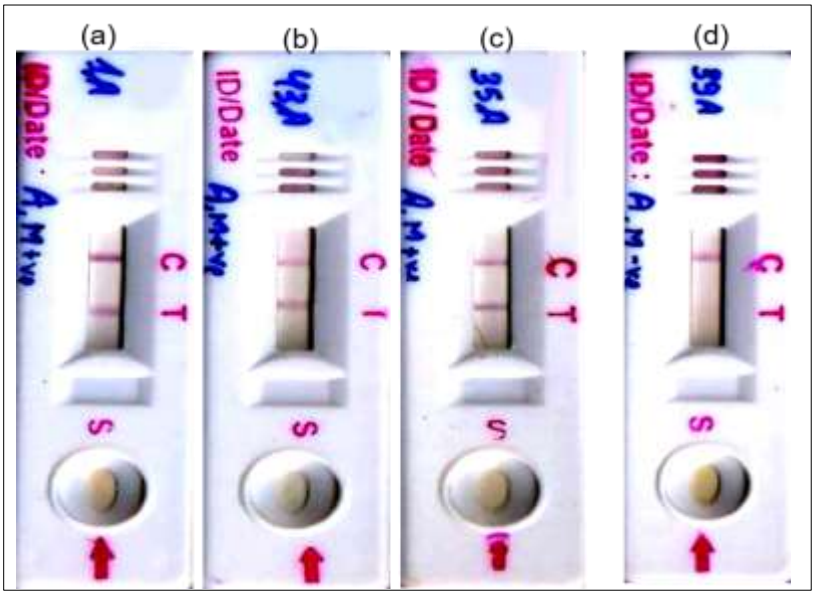


Figure 3B.4. Screening of serum samples with the $rHbpA_{34}$ - based lateral flow device. The figure includes three representative serum samples that tested positive with the lateral flow device (a, b & c) and a negative sample (d). T is the test band indicating the detection of anti-HbpA antibodies in the serum and C represents the control. 'S' represents the sample application pad.

Table. 3B.3. Performance of lateral flow vs MAT and ELISA

Test	**MAT**				**$rHbpA_{34}$-ELISA**			
		(+)	**(-)**	**Total**		**(+)**	**(-)**	**Total**
Lateral Flow	**(+)**	12	0	12	**(+)**	12	0	12
	(-)	1	7	8	**(-)**	2	6	8
	Total	13	7	**20**	**Total**	14	6	**20**

3B.1.2. Screening of serum samples for anti-sphingomyelinase antibodies

A total of 131 serum samples from the above three groups (Group I, n = 81; Group II, n = 20 and Group III, n = 30) were screened for anti-sphingomyelinase antibodies using $rSphCD_{210}$ as antigen; rSph4 was used as an additional control. LipL41, established as a diagnostic antigen was also included for comparing all the ELISA based tests against MAT. Most of the MAT-positive cases in Group I showed high levels of antibodies against $rSphCD_{210}$ and rLipL41 (Fig. 3B.5) that were statistically significant ($p < 0.001$) when compared to the Group III endemic normals. Some of the MAT negative Group II cases

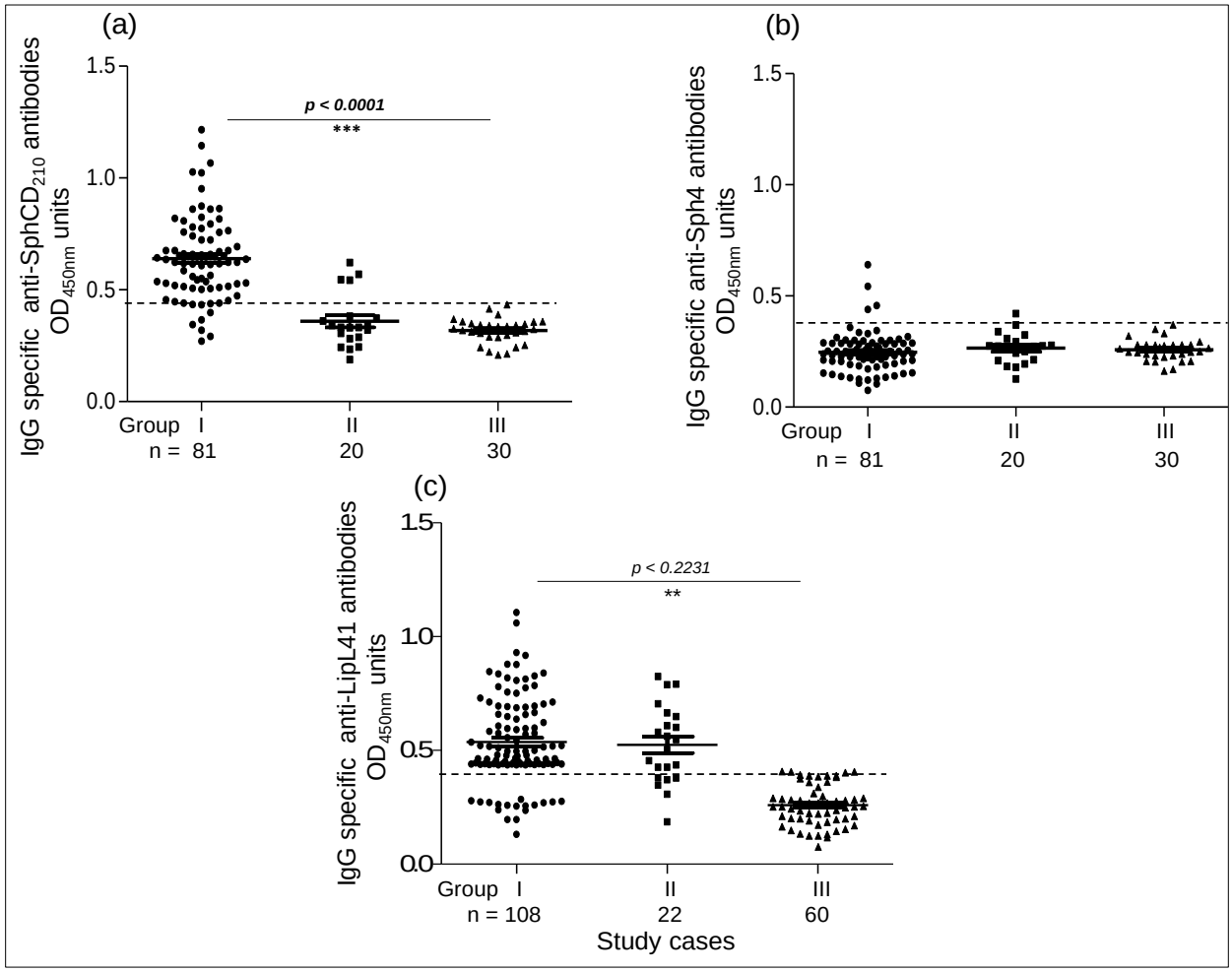


Figure 3B.5. ELISA-based detection of serum antibodies against sphingomyelinase and LipL41. Panels (a to c) shows titres of antibodies against $rSphCD_{210}$, rSph4 antibodies and rLipL41 in the different study subjects respectively. The horizontal line indicates the mean absorbance value in each group. The dotted line represents the cut-off value calculated as mentioned under Materials and Methods. Data are presented as mean ± SD of two independent experiments. The results were analyzed by non-parametric, Mann Whitney test and considered significant when $p < 0.0001$.

were above the cut-off value. The titre was low against rSph4 (lacking exo-endo phosphatase domain), an anticipated finding. As done earlier, the performance of these antigens was correlated with MAT (Table 3B.4).

Table 3B.4. Evaluation of rSphCD$_{210}$-ELISA, rSph4-ELISA and LipL41-ELISA vs MAT

Table 3B.4 showing the performance of ELISA vs MAT

Tests	**MAT**			
rSphCD$_{210}$-ELISA		**(+)**	**(-)**	**Total**
	(+)	75	5	80
	(-)	6	45	51
	Total	81	50	**131**
rSph4-ELISA		(+)	(-)	**Total**
	(+)	4	3	7
	(-)	77	47	124
	Total	81	50	**131**
rLipL41-ELISA		(+)	(-)	**Total**
	(+)	91	20	111
	(-)	17	62	79
	Total	108	82	**190**

Statistical analysis of the various ELISA tests performed with different antigens (Table 3B.5) made possible a comparison of the performance of each of these antigens. While maximal specificity was seen with the commercial PanBio ELISA (95%), both rSphCD$_{210}$ and rHbpA$_{34}$ were promising candidates, with specificity of 90 and 89% respectively; least specificity of 76% was seen with rLipL41. The in-house antigens rSphCD$_{210}$ and rHbpA$_{34}$ showed sensitivity of 92 and 95%, with the least sensitivity of 56% seen with PanBio ELISA.

Table 3B.5 Statistical analysis of ELISA tests performed with different leptospiral antigens

Antigens used in ELISA	MAT			
	Sensitivity (95% CI)	Specificity (95% CI)	Predictive values	
			Positive (95% CI)	Negative (95% CI)
rSphCD$_{210}$	92 (85.56 - 97.21)	90 (78.17 - 96.63)	94 (86.00 - 97.92)	88 (76.12 - 95.53)
rSph4	5 (1.39 - 12.18)	94 (83.43 - 98.68)	57 (18.75 - 89.58)	38 (29.35 - 47.05)
rHbpA$_{34}$	95 (88.30 - 97.93)	89 (80.18 - 94.86)	91 (85.17 - 96.23)	92 (84.20 - 97.16)
PanBio	56 (45.68 - 65.12)	95 (87.98 - 98.66)	94 (84.76 - 98.27)	62 (52.83 - 70.41)
rLipL41	84 (76.00 - 90.55)	76 (64.88 - 84.42)	82 (73.55 - 88.63)	78 (67.80 - 86.94)

3B.2. Diagnostic potential of HbpA and sphingomyelinase in the differential diagnosis of leptospirosis from dengue fever – Clinical study II

40 patients from M.O.S.C. hospital in Kolenchery, Kerala were included in this study. They were subjected to routine clinical examination and laboratory testing that included complete blood profile and laboratory investigations. All these patients showed clinical symptoms of fever and myalgia with some of them showing involvement of liver, lungs and kidneys (Table 3B.6).

Table 3B.6. Clinical and biochemical parameters of patients with symptoms of leptospirosis

No.	Age	Sex	Clinical symptoms (in addition to fever and myalgia)	Urine albumin	Hb g / dL	TC	ESR mm / h	Platelet count	sGOT	sGPT	Blood Urea mg / dl	Serum Creatinine mg /dL
UH1	46	M	Liver and renal involvement	Nil	15.3	3100	9	225000	ND	33	ND	1
UH2	40	F	Severe symptoms, shock hypotension, cardiac & lungs damage (++), no involvement of liver and kidneys	+	7.8	5800	50	32000	57	27	31	2.1
UH3	51	F	Diarrhoea, low blood pressure	Trace	13.1	11900	39	218000	ND	52	33	1.9
UH4	51	F	Liver involvement	Trace	12	4400	56	80000	42	26	12	1
UH5	49	M	Diarrhoea, alcoholic renal calculus	Trace	11.8	12300	53	110000	37	39	78	2.4
UH6	41	F	Anemia, liver involvement	Nil	7.4	7400	60	150000	105	102	27	1
UH7	55	M	Alcoholic liver damage	+	14.4	5700	56	259000	92	12	ND	0.9
UH8	21	M	Renal damage, pancreatitis	+	8.8	11100	45	30000	78	56	68	3
UH9	38	M	Mild kidney damage	Trace	13.3	7000	33	147000	ND	41	ND	1.2
UH10	17	M	Joint pains	+	14.3	10100	26	232000	ND	37	ND	1
UH11	38	F	Joint pains, ↓ Ca^{2+} & Mg^{2+}	+	11	8800	79	42000	63	107	93	1.9
UH12	50	F	Mild diabetes	Trace	11.3	12400	40	75000	44	93	59	1.7
UH13	32	M	Cough, vomiting	Nil	14.7	15000	6	275000	ND	123	ND	0.9
UH14	85	F	Chills	Nil	11.6	8100	19	209000	ND	108	ND	3.7

No.	Age	Sex	Clinical symptoms (in addition to fever and myalgia)	Urine albumin	Hb g / dL	TC	ESR mm / h	Platelet count	sGOT	sGPT	Blood Urea mg / dL	Serum Creatinine mg /dL
UH15	31	M	Viral infection	Nil	15.4	7100	18	18000	ND	93	ND	1
UH16	54	M	Hepatitis B with leptospirosis	Nil	14.1	7500	40	104000	123	1726	ND	1
UH17	27	M	Prior history of dengue (2 months	++	16.7	15400	19	30000	ND	106	ND	1
UH18	44	M	Renal failure	Trace	12.2	9800	62	50000	63	87	204	6.8
UH19	31	M	Typhoid	+	16.4	5100	16	109000	63	75	ND	1.1
UH20	54	M	Diarrhoea, history of diabetes	++	15.3	16200	2	10600	609	547	34	1.7
UH21	70	M	Bronchial asthma, diabetes	++	12.5	5300	43	122000	39	42	ND	1
UH22	55	M	Alcoholism, diabetes	Nil	13.4	7600	39	153000	63	91	14	0.1
UH23	39	M	Cramps and body aches	Nil	20.5	12500	1	30000	42	76	ND	0.8
UH24	16	M	Fever, myalgia	Trace	11.4	9000	23	150000	ND	30	ND	1.1
UH25	29	M	Mild liver and renal damage	Nil	13.8	7800	4	153000	ND	39	ND	1.2
UH26	63	M	Mild liver and renal damage, loose stool	Trace	11.9	9700	64	184000	69	64	109	4.2
UH27	47	M	Vascular headache, diabetes	++	14.3	13000	58	139000	169	192	54	2.4
UH28	34	F	Old paraparesis, vomiting, ascitis,	Nil	11.4	6700	16	176000	ND	36	17	0.8
UH29	17	M	Vomiting, headache, weakly reactive for Dengue NSI Ag	Nil	14	3600	4	60000	460	136	17	0.9

Note:

- UH1 – UH23: Group I patients diagnosed as leptospirosis; they tested MAT positive

- UH24-UH29: Group II patients diagnosed as PUO; they were MAT negative
- Hb – hemoglobin; TC – total leucocyte count; ESR – erythrocyte sedimentation rate; sGOT – serum Glutamate oxaloacetate transaminase; sGPT – serum glutamate pyruvate transaminase
- Normal values: Hb: 12 -15 g / dL; TC: 4000 – 10,000 / cu.mm; ESR: ≤ 15 mm / hour; Platelet count: 1,50,0000 – 4,50,000 / cu.mm, sGOT & sGPT ≤ 40 U / mL; Blood urea: 20 – 40 mg / dL; creatinine: 0.5 -1.2 mg / dL
- ND: not determined.

3B.2.1. Study subjects

The study subjects were grouped based on clinical examination and MAT analysis (Table 3B.6). Serum and urine from these patients were used for the demonstration of anti-leptospiral antibodies and antigens respectively. Group I patients (n = 23) were confirmed cases of leptospirosis based on MAT and clinical correlation. 6 patients who were clinically indicative of leptospirosis but were MAT negative were included in Group II and referred to as PUO (pyrexia of unknown origin). 11 patients, who tested MAT negative were confirmed as suffering from dengue based on clinical diagnosis, platelet count and positivity using commercial kit (Dengue Day I test by J. Mitra and Co., India) for Dengue NS1 antigen and IgM & IgG antibodies in serum; these patients were included in Group II.

Both serum and urine samples were subjected to screening in this study. As mentioned, all the serum samples were tested for MAT and the major infecting serovar was Lai (22.5%), with the other serovars being Pomona (12.5%), Hardjobovis (7.5%), Celledoni (5%), Tarassovi (5%), Australis and Bankinang (2.5%). The titres were high in some of these patients. As mentioned below, serum was screened for IgM-specific anti-leptospiral antibodies using whole cell sonicates of pathogenic and non-pathogenic serovars. Urine was screened both for anti-leptospiral antibodies as well as leptospiral antigens based on the hypotheses that a) not only albumin could be detected due to renal damage but also antibodies and b) the shedding of leptospires from an infected individual could result in the detection of pathogen-specific antigens of diagnostic potential.

3B.2.2. Serum of Group I leptospirosis patients showed high levels of IgM-specific anti-leptospiral antibodies

It can be seen from Fig. 3B.6 that serum of Group I patients showed high levels of antibodies against whole cell sonicates of the pathogenic serovars. It was evident that the

dengue patients tested negative, clearly identifying the Group I as cases of leptospirosis. It may be pointed out that two PUO Group II patients (UH26 and UH27) showed an increase, though small in the titre of anti-leptospiral antibodies.

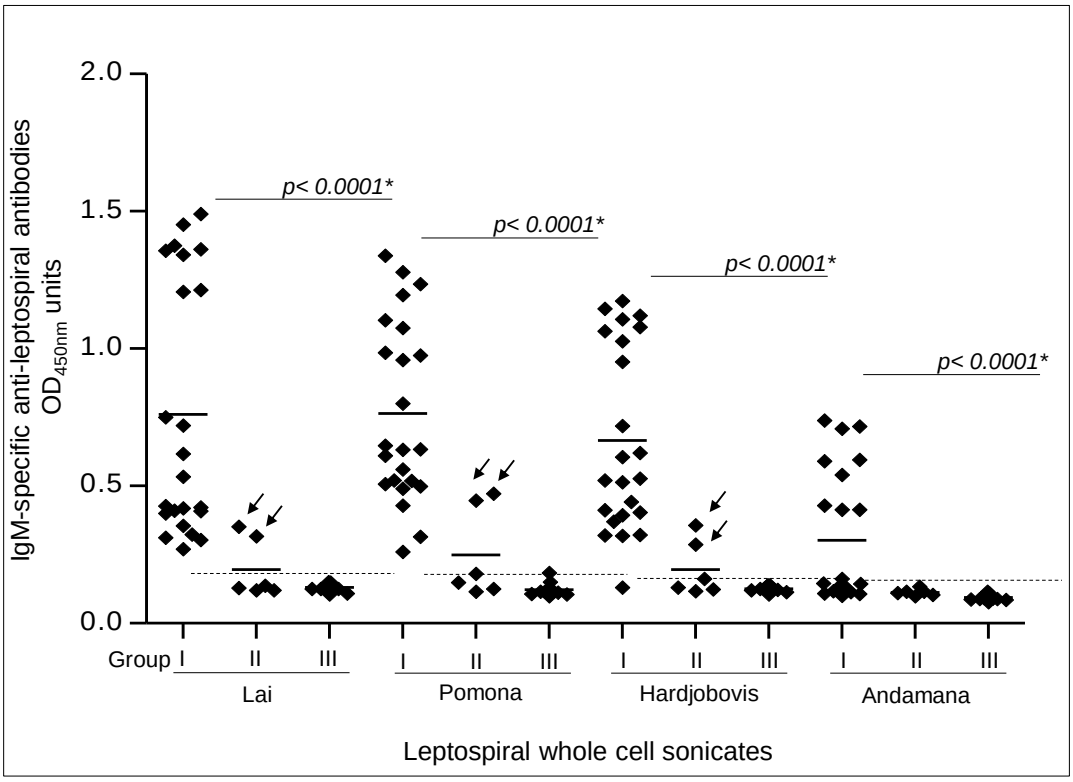


Figure 3B.6. High levels of IgM-specific antibodies against whole cell sonicates of pathogenic *Leptospira* spp. in the serum of Group I patients. Figure shows the titre of IgM antibodies against leptospiral whole cell sonicates of pathogenic serovars Lai, Pomona, Hardjobovis and the non-pathogenic serovar Andamana in Group I (MAT positive), Group II (MAT negative) and Group III (Dengue) patients respectively. The dotted line shows the cut off value calculated as mentioned in Materials and Methods. The arrows represent the two MAT negative cases that tested positive by IgM ELISA. The horizontal line indicates the mean absorbance value in each group. Data are presented as mean ± SD of two independent experiments. The results were analysed by non-parametric Mann Whitney test and considered significant when $p < 0.0001$.

The two serum samples UH26 and UH27 were additionally screened for antibodies against the pathogen-specific LipL41 and $HbpA_{34}$ antigens (demonstrated to be of diagnostic importance in our earlier Clinical Study I). The high values, shown in Fig.3B.7 confirmed these to be of leptospiral etiology.

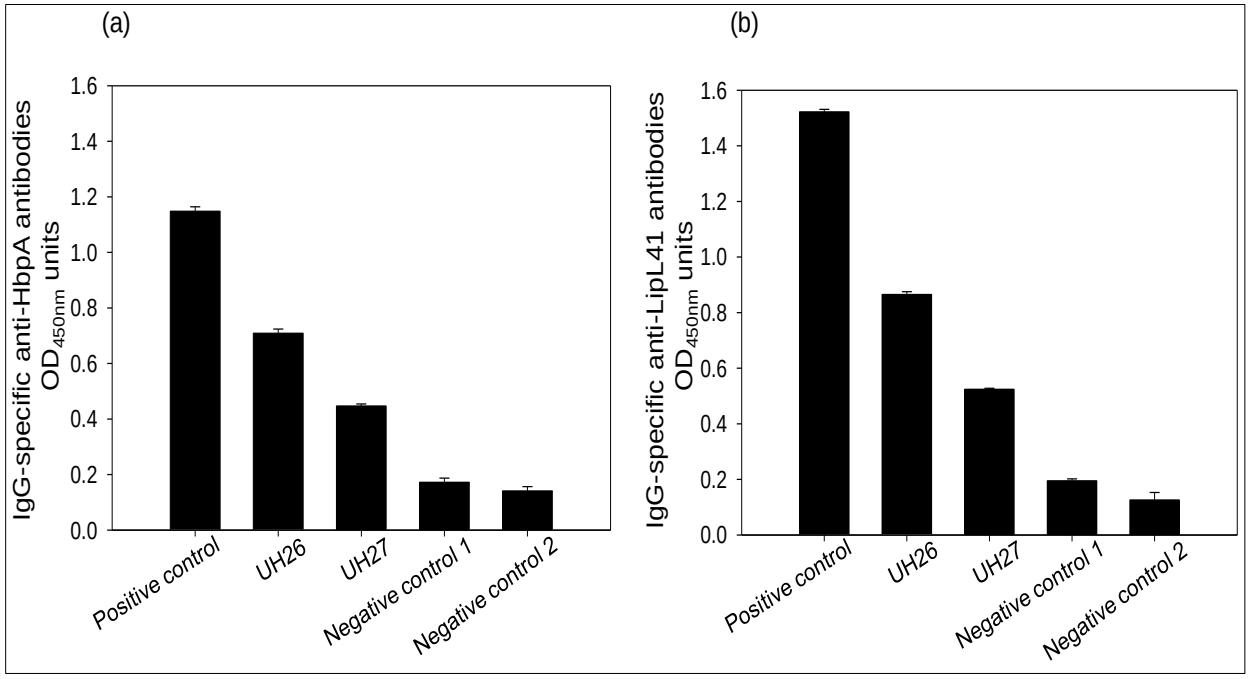


Figure 3B.7. ELISA-based demonstration of antibodies against rHbpA$_{34}$ and rLipL41 in the serum of UH26 and UH27. Panels a & b show the presence of antibodies against HbpA$_{34}$ and LipL41 in UH26 and UH27. The positive control was from a MAT positive leptospirosis patient in Group I. Negative controls included two MAT negative samples from Group III. The error bars represent SD calculated from two identical experiments performed in duplicates. The antibody and antigen blanks showed negligible OD_{450nm} values of 0.087 and 0.081 respectively.

3B.2.3. Demonstration of IgM-specific anti-leptospiral antibodies in urine of Group I patients

Urine of Group I patients showed IgM – specific antibodies against the proteins from the pathogenic serovars, though their levels were relatively much lower than that seen in the respective serum.

It is noteworthy that these antibodies did not react with the whole cell sonicate of the non-pathogenic serovar Andamana. Their complete absence in the dengue patients was a notable feature that facilitated differential diagnosis. Interestingly, they were seen in the two Group II patients' urine samples, implicating these cases as suffering from leptospirosis (Fig.3B.8).

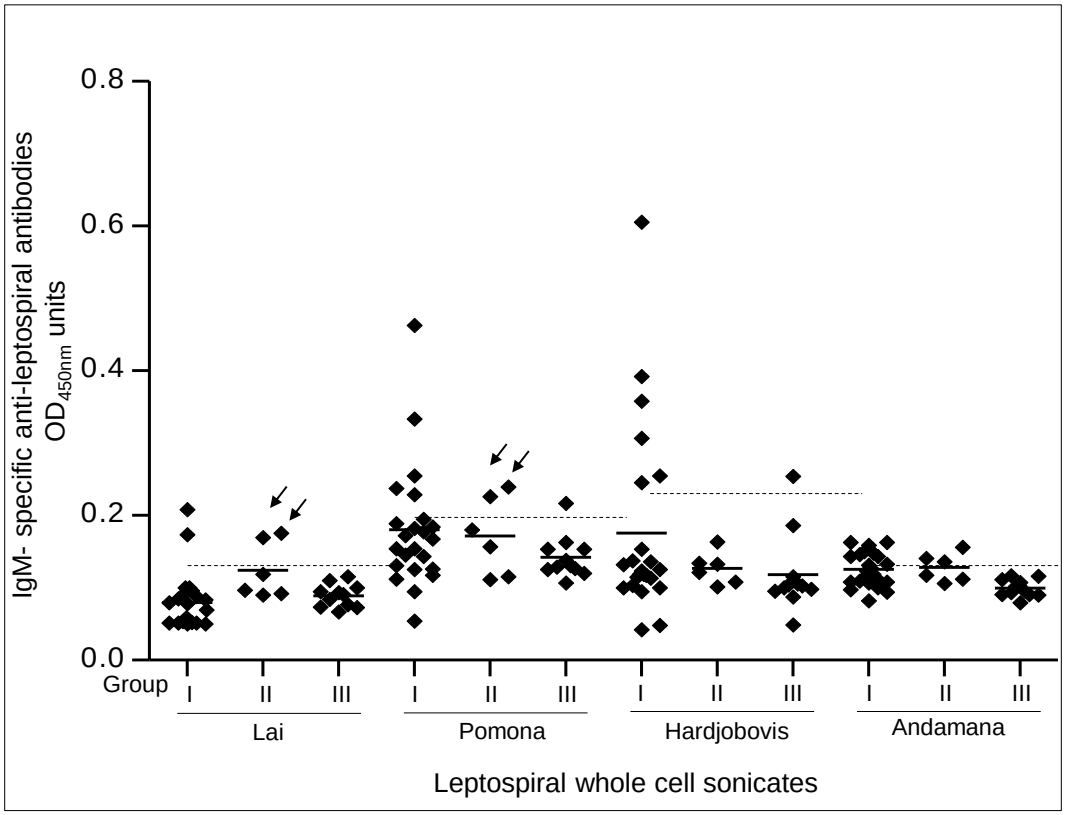


Figure 3B.8. Presence of anti-leptospiral antibodies in urine of patients with leptospirosis and not in dengue patients. ELISA done with whole cell sonicates as antigens identified low levels of urinary IgM-specific antibodies against leptospiral whole cell sonicates of pathogenic serovars Lai, Pomona and Hardjobovis in the Group I patients, with negligible reactivity with the non-pathogenic serovar Andamana. The horizontal lines indicates the mean absorbance value in each group. The dotted lines show the cut-off value calculated as indicated in Materials and Methods. The arrows point to the two Group II samples who also presented with a rise in antibody levels in their serum. Data are presented as mean ± SD of two independent experiments.

3B.2.4. Leptospiral antigens in the urine of Group I patients with leptospirosis: detection of HbpA, sphingomyelinase, LipL32 and LipL41

3B.2.4.1. Detection of leptospiral antigens by ELISA

All the 40 urine samples were screened for HbpA, sphingomyelinase, LipL32, Fla1 and LipL41 by ELISA, using the respective antibodies.

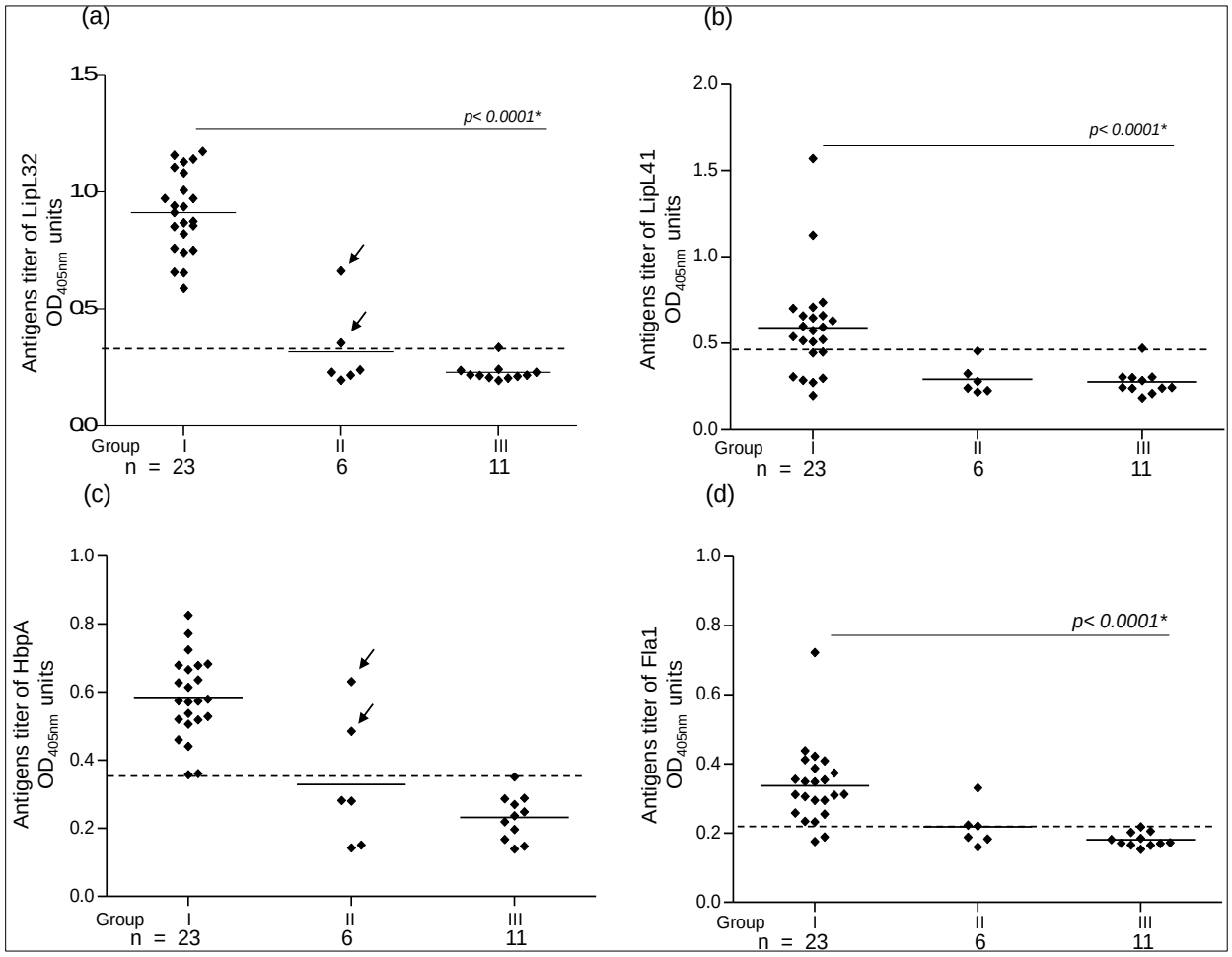


Figure 3B.9. ELISA-based detection of leptospiral antigens. Figure shows titre of leptospiral antigens LipL32 (Panel a), LipL41 (Panel b), HbpA (Panel c) and Fla1 (Panel d) in Group I (MAT positive), Group II (MAT negative) and Group III (Dengue) patients urine samples respectively. The dotted line shows the cut off value calculated as mentioned in Materials and Methods. The arrows represent the two MAT negative cases that tested positive by IgM ELISA. The horizontal line indicates the mean absorbance value in each group. Data are presented as mean ± SD of two independent experiments. The results were analysed by non-parametric Mann Whitney test and considered significant when $p < 0.0001$.

Fig.3B.9 shows the urinary detection of LipL32, LipL41, Fla1, HbpA in the Group I patients and Fig. 3B.10 shows the presence of sphingomyelinase(s) in this group; the latter detected with the specific antibodies against $SphCD_{210}$, $Sph2_{163}$ and Sph4. All the Group III dengue patients tested negative for these urinary antigens, an observation of significant

clinical relevance. UH26 and UH27 were further confirmed as leptospirosis cases as LipL32, HbpA and sphingomyelinase were present in their urine samples.

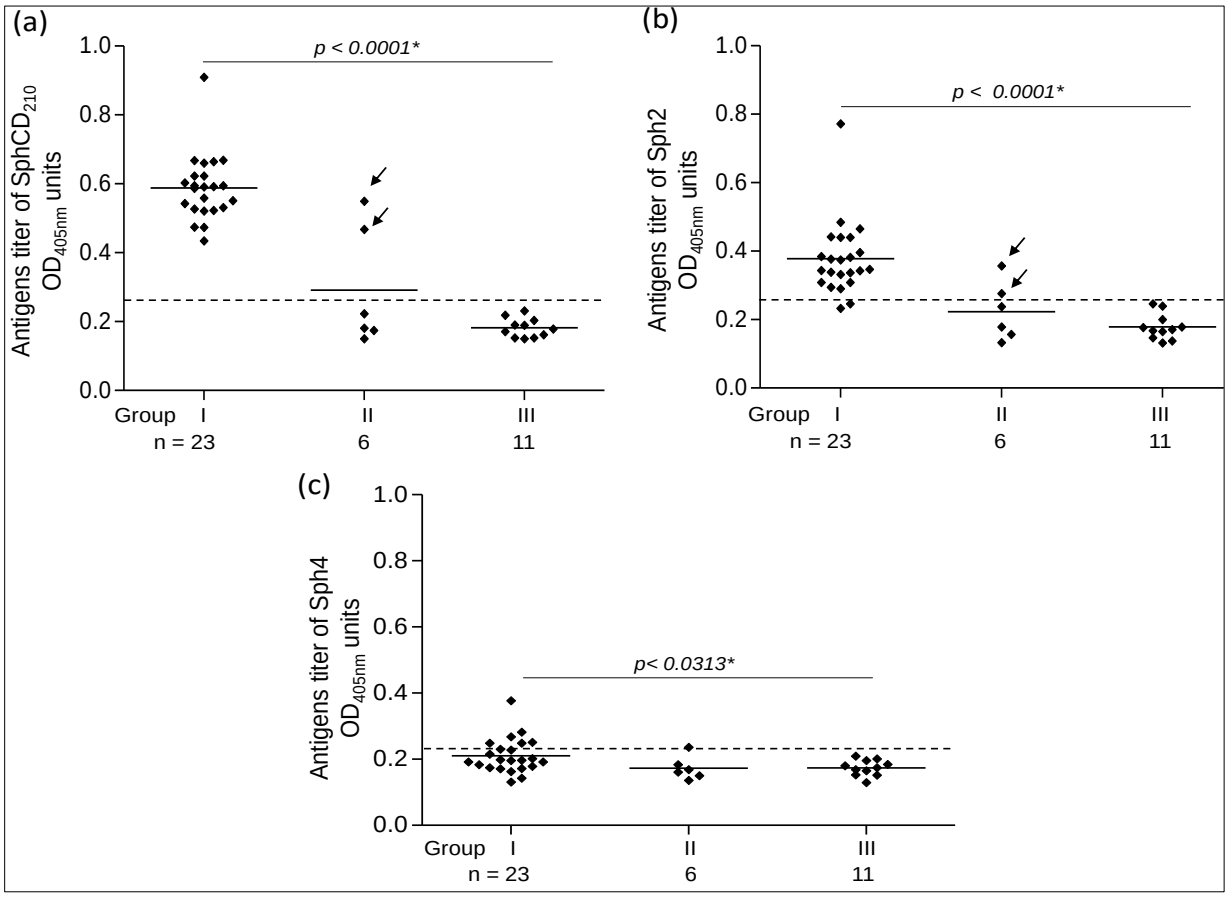


Figure 3B.10. Presence of sphingomyelinases urinary antigens in urine of patients with leptospirosis. Figure shows titre $SphCD_{210}$ (Panel a), Sph2 (Panel b) and Sph4 (Panel c) in Group I (MAT positive), Group II (MAT negative) and Group III (Dengue) patients urine samples respectively. The dotted line shows the cut off value calculated as mentioned in Materials and Methods. The arrows represent the two MAT negative cases. The horizontal line indicates the mean absorbance value in each group. Data are presented as mean ± SD of two independent experiments. The results were analysed by non-parametric Mann Whitney test and considered significant when $p < 0.0001$.

3B.2.4.2. Immunoblotting analysis of urinary leptospiral proteins

Urine of Group I patients that tested positive by ELISA were further screened by Western blot using antibodies against LipL32, HbpA, $SphCD_{210}$ and $Sph2_{163}$ respectively. Urine from UH2, UH3, UH17 and UH22 strongly reacted with anti-LipL32 antibodies and

showed a single band of 32 kDa (Fig. 3B.11a). UH22 was positive for HbpA but a 73 kDa band, smaller than the expected size was observed (Fig. 3B.11d).

Urine from UH3, showing LipL32 also showed the presence of a 42 kDa sphingomyelinase recognized by both anti-$SphCD_{210}$ and anti-$Sph2_{163}$ antibodies. Interestingly, both the antibodies identified a 58 kDa sphingomyelinase in UH1 and UH16 (Fig. 3B.11b and c). Screening for LipL41 could not be performed due to insufficient urinary protein.

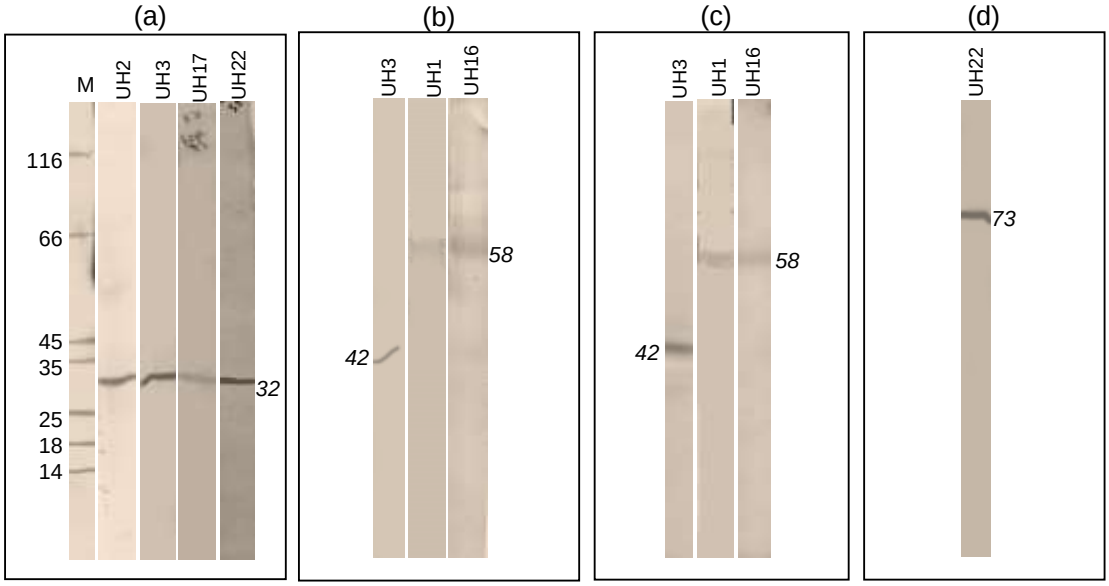


Figure 3B.11. Immunoblot of leptospiral proteins in urine. Figure shows immunoblots of urine samples with antibodies against LipL32 (Panel a), $Sph2_{163}$ (Panel b), $SphCD_{210}$ (Panel c) and HbpA (Panel d). 50 µg of urinary proteins from MAT positive cases (n = 23) were subjected on 5-20% gradient SDS-PAGE. Four identical blots of each urine sample was incubated separately with antibodies (diluted 1:1000) against LipL32, $SphCD_{210}$ and $Sph2_{163}$ and HbpA. Anti-rabbit IgG - alkaline phosphatase conjugate (diluted 1:5000) was used as the secondary antibody and the blot was developed with BCIP-NBT. M represents molecular weight marker.

3B.2.5. Identification of pathogen-specific leptospiral proteins recognized by serum / urinary antibodies from Group I patients

Antibodies present in the serum and urine of Group I patients were shown to react predominantly with antigens present in whole cell sonicates of pathogenic serovars. Western blot analysis of leptospiral cell sonicates with ten high titre serum and the corresponding urine samples demonstrated several serovar-specific antigens (Fig.

3B.12). Three samples (UH5, UH6 and UH10) that were negative for urinary anti-leptospiral antibodies were positive with the respective sera. Interestingly, these urinary antibodies were mainly against proteins from the pathogenic serovars Lai, Pomona and Hardjobovis.

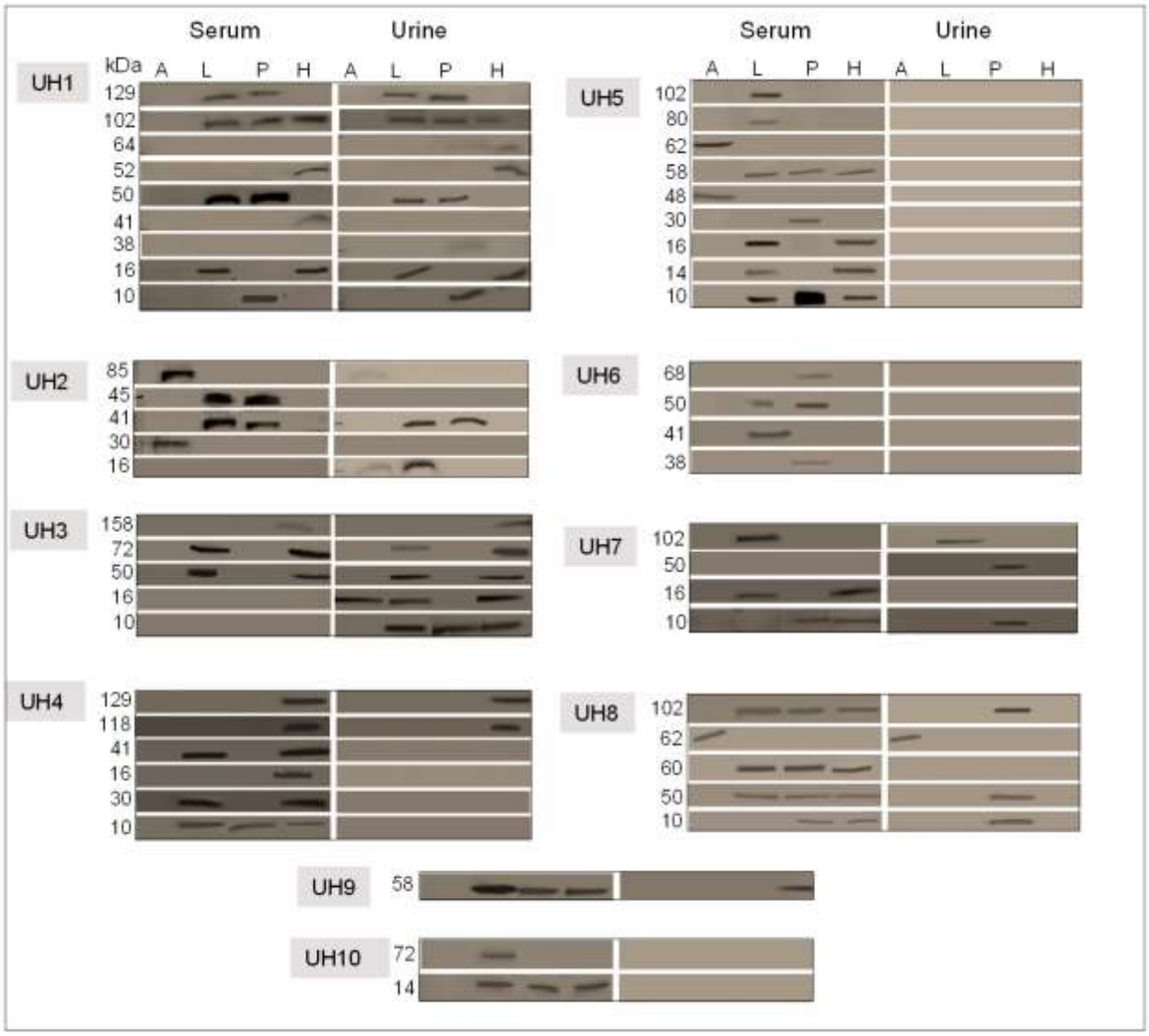


Figure 3B.12. Immunoreactivity of serum and urinary antibodies with leptospiral proteins from pathogenic *Leptospira*. 60 µg of whole cell sonicates of the serovars Lai, Pomona, Hardjobovis and Andamana were subjected to SDS-PAGE and upon transfer to nitrocellulose membrane subjected to immunoblotting with both serum and urine at a dilution of 1: 200 from ten patients in Group I (UH1 – UH 10), who showed high levels of serum anti-leptospiral antibodies, followed by the addition of anti-human IgG-alkaline phosphatase conjugate at dilution of 1:5,000. The panel shows the various bands of reactivity of the leptospiral proteins detected by both serum and urinary antibodies.

Six leptospiral proteins of approximate molecular masses of 102, 58, 50, 41, 16 and 10 kDa respectively that showed strong reactivity with the serum / urine from patients with leptospirosis were purified by preparative gel electrophoresis and subjected to MALDI-TOF MS/MS analysis. Table 3B.7 (see also Appendix II.B2) shows the identification of these six proteins.

Table 3B.7. MALDI-TOF MS/ MS analysis of six leptospiral proteins detected with serum / urinary antibodies from Group I patients

Approximate mass (kDa)	**Gene Locus**	**Annotation**	**Identified Dalton**	**MASCOT Score**
102	LA2432	Biotin requiring enzyme	102468	151
58	LA2639	Acyl-CoA dehydrogenase	61739	115
50	LA1409	Serine hydroxylmethyltransferase	45117	73
41	LA4165	Argininosuccinate synthase	45131	111
16	LA3598	Ferritin-like protein	16150	138
10	LA2654	Molecular chaperone GroES	10441	134

CHAPTER IV
DISCUSSION

The findings in this study, grouped into two parts in Chapter III will be discussed as a) Sphingomyelinases of *L. interrogans* serovar Pomona and b) Clinical significance and diagnostic potential of spingomyelinases and hemin-binding protein HbpA.

Sphingomyelinases of *L. interrogans* serovar Pomona

In general, several Gram-positive and Gram-negative bacteria produce sphingomyelinases and phospholipases that are found either on their cell surface or are secreted into the immediate environment. These two classes of enzymes hydrolyze their respective substrates sphingomyelin and glycerophospholipids present on eukaryotic cell membranes. This disrupts the integrity of the host cell membrane resulting in the lysis and release of the contents of the cell. Hence, they are considered as virulence factors that help to establish the infection and facilitate the growth and colonization of the pathogen within the mammalian host (Flores-Diaz *et al.*, 2016). The focus of this study is to identify and demonstrate the biological activity of the leptospiral sphingomyelinases produced by axenically grown *L. interrogans* serovar Pomona and upon infecting mammalian cells, with the Vero cell line being used in this study. The highlights of the study are a) justification for choosing serovar Pomona based on the protein profile and biological activity of the sphingomyelinases when compared to serovar Lai, b) demonstration of time-dependent damage and cell death of Vero cells upon infection by serovar Pomona, c) demonstration of hemolytic activity and the identification of a 42 kDa secreted sphingomyelinase in the growth medium and c) comparison of the findings in the leptospires recovered from Vero cells with the sphingomyelinases produced by these organisms grown in EMJH laboratory medium.

Several pathogenic bacteria produce hemolysins. The α toxin is a 33 kDa pore-forming molecule that plays an important role in the *S. aureus* - mediated pneumonia and sepsis. It effectively lyses several mammalian cells; the strong lysis seen with rabbit erythrocytes is the basis for laboratory testing (Bhakdi & Tranum-Jensen, 1991). In addition to its lytic activity, it plays an important role in triggering the release of several cytokines resulting in sepsis. The β toxin, a 35 kDa protein with sphingomyelinase activity is produced by certain strains of *S. aureus* (Huseby *et al.*, 2007). This toxin, unlike the α toxin is specific for sheep erythrocytes and is referred to as hot-cold hemolysin, as its hemolytic activity increases upon incubation at low temperatures. Both these toxins are

characterized by the presence of a cleavable signal sequence in the delta (δ) hemolysin, which is expressed by the majority of *S. aureus* isolates. Hemolysins have been extensively studied and reviewed in several bacterial systems, including *Streptococcus* spp., pathogenic *E. coli*, *Listeria* spp., *Bacillus cereus* and others.

As mentioned in Review of Literature (Chapter I), early reports on the hemolytic activity by pathogenic leptospires implicated the leptospiral sphingomyelinases. The direct association of these molecules with lytic activity was demonstrated later, with a report on the pore-forming effect of recombinant SphH on mammalian cells (Lee *et al.*, 2002). Subsequent reports on other recombinant sphingomyelinases and their biological activities (Zhang *et al.*, 2008; Carvalho *et al.*, 2010) were not conclusive. The expression of biologically active sphingomyelinases has been challenging due to problems of solubility of the recombinant proteins, also faced in our lab (Narayanavari *et al.*, 2012a). Due to difficulties faced with recombinant proteins and optimization of growth conditions to maximize their expression, progress in the understanding of these molecules has been slow. Much remains to be understood about these molecules and some of the unanswered questions include a) are all the *sph* genes in the genome of a serovar expressed simultaneously or are they individually controlled by environmental signals, b) are these expressed products present as inactive precursor molecules that are cleaved to yield the functional toxin and c) what triggers this cleavage? Here, our interest was to identify differences in the expression of these molecules upon infection of mammalian cells and study the difference, if any from the profile of these molecules in organisms grown in laboratory media.

Sph2 is considered as a true sphingomyelinase based on the structural similarity with other bacterial sphingomyelinases, presence of conserved amino acid residues at the enzyme active site and demonstration of hemolytic and enzymatic activity (Narayanavari *et al.*, 2012a). Conclusive evidence was provided by abolishing and restoring the biological activity of Sph2 using genetic manipulation techniques (Narayanavari *et al.*, 2015). *In silico* analysis of Sph2 of serovar Lai revealed three 19 aa repeats at the N-terminal end. It is thought that these proline-rich repeats causes structural constraints on the protein, causing it to migrate slower (Narayanavari *et al.*, 2015). Regulation of expression of *sph2*, studied in four different serovars (Narayanavari

et al., 2015) showed that the serovar Pomona expressed higher basal level of sphingomyelinase. This is thought to be due to presence of an insertion sequence-like element near the promoter region of *sph2*, with added influence of an extra 75 nucleotides stretch in Pomona, seen within the 5' end of the coding region (Artiushin *et al.*, 2004; Timoney *et al.*, 2011; Narayanavari *et al.*, 2012b). On the other hand, serovars Lai, Copenhageni and Manilae expressed Sph2 only upon supplementation with sodium chloride to levels equivalent to physiological salt concentration, a feature first demonstrated in Lai in an earlier study (Matsunaga *et al.*, 2007). The antibody used in their study was anti-$Sph2_{163}$ antibodies that was shown to react with Sph2 and SphH (Narayanavari *et al.*, 2015). While defined bands of Sph2 (89 kDa) and SphH (63 kDa) were seen in the other three serovars, multiple bands were seen in the serovar Pomona (Narayanavari *et al.*, 2015). This was intriguing, and it was not clear if they were degradation products or processed products of the full-length sphingomyelinase.

All the *sph* genes of serovar Lai, with the exception of *sph4* are present in serovar Pomona. Table 4D shows the details of the *sph* genes in the two serovars.

Table 4D. Sphingomyelinase genes in serovars Lai and Pomona

Gene	***L. interrogans* serovar Lai strain 56601**			***L. interrogans* serovar Pomona strain Pomona**		
	Locus tag	**Length (bp)**	**Molecular mass of expressed protein (kDa)**	**Locus tag**	**Length (bp)**	**Molecular mass of expressed protein (kDa)**
sph1	LA_1027	1,704	62.4	LEP1GSC014_RS219475	1,698	62.2
sph2	LA_1029	1,824	66.8	LEP1GSC014_RS219470	1,899	69.6
sph3	LA_4004	1,533	56.2	LEP1GSC014_RS213280	1,533	56.2
sphH	LA_3540	1,578	57.8	LEP1GSC014_RS221615	1,578	57.8
sph4	LA_3050	720	26.4	Absent		

The above information was obtained from https://www.ncbi.nlm.nih.gov/genome/179. NC_004342 and AFLT02000000 are the NCBI accession numbers for the genome of serovar Lai and Pomona respectively

Sph2 of serovar Pomona, characterized by 25 aa repeat has four such stretches instead of three, with the fourth repeat contributed by the 75 nucleotides additionally present in this serovar (Fig. 4D).

Sph2 of serovar Lai (LA1029)

MLFSLIRCLPEKESSYKDLFTSLLFLPNQTNS**NQVNSVSINNDPANPNPVN**PASANN**NQVNAVPENDD**
PANLNPVNPASANS**NQVNAAPENGSPADPNPAN**LASANNNQVNAVPANNYFTKEDSSNNIPKKVNSKN
VEIKVLSHNVFMLPTNLPRWGNLGHDERAKRISKSDYVKNQDVIVFEEAFDTSARKILLDNLREEYPY
QTDVVGRTKKNWDASLGNFRSYSLVNGGVVILSKWPIEEKIQYIFNDSGCGADWFANKGFVYVKINKE
GKKFHVIGTHAQSQDQNCSNLGIPNRANQFDDIRNFIYSKNIPKDETVLIVGDLNVIKESNEYYDMIS
RLNVNEPRYVGVPFTWDAKTNEIAAYYYENEEPVYLDYIFVSKSHAQPPVWQNLAYDPVSKQTWTVSG
YTSDEFSDHYPIYGFVYADPSTPTKSGHKKKYDQVSFQSAANGKYIQADPNRKNGWLKADAVIETDFT
KFNLLQEGNLNPSCIKNGLVRIESSRFLNYFWNWWLGGGSGNYGYYSKFNDASNQLEIINLSDECLEN
GSKIVFKDYDTYSRNHYYLTVWDKGNWNEHLYLWKDSISQREIFYLKLNSTPVRNWSADLIYR

Sph2 of serovar Pomona (LEP1GSC014_RS219470)

MLFSLIRCLPEKESSYKDLFTSLLFLPNQTNSNQVNSV**SINNDPANPNPVNPASANNNQVN**AV**PENDNP**
ANLNPVNPASANSNQVNAV**PENDNPANLNPVNPASANSNQVN**AA**PENGSPTDPNPANLASANNNQVN**AV
PANNYFTKEDSSNNIPKKVNSKNVEIKVLSHNVFMLPTNLPRWGNWGHDERAKRISKSDYVKNQDVIVF
EEAFDTSARKILLDNLREEYPYQTDVVGRTKKNWDASLGNFRSYSLVNGGVVILSKWPIEEKIQYIFND
SGCGADWFANKGFVYVKINKEGKKFHVIGTHAQSQDQNCSNLGVPNRANQFDDIRNFIYSKNIPKDETV
LIVGDLNVIKESNEYYDMISRLNVNEPRYVGVPFTWDAKTNEIAAYYYENEEPVYLDYIFVSKSHAQPP
VWQNLAYDPVSKHTWTVSGYTSDEFSDHYPIYGFAYADPSTPTKSGHKKKYDQVSFQSAANGKYIQADP
NRKNGWLKADAVIETDFTKFNLLQEGNLNPSCIKNGLVRIESSRFLNYFWNWWLGGGSGNYGYYSKFND
ASNQLEIINLSDGCLENGSKIVFKDYDTYSRNHYYLTVWDKGNWNEHLYLWKDSISQREIFYLKLNSTP
VRNWGADLIYR

Figure 4D. Sph2 of serovars Lai and Pomona. The three repeat sequences of 19 aa and 25 aa respectively in Sph2 of serovars Lai and Pomona are represented in red, with the boxed region (aa 71 - 96) representing the additional 25 amino acids.

In addition to these observations at the molecular level, other factors such as the basal expression (but inducible with added salt), differences in the banding pattern and hemolytic activities of the sphingomyelinases led us to choose serovar Pomona over serovar Lai for our study. Since the genome of pathogenic *Leptospira* spp. show multiple sphingomyelinase genes, two antibodies, namely anti-Sph2$_{163}$ antibody against the 163 aa (27 -190 aa) Sph2 fragment and anti-SphCD$_{210}$ raised against a 210 aa (123-332 aa) Sph3 fragment were used. Anti-Sph2$_{163}$ antibodies (gifted by Prof. Haake at UCLA) recognized two closely migrating bands (59 and 54 kDa) in serovar Lai, with the banding pattern showing variation in both size and number in serovar Pomona (90, 62 and 52 kDa). Since this antibody reacts with both Sph2 and SphH, it can be inferred that the

above bands seen in the two serovars are either Sph2 or SphH. Surprisingly, the 90 and 52 kDa sphingomyelinase bands in serovar Pomona was recognized by anti-rSphCD$_{210}$ antibodies, which additionally reacted with two low molecular weight proteins (38 and 12 kDa) in both the serovars. The differences in the molecular sizes observed here and the previously reported study (Narayanavari *et al.*, 2015) is due to the use of different strains. Also, it may be mentioned that it is unclear as to which of the encoded sphingomyelinase(s) is expressed and further, these molecules are highly likely to undergo processing, possibly by proteolytic cleavage to yield smaller biological potent molecules. The above possibilities account for the variation in the size of the fragments recognised by these antibodies.

Another important reason for choosing the serovar Pomona was the salt-induced high (48.7%) hemolytic activity of the spent growth medium that was not seen with the serovar Lai. This finding clearly indicated a sphingomyelinase that was secreted into the growth medium. The observed enhanced hemolytic activity exhibited by the serovar Pomona could account for the hemolytic anemia and hemoglobinuria observed in ruminants infected with Pomona strains (Smith & Armstrong, 1975).

A salient finding of this study is the identification of the secreted form of sphingomyelinase as a 42 kDa protein from the growth medium of Pomona-infected Vero cells (discussed below). We could not perform the protein separation and Western blotting of the spent growth medium of axenically grown organisms due to interference of the large amount of BSA added in the medium of growth. The 42 kDa sphingomyelinase was reported from our lab, in the context of iron limitation triggering its expression in the serovar Lai (Velineni *et al.*, 2009). This 42 kDa protein was detected with anti-SphCD$_{210}$ antibodies in the outer membrane vesicles of serovar Lai grown in low iron medium. The cultures, subjected to iron-regulated growth were incubated for 5 hours at 37°C, prior to analysis, a procedure that was optimized for the expression of the iron-regulated protein HbpA (Asuthkar *et al.*, 2007). A similar finding was observed in our earlier studies on sphingomyelinases (Narayanavari, 2012). The outer membrane vesicles of iron-limited serovar Lai showed the 42 kDa band when grown at 37°C and not at 30°C. This protein was confirmed as SphH by Western blotting with SphH-specific antibodies (experiments

performed in the lab of Prof. Haake, UCLA using antibodies raised against SphH-specific peptides). Increased levels of SphH correlated with the high *sphH* transcripts seen under these conditions.

Infection of mammalian cell line by serovar Pomona was done to observe the time-dependent damage of the host cell and understand the role of sphingomyelinase in this damage. In natural infections, it is well known that *Leptospira* colonize and thrive in the renal tubules. Hence, monkey kidney epithelial Vero cell line was selected for infection studies. This cell line maintained as a monolayer was infected with the serovar Pomona and time-dependant observations of the infected Vero cells was performed both by scanning electron microscopy and flow cytometry to monitor cell damage and death. Within 30 minutes upon addition of leptospires, scanning electron microscopy revealed the attachment of these bacteria to the surface of the Vero cells. The attachment of several leptospires to a single host cell is known (Liu *et al.*, 2007), possibly to ensure that they gain entry into the host cells. The adherent Vero cells slowly rounded and detached from the substratum, a process that was faster upon infection with serovar Pomona than Lai. The sphingomyelinase-deficient non-pathogenic serovar Andamana did not cause any cell damage and merely adhered to the glass surface, as reported with *L. biflexa* serovar Patoc (Ballard *et al.*, 1986). This strengthened the role of sphingomyelinases in the pathogen-induced cell damage. The cell damage was more pronounced when the infecting leptospires were induced with sodium chloride prior to infection, again reflecting the up-regulation of the sphingomyelinases by salt. In addition, serum in the culture medium is also likely to influence the expression of the sphingomyelinases. More work is required to understand the role of serum, specifically with reference to the iron-chelating nature of the transferrin in the serum that is known to lower the available free iron.

With time, the leptospires invaded the host cells and by an hour, they were not detected on the cell surface. At the same time, the damage to the cell surface became increasingly evident and by 2 h, there was significant damage to the outer cell surface of the Vero cells. These time-dependent changes were unambiguously captured by scanning electron microscopy. It was evident from these observations at the early time-points after infection that cell death was imminent. Flow cytometry analysis revealed that the viability of the host cells was reduced drastically at 4 hours after infection. The salt-

treated leptospires were more virulent, reflected by only 0.44% viable cells compared to 12.7% viable cells when infected with serovar Pomona minus salt. These observations indicated the role of the induced sphingomyelinases as the mediators of cell damage and death. Experimentally, we established this by increasing the viability of the Vero cells by prior incubation of the leptospires with anti-SphCD$_{210}$ antibodies. The % of early apoptotic cells was high (74.8%) and with time shift to the late apoptotic population. Such apoptotic processes have been demonstrated in experimental animal studies (Brihuega *et al.*, 2011). When BALB/c mice were infected with pathogenic *Leptospira,* many features such as decrease in the volume of the cytoplasm, the appearance of rounded cells and shrinkage of mitochondria, characteristic of apoptosis were noted in lung, liver and kidneys (Marinho *et al.*, 2015).

The role of sphingomyelinases in host cell death was evident from our observations. First, we demonstrated, using confocal microscopy the expression of this toxin by the infecting pathogen. Second, we demonstrated both at the transcript level and protein level the elevated expression of this molecule. Further studies are needed understand if the increase in mRNA level is due to increased expression or decreased degradation. Third, we demonstrated the hemolytic and enzymatic activity of the secreted form of the protein, which we identified as a 42 kDa band by immunoblot analysis. The cell damaging effect of the growth medium containing this 42 kDa sphingomyelinase on a fresh batch of Vero cells strengthened our conviction on the role of the sphingomyelinase, with the conclusive evidence provided by the recombinant protein causing membrane damage and holes in the host cells established by scanning electron microscopy.

In conclusion, this study has emphasized the role of sphingomyelinases as virulence factors. Their expression in laboratory medium and upon infecting mammalian cells has contributed further to our knowledge on these toxins, with a salient finding being the identification of the secreted form of a 42 kDa band that has cell-damaging potential by virtue of its hemolytic and enzymatic activity. Much remains to be understood about these molecules, as we are faced with difficulties in unravelling their structural heterogeneity, factors influencing their expression among the different serovars, possibly host-dependent processes, their biological activity and resultant impact in different organs

in-vivo. The expression of these molecules *in-vivo*, detailed below clearly highlights their essentiality for survival *in-vivo*.

Clinical significance and diagnostic potential of sphingomyelinases and hemin-binding protein HbpA

Specific diagnosis of leptospirosis symptomatically by clinical examination has been challenging. Also, laboratory diagnosis has also been of limited use, either due to economical constraints of commercial kits or the technical skill needed for performing the test, as in MAT. Thus, there is a need to develop simple, economical and easy-to-do tests that can be used in routine laboratories. ELISA and point-of-care device like the lateral flow test are two such tests and they are the focus of this study. The challenging feature of developing these tests is the identification of pathogen-specific antigen(s) that is expressed by all serovars, making possible the disease diagnosis, irrespective of the infecting serovar in the various geographical locations. Our earlier and current studies established the diagnostic implications of the iron-regulated hemin-binding protein HbpA. In this study, we developed both an ELISA-based test and a lateral flow device by cloning and expressing a 34 kDa fragment of HbpA ($HbpA_{34}$), as against $HbpA_{55}$, a 55 kDa fragment used in our earlier studies. Sphingomyelinase(s) expressed as virulence factors by pathogenic leptospires has been another major focus in our clinical studies. Our findings highlight both HbpA and sphingomyelinase as highly promising candidates for diagnosis, as discussed below.

HbpA is an 81 kDa iron-regulated outer membrane protein that binds hemin that facilitates acquisition of iron by the direct acquisition machinery (Asuthkar *et al.*, 2007). The presence of circulating antibodies against HbpA in patients with leptospirosis (Velineni *et al.*, 2007a; Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012) not only shows that it is expressed *in-vivo* but implies the iron-limiting conditions faced by the pathogen in the human host, a well-established phenomenon called ‘nutritional immunity’(Kochan, 1976). $HbpA_{55}$, shown to be specific for *Leptospira*, with negligible cross-reactivity with other bacterial proteins (Sivakolundu *et al.*, 2012) was demonstrated as a promising candidate in the screening of both human and bovine samples (Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012). The performance of the protein was comparable with LipL32

and LipL41, two well-studied diagnostic antigens for the screening of this disease. However, it was seen that the specificity of all these ELISA-based tests were relatively lower than the commercial PanBio ELISA kit (Sivakolundu *et al.*, 2012).

In the current clinical study I, the focus was to screen serum antibodies against both HbpA and sphingomyelinase and compare their performance with the gold standard MAT and the commercial PanBio ELISA. The focus to increase the specificity of HbpA-ELISA and simultaneously assess the *in-vivo* expression of the virulent sphingomyelinase and assess its potential as a diagnostic antigen. The chosen HbpA fragment is a 34 kDa fragment ($HbpA_{34}$; aa 470 to 710) with nine potential B-cell epitopes that overlapped partially with $HbpA_{55}$ (amino acids 229 to 710). The performance of $HbpA_{34}$ as an antigen in both ELISA and the lateral flow device was found to be satisfactory, as discussed below. Sphingomyelinases have posed several difficulties, due to the presence of several sphingomyelinases in a particular strain and the sequence heterogeneity, not only among different leptospiral species but within a species. In addition, it is not known if they are expressed as pro-proteins and if so, whether they serve as substrates for proteolytic cleavage to yield potent, biologically active molecules. As detailed in Methods, *L. interrogans* serovar Lai shows the presence of five *sph* genes, whose expression by the organism in both *in-vitro* grown cultures and *in-vivo* remains to be understood. The sequence and structure of a biologically active form of one or more of these sphingomyelinases remains an enigma. We reported a 42 kDa sphingomyelinase in serovar Lai grown under iron-limiting conditions using immunoblot analysis but did not present evidence for its functionality (Velineni *et al.*, 2009). Considering the lacunae in these molecules, we used antibodies raised against the common enzymatic domain (anti-SphCD_{210}; common domain) present in four of the five sphingomyelinases. Also, we included antibodies against Sph4, a sphingomyelinase lacking the enzymatic domain that served as an additional control.

Of the 108 MAT positive samples (out of a total 130 cases of suspected leptospirosis), there was good correlation of $HbpA_{34}$-ELISA with MAT. 102 samples tested positive by both the tests, with $HbpA_{34}$-ELISA failing to detect 6 of the MAT - positive samples. The sensitivity of $HbpA_{34}$ as an antigen was 95% and was superior to the commercial PanBio ELISA; the latter detected only 60 samples and showed sensitivity of

only 56%. Among the commercial kits for the sero-diagnosis of leptospirosis, PanBio IgM ELISA, recommended by the World Health Organization (WHO, 2003) is the most commonly used. The usefulness of this assay, with variation in the specificity (65 - 98.0%) and sensitivity (36 - 60.9%), particularly in endemic areas has been reported (Effler *et al.*, 2002; Wagenaar *et al.*, 2004; Blacksell *et al.*, 2006). It was inferred from these studies that the low performance was probably due to the endemicity and the presence of high basal titres of antibodies in the healthy population (Desakorn *et al.*, 2012). The test thus requires standardisation by determining the basal titres and calculating a cut-off value in the specified geographical location. This was also found to be true when we compared PanBio ELISA and $HbpA_{55}$-ELISA with MAT in screening for leptospiral uveitis (Sivakolundu *et al.*, 2012); it was seen that the sensitivity and specificity of PanBio IgM ELISA was 90 and 54% respectively. The lower specificity seen with $HbpA_{55}$ - ELISA [58% and 63% in human and bovine samples (Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012)] increased to 89% with $HbpA_{34}$, with these comparisons made against the gold standard MAT. $HbpA_{34}$ was effective not only in ELISA but also in the point-of care lateral flow device. Twelve of the twenty samples tested positive in the lateral flow device. The clear band in the lateral flow device reflected the potential of HbpA as an antigen. The evaluation, however needs to be carried out on a larger sample size to study the application of this device in screening samples in a clinical setting.

Among the 131 samples screened for anti-sphingomyelinase antibodies, 75 of the 81 MAT-confirmed samples were positive for anti-$SphCD_{210}$ antibodies, with 77 samples testing negative for antibodies against rSph4, as anticipated finding due to the truncated nature of this antigen. A comparative evaluation the various antigens in the in-house ELISA, including LipL41 with MAT showed the potential of ELISA as an alternative screening method. The reason for MAT negativity for ELISA-positive samples is possibly due to the exclusion of the prevalent serovar in that geographical region and not due to the test *per se*. However, despite its high specificity, alternate tests are needed considering the inherent disadvantages of MAT.

Considering the choice of suitable antigens for ELISA-based tests, several outer membrane proteins, by virtue of their surface exposure and direct contact with the immediate environment have been evaluated as potential candidates for the sero-

diagnosis of leptospirosis. Several proteins including LipL32 and LipL41 have been highlighted in Chapter I. In this study, we showed that both HbpA and sphingomyelinase are highly promising candidates for diagnosis, with good correlation with LipL41 and equivalent / superior performance to the commercial PanBio ELISA kit in terms of sensitivity and specificity.

In the Clinical study 2, the objective of the study was to evaluate urine as a biological sample for screening of leptospirosis. Since we included samples from dengue infection, there was an additional advantage of comparing our findings with a disease with similar symptoms. Dengue is an arboviral disease of public concern that is transmitted by mosquitoes (Jaenisch *et al.,* 2016). In this study, though performed on a small number of 40 patients, we included discrete group of patients with either leptospirosis or dengue and did not consider co-infections. Both these diseases rise sharply during monsoon seasons and in India, by virtue of its natural features such as climate, geological setting and frequent floods, there is high incidence of dengue, leptospirosis, malaria and enteric fever (Shanbag 2010). Considering the increasing reports on co-infection of these two diseases (Rele *et al.*, 2001; Zaki & Shanbag, 2010; Mishra *et al.*, 2013; Suppiah *et al.*, 2017), our findings on the differentiation of these two diseases is of considerable clinical significance.

The laboratory diagnosis of all the 23 cases in Group I was leptospirosis, the evidence being high serum titres of IgM-specific anti-leptospiral antibodies, MAT positivity and presence of urinary leptospiral antigens coupled with low levels of IgM antibodies. Our findings of low sero-reactivity with the antigens from the serovar Andamana ruled out the exposure to non-pathogenic *Leptospira* spp. Disease diagnosis was confirmed upon detection of HbpA, sphingomyelinase and the other leptospiral antigens LipL32, LipL41 and Fla1 in the urine of these patients; patient UH3, showing severe symptoms of the disease showed the highest titre of these antigens. None of the above findings were seen in the 11 dengue fever patients in Group III. The diagnostic potential of the urinary antigen screening was evident in the identification of the two PUO cases in Group II, who despite being MAT negative and presenting a marginal rise in serum titre of anti-leptospiral antibodies excreted the above-mentioned leptospiral proteins in their urine.

Proteins are not present in the urine of healthy individuals and its presence, specifically albumin is indicative of renal damage. The involvement of the kidneys in

leptospirosis can be mild with proteinuria that can become severe, called as acute kidney injury (AKI). In tropical countries, specifically in regions endemic for leptospirosis, AKI incidence is often high (Sitprija V *et al.*, 1997). In this condition, there is raised serum urea and creatinine and often associated with jaundice. It is thought that the high serum bilirubin levels due to liver injury causes the renal damage, which is often associated with poor urine output (oliguria), with anuria seen in the worst cases (Daher Ede *et al.*, 2010). The damaging effect is thought to be directly due to the organisms themselves and due to the toxin-induced immunological reactions (Daher Ede *et al.*, 2010). The organisms and several outer membrane proteins, specifically the abundantly expressed LipL32 have been identified and it is thought that several outer membrane components, including proteins and LPS contribute to the tubular necrosis. In India, considerable work on renal damage due to leptospirosis has been done by Muthusethupathi and his group (Muthusethupathi *et al.*, 1994). Renal damage in animals have been reported (Monahan *et al.*, 2009; Llewellyn *et al.*, 2016). In our study, we observed both IgM and IgG-specific antibodies, the latter in high levels in the urine of patients with leptospirosis (Thresiamma *et al.*, 2017).

Of greater emphasis is the usefulness of detecting pathogen-specific leptospiral antigens, including HbpA and sphingomyelinase in the urine of patients with leptospirosis and not in dengue patients. In an effort to understand the clinical significance of the urinary leptospiral antigens demonstrated in this study, the two well-studied antigens LipL32 and LipL41 deserve mention first. LipL32 is an outer membrane protein present in all pathogenic *Leptospira* spp. (Haake *et al.*, 2000; Cullen *et al.*, 2004; Malmstrom *et al.*, 2009). The high level of expression of this protein, its presence in the tissues of experimentally-infected animals (Haake *et al.*, 2000; Yang *et al.*, 2002; Lowanitchapat *et al.*, 2008) and urine (Grevemeyer *et al.*, 2017) reflects the clinical relevance of this protein. The vaccine potential of this protein remains to be ascertained as the role of this protein is still unknown, with no evidence of its essentiality seen in *LipL32* mutants (Murray *et al.*, 2009). But, its diagnostic potential has been well explored (Flannery *et al.*, 2001; Bomfim *et al.*, 2005; Chalayon *et al.*, 2011), with efforts made to improve the specificity of detection using defined LipL32 peptides (Aviat *et al.*, 2010). In this study, ELISA identified the protein in the urine of all Group I patients. The specificity of detection in the urine was

evident from the discrete band seen upon immunoblotting with anti-LipL32 antibody in four of the samples. The failure to detect them in the other samples could be due to the lower sensitivity of Western blotting when compared to ELISA. LipL41, due to the paucity of the samples could be demonstrated only by ELISA and not by immunoblotting in the 23 Group I cases. Both LipL32 and LipL41 were high in the urine of the severely affected UH3 patient.

Analysis of the leptospiral sphingomyelinases revealed that they are expressed *in-vivo* and be seen within a few days upon infection, as the patients came to the hospital immediately upon development of symptoms. The immunoblot profile of these molecules in the urine of infected patients showed the presence of a 58 kDa band in two samples and a 42 kDa band in UH3, presenting with severe symptoms. Both anti-SphCD$_{210}$ and anti-Sph2 antibodies recognized the 58 kDa and 42 kDa proteins, affirming they are sphingomyelinases. The size variation reflects two observations, namely a) these proteins are smaller in size when compared to the protein deduced from the *sph* gene sequences and b) the processing of the pro-protein differed among the patients implicating the possible role of the immediate environment of the human host effecting their cleavage. We hypothesise that iron levels regulated the expression of the 42 kDa sphingomyelinase. Earlier, we demonstrated the presence of this band in the vesicles of iron limited serovar Lai (Velineni *et al.*, 2009). Iron acts as a regulatory molecule in the expression of several bacterial toxins (Sritharan, 2000) and it is highly likely that the iron-withholding phenomenon of the human host accounted for the presence of this sphingomyelinase. It may also be recalled that the 42 kDa secreted form of sphingomyelinase was detected in the growth medium of Vero cell line infected with leptospires.

Expression of HbpA, demonstrated by ELISA in the leptospirosis patients is justified considering the iron-limiting conditions faced by these pathogens. Specificity of the protein to leptospiral infections is established by its absence in dengue patients. While ELISA identified the protein, Western blotting analysis detected the protein in a single sample. The smaller size of the protein (73 kDa *vs* the predicted size of 81 kDa) and the failure to detect it in UH3 by immunoblotting despite the high levels seen in ELISA warrants further analysis.

ELISA of urinary proteins for diagnosis of leptospirosis was made possible by the availability of specific antibodies against the above antigens. There was no elaborate processing of the urine as was demonstrated for the detection of urinary leptospiral LPS (Saengjaruk *et al.*, 2002; Widiyanti *et al.*, 2013). The test is non-invasive and easy-to-perform. The usefulness of the test was evident from the diagnosis of the two cases, presenting with marginal rise in the serum anti-leptospiral antibodies that was also MAT negative. The urine of these two patients showed antigens thus demonstrating that they are leptospirosis cases despite being MAT negative. However, the test must be validated on a large population of individuals including normal population and infected patients, including several tropical diseases like malaria, viral flu, dengue and others.

Using the serum of infected patients as source of anti-leptospiral antibodies, we identified leptospiral proteins expressed within the human host. With most of them identified as proteins associated with metabolic pathways, further work is needed to study their association with leptospiral etiology. In conclusion, the study has yielded considerable information on the potential of the sphingomyelinase toxin, HbpA and other outer membrane proteins in disease diagnosis. Future work will focus on the effectiveness of using combination of these antigens for diagnosis of leptospirosis.

CHAPTER V

SALIENT FINDINGS FROM THE STUDY

I. Expression of sphingomyelinases in *L. interrogans* serovar Pomona

- Sph2 of serovar Pomona has additional 25 amino acids when compared to the corresponding homologue in serovar Lai.
- Serovar Pomona showed higher basal levels of sphingomyelinase, which is induced with 120 mM of sodium chloride.
- Highest hemolytic activity (48.7 %) was seen with spent growth medium of serovar Pomona.
- Increased transcript and elevated expression profile with diverse banding patterns were evident in leptospires recovered from infected Vero cells compared to axenically grown organisms.
- The banding patterns of the sphingomyelinases were identical in the immunoblots developed with anti-$Sph2_{163}$ and anti-$SphCD_{210}$ antibodies.
- The attachment and invasion of Vero cells by the serovar Pomona was evident from scanning electron microscopy. Cell death was confirmed by flow cytometry. Upon pre-incubation of the infecting pathogen with anti-$SphCD_{210}$ antibodies, there was increased viability of Vero cells.
- Sphingomyelinases in the infected Vero cells were demonstrated by confocal microscopy using anti-$SphCD_{210}$ antibodies.
- A secreted 42 kDa sphingomyelinase was identified in the growth medium of Vero cells infected with serovar Pomona. Hemolytic and enzymatic activity were demonstrated with this growth medium unlike the uninfected Vero cells.
- Cytotoxic activity of the growth medium on a fresh batch of uninfected Vero cells was evident by scanning electron microscopy.
- Cytolytic role of sphingomyelinase was confirmed by adding $rSphCD_{210}$ to Vero cells. The cell damage noted with $rSphCD_{210}$ was not seen upon addition of rSph4, a biologically inactive molecule lacking the exo-endophosphatase domain.

II. Sero-diagnosis of leptospirosis and demonstration of pathogen-specific leptospiral proteins in the urine of patients with leptospirosis and not in dengue patients

Clinical study I

- $HbpA_{34}$ (sensitivity 95%; specificity 89%) was effective not only in ELISA but also in the point-of care lateral flow device.
- The sensitivity and specificity of $SphCD_{210}$-ELISA was good with values of 92 and 90% respectively.
- The study has been satisfactory and promising, leading to future prospects of developing an in-house ELISA with $rHbpA_{34}$ and $rSphCD_{210}$ in screening of samples with leptospirosis.

Clinical study II

- The presence of pathogen specific proteins such as LipL32, LipL41, Fla1, HbpA and sphingomyelinase(s) in urine of all the Group I cases as leptospirosis and the notable absence in dengue patients facilitated the differential diagnosis of leptospirosis from dengue. This showed good correlation with MAT and serum ELISA for anti-leptospiral antibodies.
- Identification of two PUO cases (UH26 and UH27) based on urine and serum analysis is a good indicator for urine analysis
- Several leptospiral proteins were detected using the patients' serum samples as sources of antibodies. Further studies are needed to assess their diagnostic significance.
- The findings also provide scope for the development of point of care devices for leptospiral antigen detection in urine.

CHAPTER VI

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CHAPTER VII

APPENDIX I AND II

A1. List of reagents and suppliers

Name of Chemicals	Cat. Log No.	Name of Supplier
EMJH base	279410	Becton Dickinson and Company (MD, USA)
EMJH enrichment	279510	
Noble agar	214220	
FITC Annexin V Apoptosis Detection Kit I	556547	
Lyophilized Dulbecco's Modified Eagle Medium (DMEM)	D5648	Sigma Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	D2438	
Agarose	A9539	
Imidazole	I2399	
Bovine serum albumin (BSA)	A2153	
Acrylamide	A3553	
Bisacrylamide	M7279	
NaCl	S7653	
Urea	U0631	
Isopropanol	I9039	
Ethylenediamine-*N*,*N'*-diacetic acid (EDDA)	158186	
BCIP / NBT liquid substrate system	B1911	
3, 3′-diaminobenzidine (DAB)	D5637	
Bicinchoninic acid (BCA) protein estimation kit	B9643	
Sphingomyelinase C from *Staphylococcus aureus*	S8633	
Ceramide	22244	
3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB tablet)	T3405	
4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP tablet)	N9389	

Propidium Iodide solution	P4886	
DAPI (4',6-diamidino-2-phenylindole)	D9542	
Paraformaldehyde	15812-7	
Goat anti-human IgG (Fc specific)-alkaline phosphatase antibody	A9544	
Goat anti-human IgG (Fc specific)-peroxidase conjugate	A0170	
Goat anti-human IgM (µ-chain specific)-peroxidase conjugate	A0420	
Goat anti-rabbit IgG conjugate with alkaline phosphatase	A3687	
Goat anti-rabbit IgG conjugate with FITC	FTC21	Merck Life Science Pvt. Ltd. Darmstadt, Germany
IPTG (Isopropyl-β-D thiogalactopyranoside)	094866	Thermo Fisher Scientific Incorporation (MA, USA)
Dream Taq DNA polymerase	EP0701	
T4 DNA ligase	EL0331	
SalI restriction enzyme	ER0641	
NdeI restriction enzyme	ER0581	
DNA molecular weight marker	SM0333	
Unstained protein molecular weight marker	26610	
6X Loading buffer	R0611	
1 Kb ladder	SM0331	
Tris-base	15965	Qualigens (Qualigens Fine Chemicals Pvt. Ltd., India)
Ethidium Bromide	054817	SRL Life Sciences, India
Plasmid extraction kit	K210010	
PCR gel extraction kit	K220001	

RNeasy Mini kit	155596018	Invitrogen (CA, USA)
Trizol	15596026	
SuperScript III First-Strand Synthesis System	18080051	
TLC Silica gel 60 F_{254}	1.05554.0007	
Fetal bovine serum	26140079	
Antibiotic-antimycotic solution (100X)	15240062	
Trypsin with 0.25% EDTA	25200056	
Turbo DNase	AM1907	Ambion, Texas, USA
Ni-Sepharose resin	17526801	GE Healthcare (Little Chalfont, United Kingdom)
Glutaraldehyde solution	RM5928	HiMedia, India
Sphingomyelin	TC279	
Anti-His antibody	SC-8036	Santa Cruz Biotechnology, Dallas, Texas, USA
Commercial Pan Bio IgM ELISA kit	E-LEP01M	Inverness Medical Innovation Pty. Ltd., Australia

A2. 0.1 M Phosphate buffered saline (PBS)

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 700 mL of double-distilled water, adjusted pH to 7.2 with 0.1N HCl and made up the volume to 1 L. The solution was autoclaved at 15 lbs / sq. inch for 20 min.

A3. Alsever's solution

Dissolved 2.05 g glucose, 0.89 g sodium citrate, 0.42 g sodium chloride and 0.05 g citric acid in 70 mL double distilled water and made up to 100 mL of Alsever's solution. 5 mL

was aliquoted in McCartney bottles, added some glass beads and autoclaved at 15 lbs / sq. inch for 20 min.

A4. Defibrination and preparation of 10% sheep blood

10 mL of blood was collected in Alsever's solution. The tube was continuously shaken to defibrinate, taking care to prevent lysis of RBC. RBC was collected by transferring the blood to a 15 mL Falcon tube and centrifuging at 2500 rpm for 10 min. The plasma was removed, added 0.9% saline, same volume mixed gently by tilting the tube 3-4 times to get a uniform suspension of cells. Washed the RBC twice with 0.9% saline. The supernatant was removed, 1 mL of RBC from the intact pellet was taken into another tube and made to 10 mL with 0.9% saline to get 10% cell suspension.

A5. Preparation of DMEM media

To a vial of DMEM lyophilized powder (with glutamine and without $NaHCO_3$; Sigma Aldrich, St. Louis, MO, USA), added a small volume of autoclaved milliQ water. Dissolved the contents and transferred it to 700 mL milliQ water in a 1 L flask. A small amount of water to the vial and repeated the process till there was quantitative transfer of all the medium contents into the flask. Weighed 2 g $NaHCO_3$ (Sigma Aldrich, St. Louis, MO, USA), added to DMEM solution, adjusted pH to 7.2 and the volume was made up to 1 L. The medium was filter sterilized using 0.2 µm pore size GVWP filters (Millipore Corporation, MA, USA) and stored at 4°C.

A6. Heat inactivation of Fetal Bovine Serum (FBS)

Removed a bottle of FBS (500 mL) from –20°C freezer and placed it in a 37°C water bath with a water level higher than the serum level in the bottle. This may take more 2 hours or more. Intermittently mixed by inversion. When serum was completely thawed, incubated for an additional 15 min to allowed serum to equilibrate at 37°C water bath. Raised the temperature setting of the water bath to 56°C from 37°C. During this incubation, bottle was inverted to mix the serum at every 10 min. Once water bath reached 56°C, incubated serum for 30 min. Bottle was inverted at every 10 min. Bottle was removed from water bath and allowed to cool it at room temperature for 30 min. Filtered

with 0.22 µm pore size GVWP filters (Millipore Corporation, MA, USA) and aliquoted 50 mL of heat inactivated serum into sterile 50 mL Falcon tubes and stored at -20°C.

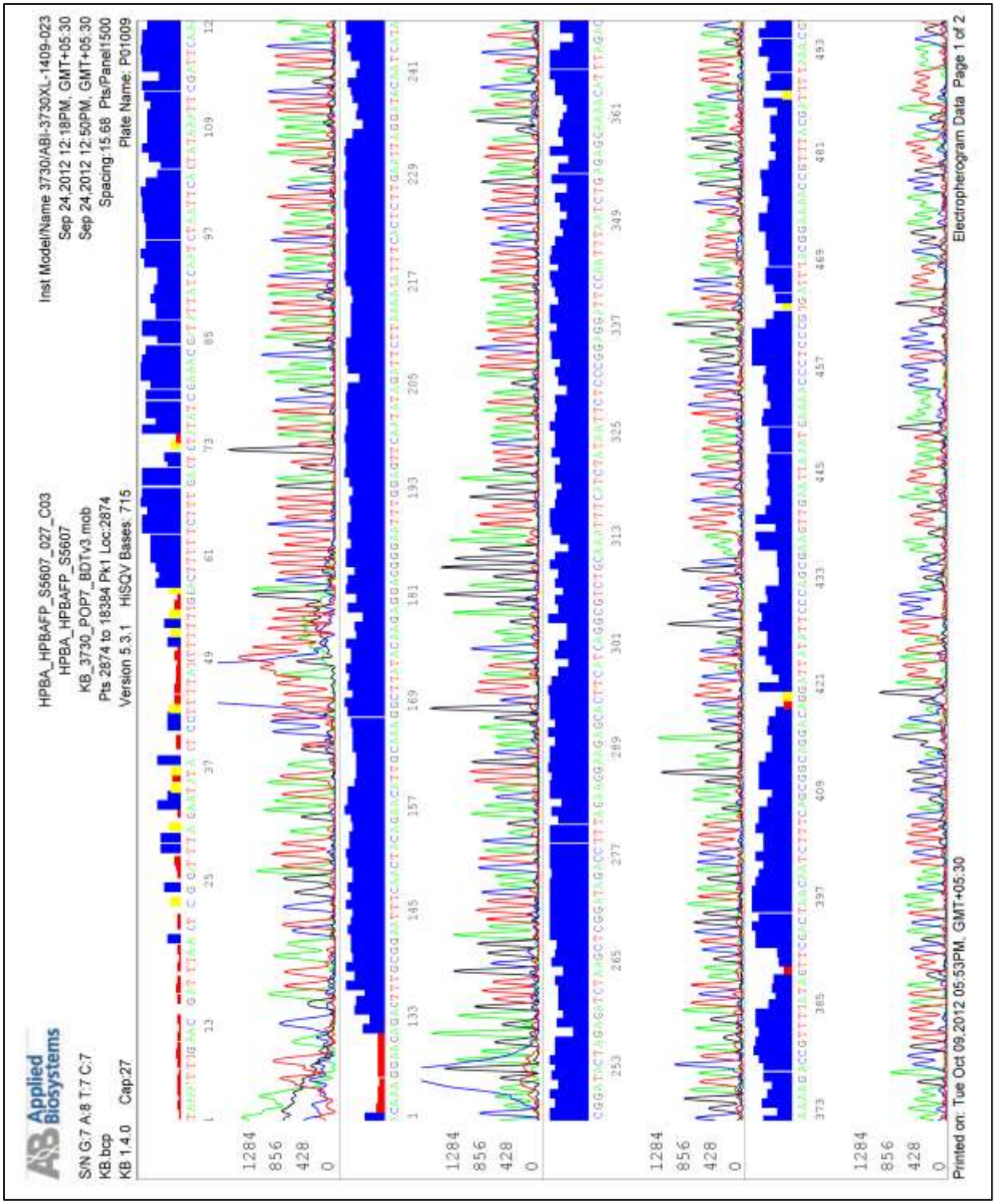
A7. Enumeration of *Leptospira* under the dark field microscope

Suspensions of well-grown *Leptospira* serovars were harvested by centrifugation at 13,000 rpm for 20 min. Pellet was washed twice with PBS. A 1:100 dilution of culture was prepared using PBS in order to adjust the cell amount to be within a countable range. 10 µL of diluted sample was loaded on glass slide and covered with 18 mm square glass coverslip. The total number of *Leptospira* were counted by taking the average of 4 corner and center at 100x magnification under dark field microscopy

Number of Leptospires = Total no. of Leptospires x dilution factor x 10^4

II.B1. Sequence analysis of *hbpA* from *L. interrogans* serovar Lai strain 56601.

(i) Electropherogram data of the sequencing of the recombinant plasmid pMS508 containing the 720 bp insert of *hbpA* (Fig. II.1)



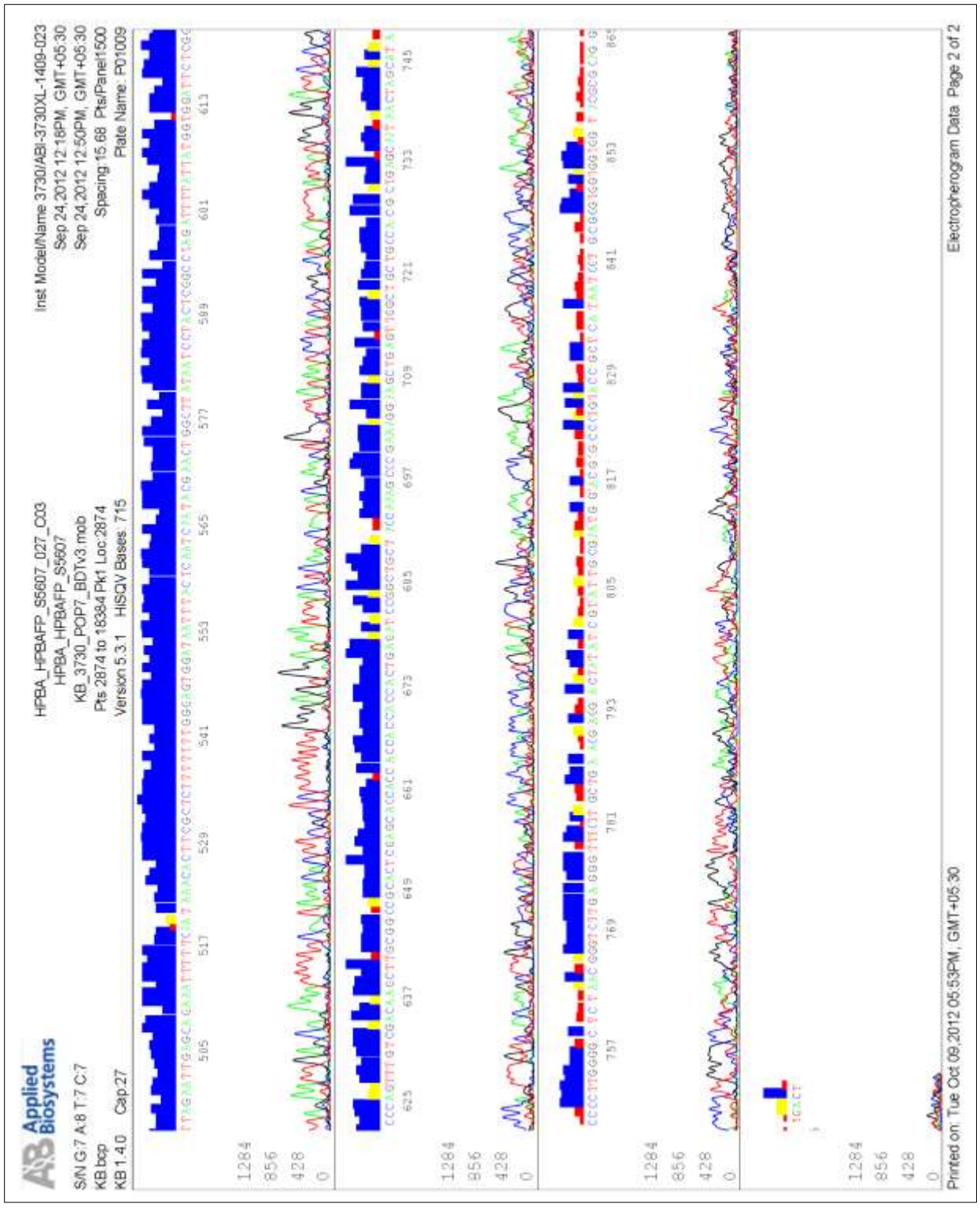


Figure II.1. Figure shows electropherogram data of the sequencing of the plasmid pMS508 using *hbpA*$_{720}$ gene-specific primers. The graph was generated using ChromasPro (Version 2.4, Technelysium Pty. Ltd., Australia).

(ii) Sequence alignment: Figure II.2 shows the alignment of the 720 bp fragment of *hbpA* with the 2310 bp full length sequence.

(a) FASTA sequence of *hbpA*$_{720}$

```
CTTTTTCTTTGAGTGTATATCGAAACGATATTATCAATCTAATTCAGTATAAATTCGA
TTCAAACAAAGGAAGAGAGTTTGCGGAATTTCAACTACAGAACATTGCAAAGGCTT
ATACAAGAGGAGGGGAATTTGGAGTTCAATATAGATTCTTAAAATATTTCACTCTTG
AATTAGGATACAATCATACGGATACTAGAGATCTAAGCTCGGATAGACCTTTAGAAGG
AAGAGCACTTCATCAGGCGTCTGCAAATTTCATCTATAATTCTCCCGGAGGATTCCA
ATTTAATCTGAGAGGAAAACATTTAGACAAAAGACCGTTTTATAGTTCGACTAACAA
TCTTTCAGCGGCAGGACAGGATTATATTCCCAGCGAAGTTGAATTAAATGAAAACCC
TCCCGTGATTTACGGAAAACCGTTTACGATTTTAAACGTTAGAATTGAGCAGAAATT
TTTCAATAAACACTTCGCTCTTTTTTTGGGAGTGGATAATTTACTCAATCAATACGA
ACTGGCTTATAATCCTACTCGGCCTAGATTTTATTATGGTGGATTCTCGGCCCAGTTT
```

(b)

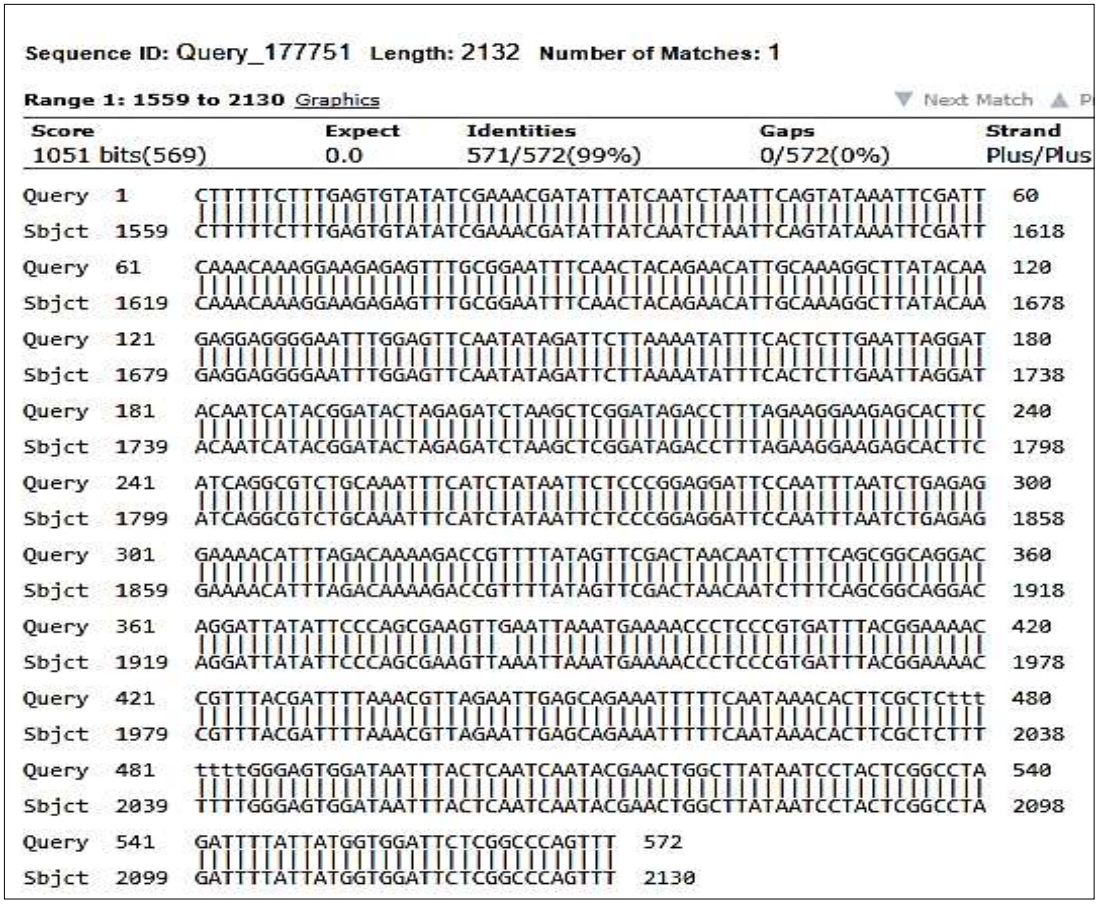
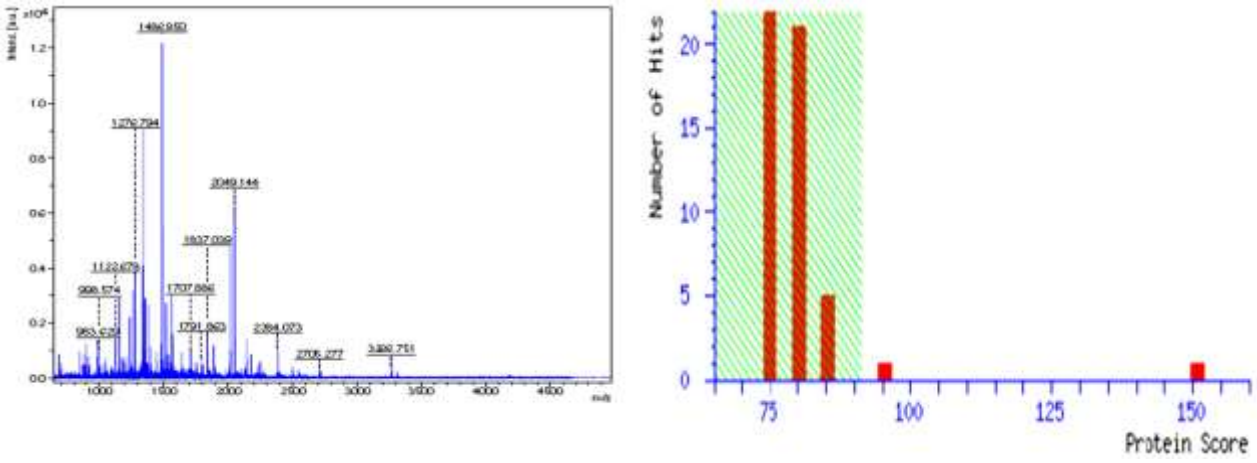


Figure II.2. Analysis of the *hbpA*$_{720}$ insert. The cloned DNA was sequenced using *hbpA*$_{720}$ gene-specific primer. Panel (a) shows the insert sequence obtained from sequencing. Panel (b) shows the comparison of the insert sequence with the LB191 sequence from genome.

II.B2. MALDI-TOF MS/MS. Six leptospiral proteins identified by immunoblotting were subjected to MALDI MS / MS analysis (Fig.II.3). The figures below show the peptide mass fingerprints and analysis by the software MASCOT showing the significance of the identification.

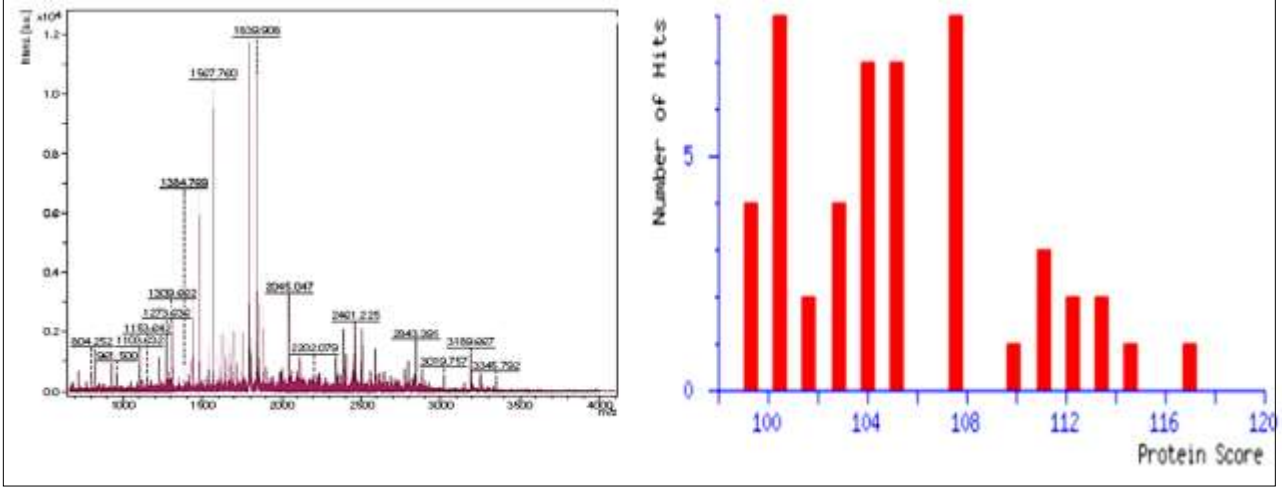
i. Biotin requiring enzyme (LA2432): Molecular mass of 102 kDa

(a) (b)



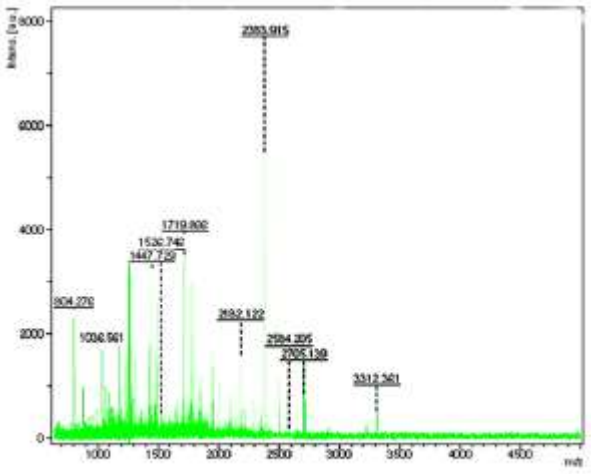
ii. Acyl-CoA dehydrogenase (LA2639). Molecular mass of 58 kDa

(a) (b)

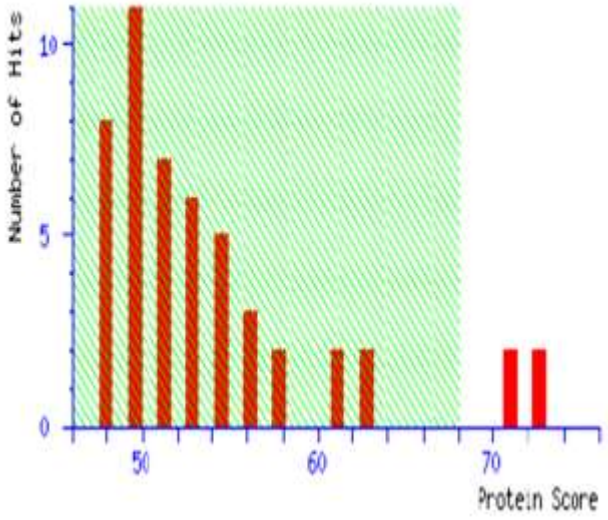


iii. Serine hydroxyl methyl transferase (LA1409): Molecular mass of 50 kDa

(a)

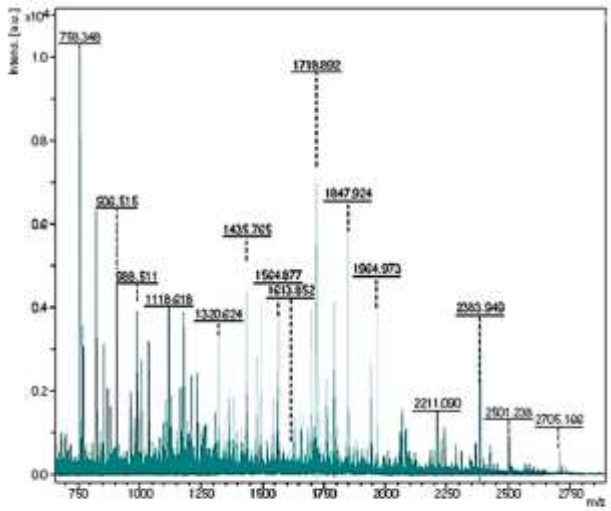


(b)

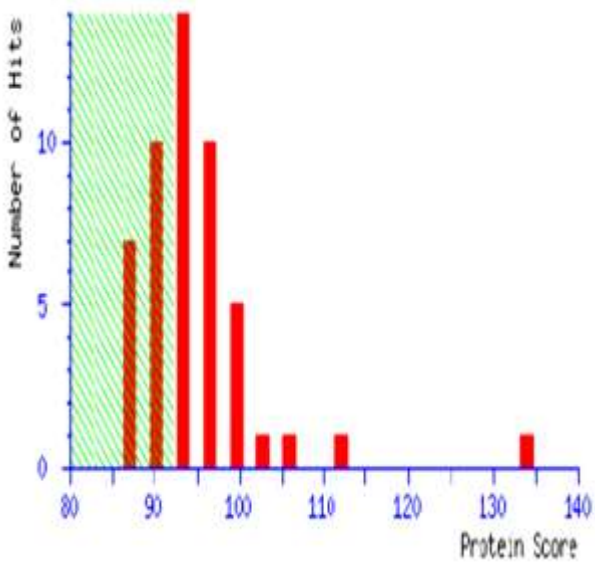


iv. Argininosuccinate synthase (LA4165): Molecular mass of 41 kDa

(a)

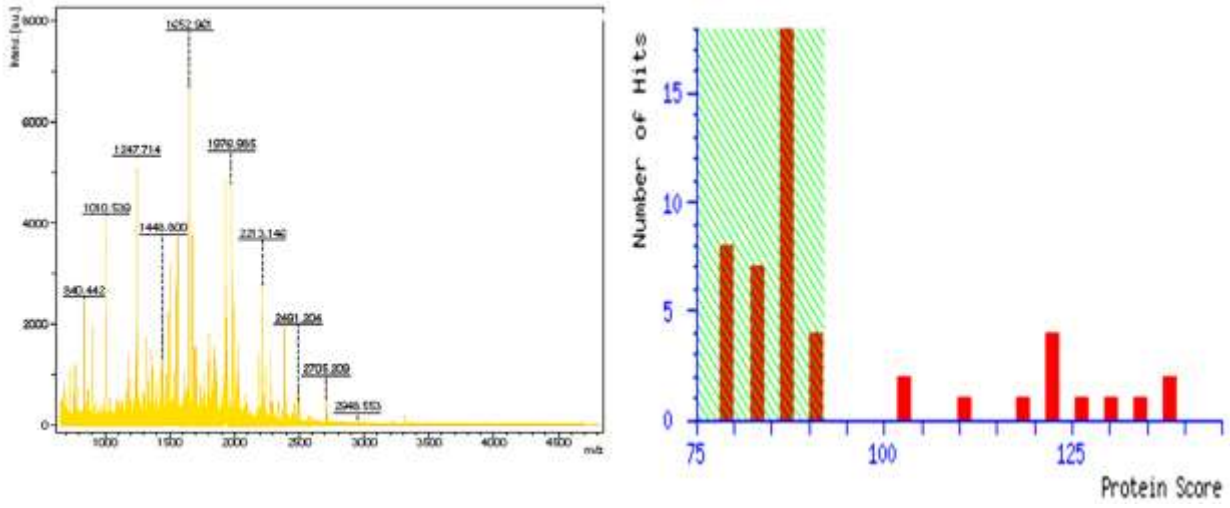


(b)



v. Ferritin-like protein (LA3598): Molecular mass of 16 kDa

(a) (b)



vi. Molecular chaperone GroES (LA2654): Molecular mass of 10 kDa

(a) (b)

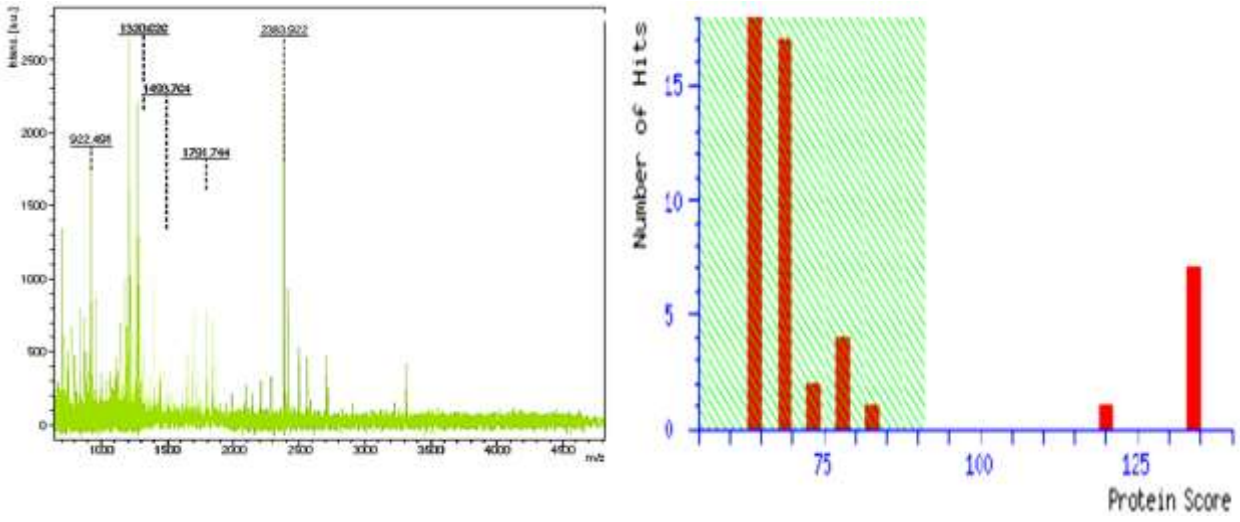


Figure II.3: Identification of six selected proteins by MALDI-TOF-MS/MS. Figures (i to vi) shows six identified proteins as Biotin requiring enzyme (LA2432), Acyl-CoA dehydrogenase (LA2639), Serine hydroxyl methyl transferase (LA1409), Argininosuccinate synthase (LA4165), Ferritin-like protein (LA3598) and Molecular chaperone GroES (LA2654) respectively. Panel (a) shows the peptide mass fingerprint of each of these proteins and Panel (b) shows the MASCOT score.

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ORIGINAL ARTICLE

Pathogen-specific leptospiral proteins in urine of patients with febrile illness aids in differential diagnosis of leptospirosis from dengue

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Abstract

Leptospirosis and dengue are two commonly seen infectious diseases of the tropics. Differential diagnosis of leptospirosis from dengue fever is often difficult due to overlapping clinical symptoms and lack of economically viable and easy-to-perform laboratory tests. The gold standard for diagnosis is the microscopic agglutination test (MAT). In this study, the diagnostic potential of screening for pathogen-specific leptospiral antigens in urine samples is presented as a non-invasive method of disease diagnosis. In a study group of 40 patients, the serum was tested for anti-leptospiral antibodies by MAT and enzyme-linked immunosorbent assay (ELISA). Urine of these patients was screened for leptospiral antigens by ELISA using specific antibodies against LipL32, LipL41, Fla1, HbpA and sphingomyelinase. Group I patients ($n = 23$) were classified as leptospirosis-positive based on MAT and high titres of circulating IgM-specific anti-leptospiral antibodies. All of these patients excreted all five leptospiral antigens in the urine. The 17 MAT-negative cases included six patients with pyrexia of unknown origin (PUO; Group II) and 11 confirmed dengue patients (Group III). The latter tested negative for both serum anti-leptospiral antibodies and urinary leptospiral antigens. A salient outcome of this study was highlighting the usefulness of screening for urinary leptospiral antigens in disease diagnosis, as their presence confirmed leptospiral aetiology in two PUO patients. Immunoblots of urinary antigens identified well-defined bands corresponding to LipL32, HbpA and sphingomyelinase; the significance of the 42- and 58-kDa sphingomyelinase bands is discussed.

Introduction

Leptospirosis is a zoonotic disease seen commonly in tropical regions during monsoon seasons. The disease dynamics, host preferences and pathogenesis are well documented in several reviews [1–5]. The organisms preferentially reside and multiply in the proximal convoluted tubules of the kidney and are periodically shed in the urine of infected hosts. The contaminated environment serves as a source of infection to other animals and humans. In humans, who are often accidental hosts, the disease is acute and usually self-limiting, but can develop into the severe fatal form called Weil's syndrome, involving the lungs, liver and kidneys.

The clinical manifestations of fever and myalgia in this disease is also seen in several viral and bacterial infections. In this study, the focus is on the differential diagnosis of leptospirosis from dengue fever, a vector-borne viral infection. Globally and in India, there are several reports on the co-incidence of these two diseases [6–10]. Differential diagnosis of leptospirosis is challenging, not only due to the lack of defined clinical presentations but also because of limitations in screening tests. With fever and myalgia seen in a majority of patients in regions endemic for these two diseases, there is a need for economical, easy-to-perform tests for early and specific diagnosis. The 'gold standard' for leptospirosis is the microscopic agglutination test (MAT) [11]. With the identification of leptospiral- and pathogen-specific antigens, enzyme-linked immunosorbent assay (ELISA) is increasingly being used as it is reported to give results comparable to MAT. LipL32 [12–15], LipL41 and Omp1 [16], iron-regulated hemin-binding protein HbpA [17–19], Lsa63 [20] and LigB [21] are antigens of diagnostic potential. In

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addition, there are reports of host biomarkers that help to differentiate the two diseases [22, 23].

Recently, we reported the presence of anti-leptospiral antibodies in the urine of leptospirosis patients [24]. In this study, we report the detection of the leptospiral antigens LipL32, LipL41, HbpA and sphingomyelinase in the urine of MAT-positive leptospirosis patients and discuss their diagnostic significance in the light of their absence in patients with dengue fever.

Materials and methods

Study group and sample collection

The study group included 40 patients at MOSC Medical College Hospital in Kolenchery, Kerala, India. They were subjected to routine clinical examination and laboratory testing that included complete blood picture and biochemical parameters. Eleven of these patients were confirmed cases of dengue, all of whom tested positive with the commercial Dengue Day 1 Test (J. Mitra & Co Pvt. Ltd, India) that detects dengue NS1 antigen and IgM and IgG antibodies to dengue virus in human serum. The clinical symptoms and biochemical parameters of the remaining 29 patients (Table S1 in the supplementary material) was indicative of leptospirosis, with most of these patients presenting with fever and myalgia. Other symptoms, found to be varying among the patients, included jaundice, low blood pressure, diarrhoea, bronchial asthma, pancreatitis, anaemia etc.; involvement of the liver, lungs and kidneys was seen in some of them.

Blood (3 mL) and urine (5 mL) samples were collected from these patients. Ethical clearance and informed consent from the study subjects were obtained from the hospital and clearance from both the Institutional Ethical Committee and Institute Biosafety Committee from the University of Hyderabad were obtained for the study.

Bacterial strains, growth and preparation of whole cell sonicates

Leptospira reference strains were obtained from the Regional Medical Research Centre (ICMR), Port Blair, Andaman and Nicobar Islands. The following were used for MAT analysis: *L. interrogans* (Australis strain Ballico, Bangkinang strain Bangkinang I, Canicola strain Hond Utrecht IV, Hebdomadis strain Hebdomadis, Lai strain 56601, Pomona strain Pomona and Hardjo strain Hardjoprajitno), *L. weilii* (Celledoni strain Celledoni), *L. borgpetersenii* (Hardjo-bovis strain L550, Tarassovi strain Perepelicin), *L. biflexa* (Andamana strain CH11, Patoc strain Patoc I). The organisms were grown at 30 °C in liquid EMJH medium supplemented with 10% enrichment medium (Difco Laboratories, USA).

Cell-free whole cell sonicates for ELISA and Western blotting were prepared from the pathogens *L. interrogans* serovars Lai and Pomona, *L. borgpetersenii* serovar Hardjo-bovis and the non-pathogen *L. biflexa* serovar Andamana. The organisms were grown to a cell density of 2×10^8 cells/mL in liquid EMJH-BSA medium, supplemented with 120 mM NaCl and incubated for 4 h to induce the expression of sphingomyelinases [25]. They were harvested, washed, sonicated and the debris removed by centrifugation at 13,000 rpm for 20 min. Protein concentration in the cell-free whole cell sonicates was estimated by the BCA kit (Sigma Aldrich, St. Louis, MO, USA) and stored at − 80 °C till use.

MAT

MAT was performed as described earlier [18] with the panel of live serovars listed above. The end point was taken as the highest dilution of the serum in which 50% of the organisms showed agglutination or there was a 50% reduction in the number of organisms as compared to the control. Titres ≥ 100 were considered positive.

Screening of serum IgM-specific anti-leptospiral antibodies by ELISA

ELISA was performed using leptospiral whole cell sonicates mentioned above. The protocol was essentially as reported earlier [18]. Five hundred nanogrammes of cell-free sonicates of the four *Leptospira* serovars were coated in a 96-well microtitre plate. Each set of antigens was incubated with the patients' serum (1:200 dilution), goat anti-human IgM (μ-chain specific)–peroxidase conjugate (1:5000; Sigma Aldrich, St. Louis, MO, USA) and 100 μL of the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO, USA) and 3 μL of H_2O_2. The plate was read in an ELISA reader (Model 680 XR, BioRad, USA) and the OD_{450nm} was recorded. The controls included known positive and negative serum samples and antigen and antibody blanks. Statistical analysis and graphs were generated with GraphPad Prism (version 6). The results were analysed by the non-parametric Mann–Whitney test and were considered as statistically significant when $p < 0.001$.

Processing of urine samples

Urine was processed as follows for all the studies detailed below. Five millilitres of urine was precipitated with 5 mL of saturated ammonium sulphate, allowed to stand for 20 min at room temperature and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 200 μL distilled water and dialysed against phosphate buffered saline (0.1 M phosphate buffer, pH 7.4, 0.9%

NaCl). The protein concentration was estimated with the BCA kit (Sigma Aldrich, St. Louis, MO, USA).

ELISA-based detection of leptospiral antigens in urine samples

ELISA was done with antibodies against LipL32, Lip41, Fla1 and $Sph2_{163}$ (all of these were provided by Prof. David Haake, UCLA), hemin-binding protein HbpA, $rSphCD_{210}$ and rSph4 (lab collection). Antibodies against $Sph2_{163}$ was against a 163 aa fragment comprising aa 27–190 (full-length Sph2 is 607 aa) and anti-$SphCD_{210}$ antibodies were against a 210 aa fragment of Sph3 containing aa 123–332 (full-length Sph3 is 510 aa).

ELISA was done as reported earlier [18]. Four hundred nanogrammes of total urinary proteins was coated on the microtitre plate, all of the above-listed antibodies were added at 1:1000 dilution and goat anti-rabbit IgG (Fc specific)–alkaline phosphatase conjugate was used at 1:5000 dilution (Sigma Aldrich, St. Louis, MO, USA). The colour was developed with 100 μL of p-nitrophenyl phosphate (Sigma Aldrich, St. Louis, MO, USA) for 20 min and the OD_{450nm} was read in an ELISA reader (Model 680 XR, BioRad, USA). The results were analysed as described above for serum antibodies.

SDS-PAGE and Western blotting: detection of LipL32, HbpA and sphingomyelinase in patients' urine samples

50 μg of total urinary proteins from Group I MAT-positive cases (n = 23) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (5–20% gradient) and immunoblot analysis was performed using standard procedures. Four identical blots of each urine sample were incubated separately with antibodies (diluted 1:1000) against LipL32, $SphCD_{210}$, $Sph2_{163}$ and HbpA, respectively. Anti-rabbit IgG–alkaline phosphatase conjugate (diluted 1:5000) was used as the secondary antibody and the blot was developed with NBT-BCIP (Sigma Aldrich, St. Louis, MO, USA).

Western blot analysis of whole cell sonicates of *Leptospira* spp. with serum and urine of leptospirosis patients

Serum and urine from ten Group I patients were selected based on the high titre of anti-leptospiral antibodies in serum. Urine was also included as urinary antibodies of both IgG and IgM classes against leptospiral proteins was reported in our earlier study [24]. 60 μg of the four leptospiral whole cell sonicates (Lai, Pomona, Hardjo-bovis and Andamana) were subjected to SDS-PAGE and immunoblotting with serum and urine, used at a dilution of 1:200, followed by the addition of anti-human IgG–alkaline phosphatase conjugate (diluted 1:5000; Sigma Aldrich, St. Louis, MO, USA). The reacting bands were detected by addition of the substrate NBT-BCIP solution (Sigma Aldrich, St. Louis, MO, USA).

MALDI-TOF-MS/MS analysis

Six leptospiral proteins in leptospiral whole cell sonicates detected with serum and urinary antibodies were purified by preparative gel electrophoresis, re-run and confirmed by immunoblotting. The approximate molecular mass of these proteins was determined using UVP VisionWorks LS Image Acquisition and Analysis Software. They were subjected to matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) analysis (Sandor LifeSciences Pvt.Ltd., Hyderabad, India) and identified using the Mascot software.

Results

MAT-based identification of patients with leptospirosis

All of the Group I patients (n = 23) tested positive by MAT; Lai and Pomona emerged as the major infecting serovars, with Hardjo-bovis, Celledoni, Tarassovi, Australis and Bangkinang being the other serovars. Group II (pyrexia of unknown origin, PUO, n = 6) and Group III dengue patients were MAT-negative.

Elevated levels of IgM-specific serum anti-leptospiral antibodies in Group I MAT-positive patients with leptospirosis

Sero-diagnosis of leptospirosis of Group I patients was confirmed by demonstrating high levels of IgM-specific anti-leptospiral antibodies in the serum of all 23 patients (Fig. 1). The titres were high, with antigens from the pathogenic serovars Lai, Pomona and Hardjo-bovis and, while not negligible, reactivity was low with the serovar Andamana. This was an expected observation due to the presence of cross-reacting antigens. A clear differentiating feature was that Group III patients with dengue fever showed negligible titres. Two PUO cases in Group II (UH26 and UH27) showed a small increase in the levels of anti-leptospiral IgM antibodies (indicated by arrows in Fig. 1), the significance of which is correlated with the presence of urinary leptospiral antigens (discussed below).

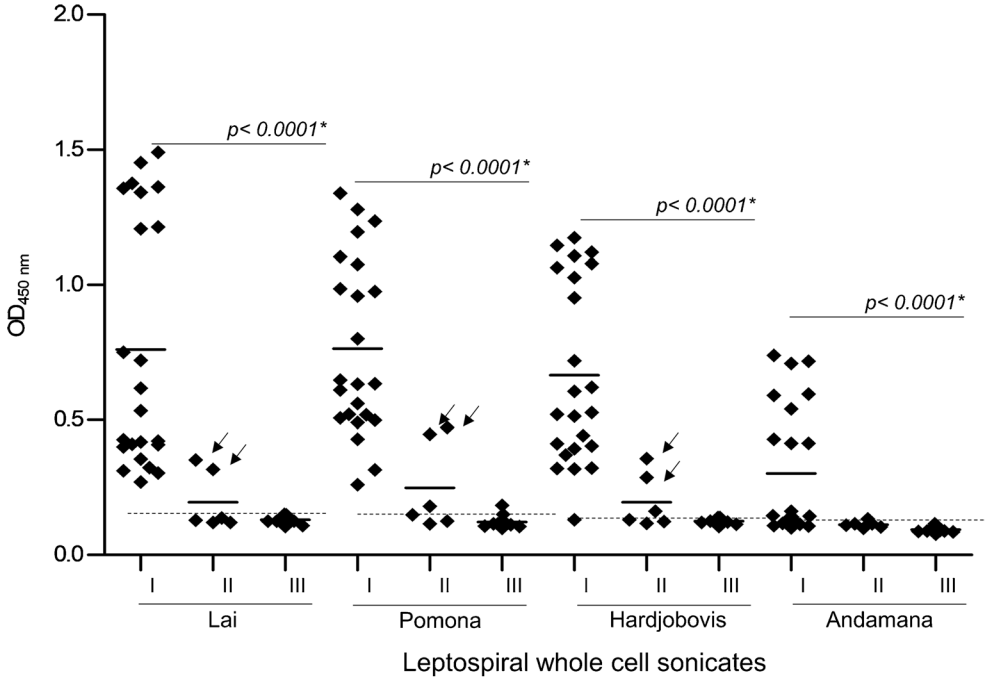

Fig. 1 Enzyme-linked immunosorbent assay (ELISA)-based screening of IgM-specific anti-leptospiral antibodies in serum samples. Whole cell sonicates of the pathogenic serovars Lai, Pomona and Hardjo-bovis and the non-pathogenic serovar Andamana were used as antigens. The test was done with serum from all three groups, as detailed in the Materials and methods section. The results were considered statistically significant when $p < 0.001$. The arrows indicate the two pyrexia of unknown origin (PUO) samples (UH26 and UH27) that showed values higher than the cut-off value, represented by the dotted line. The cut-off was calculated by adding two times the standard deviation (SD) to the mean value obtained with Group III dengue patients

Pathogen-specific leptospiral proteins in the urine of Group I patients with leptospirosis

As it is well known that leptospires migrate to the kidneys and are shed in the urine of infected animals and humans, we screened the urine samples for leptospiral antigens. ELISA of all 40 samples with the respective antibody against LipL32, LipL41, Fla1, HbpA, $SphCD_{210}$, $Sph2_{163}$ and Sph4 is presented in Fig. 2. A notable and differentiating feature was that none of the leptospiral antigens were detected in the urine of Group III dengue patients (Fig. 2a, b) but were present at high levels in Group I patients with leptospirosis. This observation is of clinical significance and relevance to the diagnosis of leptospirosis. Interestingly, the urine of the two PUO cases (UH26 and UH27 showing slightly elevated levels of serum IgM anti-leptospiral antibodies) showed the presence of LipL32, HbpA and $SphCD_{210}$. The absence of these leptospiral proteins in the urine of the remaining four patients with PUO led us to confirm UH26 and UH27 as suffering from leptospirosis and not dengue fever, in spite of testing negative for MAT. Leptospiral sphingomyelinases deserve special mention here. Whole-genome sequencing has shown the presence of three, five or seven *sph* genes encoding sphingomyelinases in *L. borgpetersenii* [26], *L. interrogans* [27] and *L. santarosai* [28], respectively. It is unclear if some or all of these genes are expressed in vivo and if the expressed proteins undergo proteolytic cleavage to yield functionally active molecules. Therefore, sphingomyelinases were detected using two different antibodies, one raised against the exo-endophosphatase domain ($SphCD_{210}$), common to all sphingomyelinases (with the exception of Sph4), and the second raised against the N-terminal fragment of Sph2 ($Sph2_{163}$), considered to be a true sphingomyelinase [29]. Figure 2b showed the better performance of antibodies against $SphCD_{210}$ than $Sph2_{163}$, and it is also evident that Sph4 in urine was negligible, implicating that it was not expressed, being a non-functional sphingomyelinase.

Immunoblots of patients' urine, developed with the above two anti-sphingomyelinase antibodies (anti-$SphCD_{210}$ and anti-$Sph2_{163}$ antibodies) revealed the presence of two different sphingomyelinases of approximate molecular masses of 42 and 58 kDa, respectively (Fig. 3). The 42-kDa band was seen in UH3, a patient whose urine, tested by ELISA, showed high levels of all four leptospiral antigens; LipL32 was also seen as a discrete band in the immunoblot. Two patients (UH1 and UH16) showed the presence of a 58-kDa sphingomyelinase, with the 42-kDa sphingomyelinase absent in these two samples. LipL32 was visualised in the immunoblots of three additional samples (Fig. 3), including UH22, the latter also showing the presence of a 73-kDa HbpA protein. Immunoblots for LipL41 were not done due to low urine sample availability.

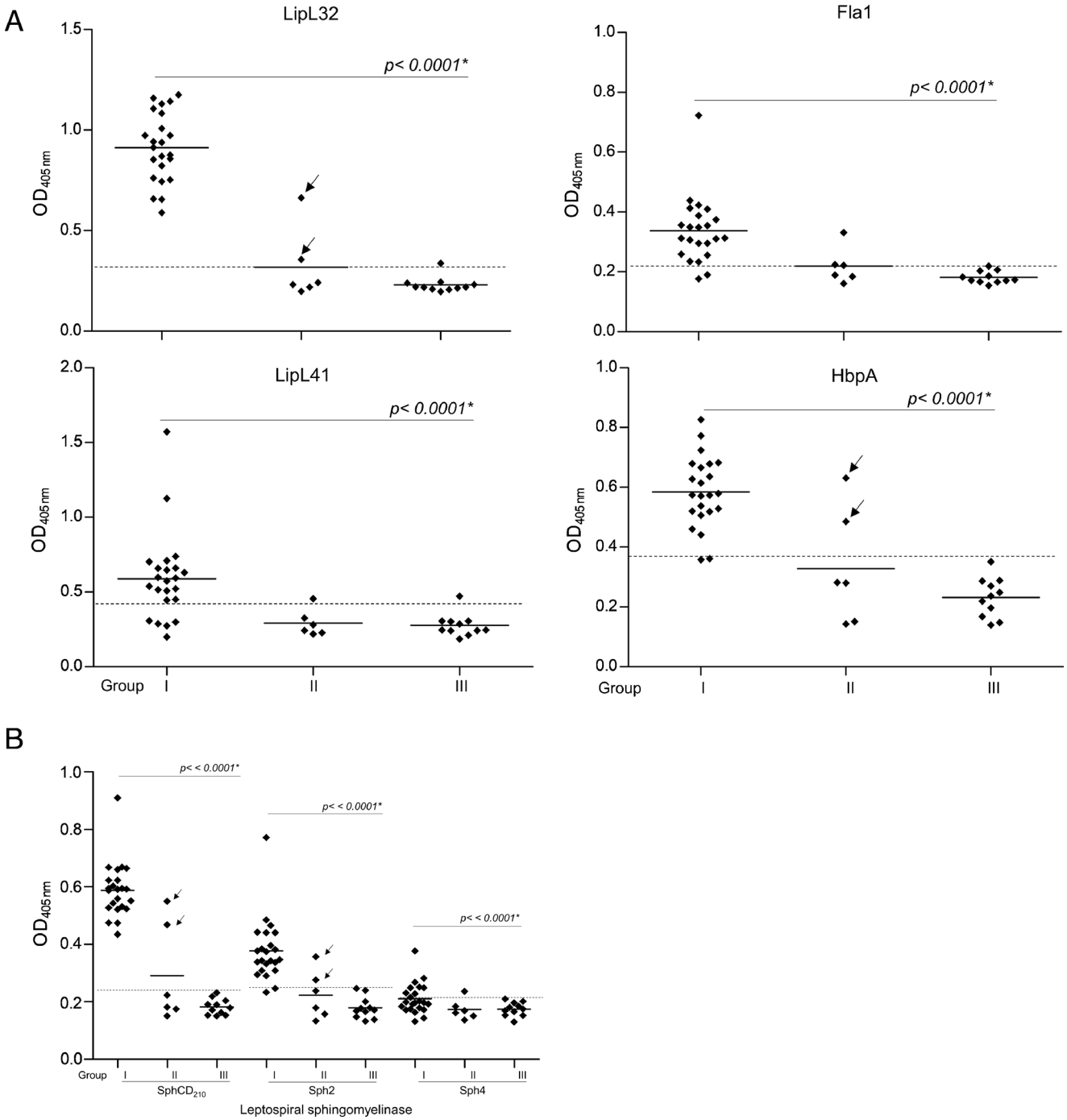

Fig. 2 Detection of leptospiral antigens in urine samples by ELISA. **a** The detection of LipL32, LipL41, Fla1 and HbpA in the urine of all three study groups using the respective antibodies. **b** Sphingomyelinase detected using anti-$SphCD_{210}$ and anti-$Sph2_{163}$ antibodies, respectively. It also shows the low titres seen with antibodies against rSph4, a sphingomyelinase lacking the exo-endophosphatase domain. The dotted line in each graph represents the cut-off calculated by adding two times the SD to the mean value obtained with Group III dengue patients

Western blotting of leptospiral whole cell sonicates with serum and urinary antibodies of patients with leptospirosis

The objective of this test was to identify the immunogenic leptospiral proteins expressed by the infecting pathogenic serovars using serum and urine antibodies from patients with leptospirosis. Leptospiral protein extracts from the three pathogenic serovars and the non-pathogenic serovar Andamana, when subjected to Western blotting with serum and urinary anti-leptospiral antibodies from ten Group I patients, showed strongly reacting bands (Fig. 4) that were seen predominantly in the pathogens. While there were some cross-reacting bands in the non-pathogenic serovar Andamana, they were not

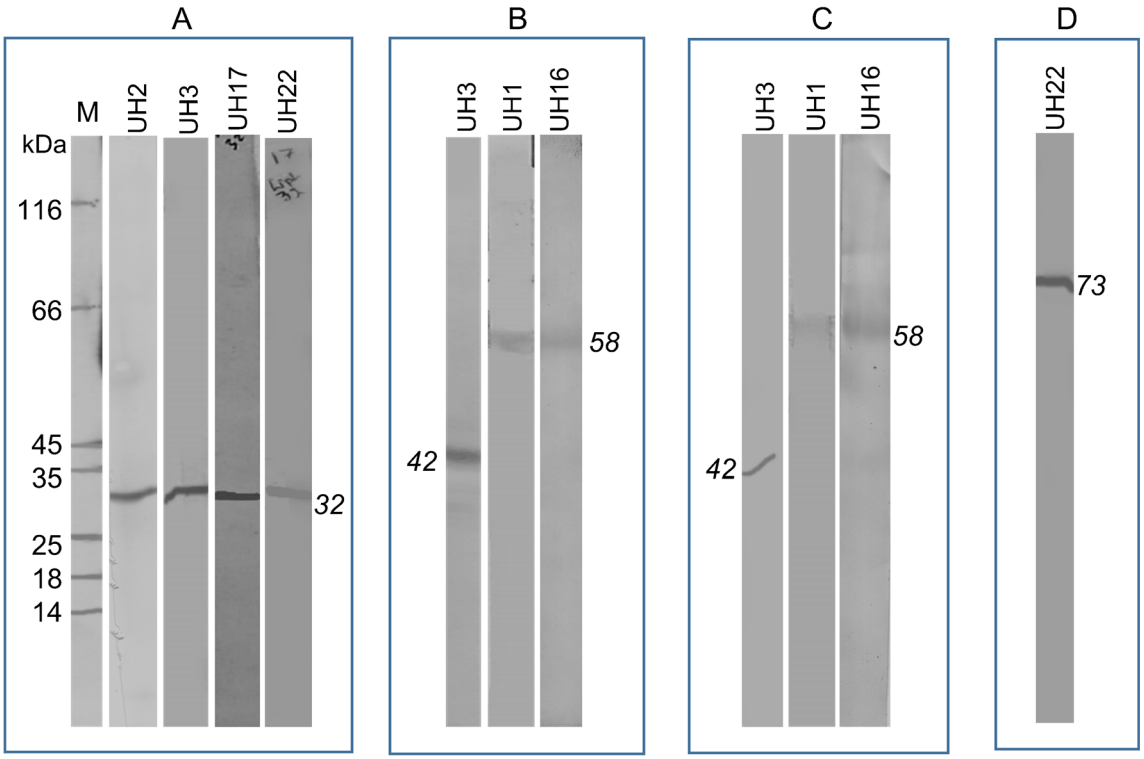

Fig. 3 Western blot analysis for the detection of leptospiral antigens in urine samples from patients with leptospirosis. Urine samples from Group I leptospirosis patients were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using antibodies against LipL32 (A), $Sph2_{163}$ (B), $SphCD_{210}$ (C) and HbpA (D), respectively. The blots were developed as detailed in the Materials and methods section. The different samples are represented by the patient number. M represents the molecular weight marker

considered for analysis. The notable observations from these immunoblotting studies were as follows: first, the circulating anti-leptospiral antibodies were excreted in the urine of some of the patients; second, these antibodies recognised pathogen-specific antigens, with antigenic similarity seen among the three serovars; and third, the immunogenic leptospiral proteins expressed in vivo (as inferred indirectly by the respective antibodies in serum and urine) varied among the different patients. Six of these leptospiral proteins that reacted strongly with antibodies present in serum and urine were of approximate molecular masses of 102, 58, 50, 41, 16 and 10 kDa, respectively. Upon subjecting to MALDI-TOF MS/MS analysis, they were identified as biotin-requiring enzyme (LA2432), acyl-CoA dehydrogenase (LA2639), serine hydroxyl methyl transferase (LA1409), argininosuccinate synthase (LA4165), ferritin-like protein (LA3598) and the molecular chaperone GroES (LA2654), respectively.

Discussion

In a tropical country like India, the aetiology of febrile illness varies and diagnosis often depends on the correlation of clinical symptoms with laboratory tests. With fever and body aches commonly seen in illnesses like influenza, dengue, malaria and chikungunya, there is a need to rule out these diseases while diagnosing leptospirosis. The salient outcome of this study is the correlation of the presence of pathogen-specific leptospiral proteins in the urine of leptospirosis patients with MAT positivity and high titres of circulating IgM-specific anti-leptospiral antibodies. The leptospiral proteins LipL32, LipL41, Fla1, HbpA and sphingomyelinase(s), expressed only by pathogenic *Leptospira* spp., were excreted in the urine of leptospirosis patients in Group I cases and were absent in dengue patients, reflecting their potential in the differential diagnosis of leptospirosis from dengue. Their clinical relevance stems from the finding that two PUO patients in Group II, who tested MAT-negative, excreted these antigens in their urine, thus confirming leptospiral aetiology and explaining the rise, though small, in the levels of serum anti-leptospiral antibodies.

In this study on 40 human patients, Groups I and III were confirmed cases of leptospirosis and dengue, respectively. The former were confirmed as leptospirosis based on clinical symptoms and MAT positivity. These patients showed high levels of IgM antibodies against the proteins from pathogenic *Leptospira*, with marginal levels of cross-reactivity with the non-pathogenic serovar Andamana. The Group III dengue patients, whose diagnosis was confirmed with a commercial kit presented markedly low levels of serum IgM-specific anti-

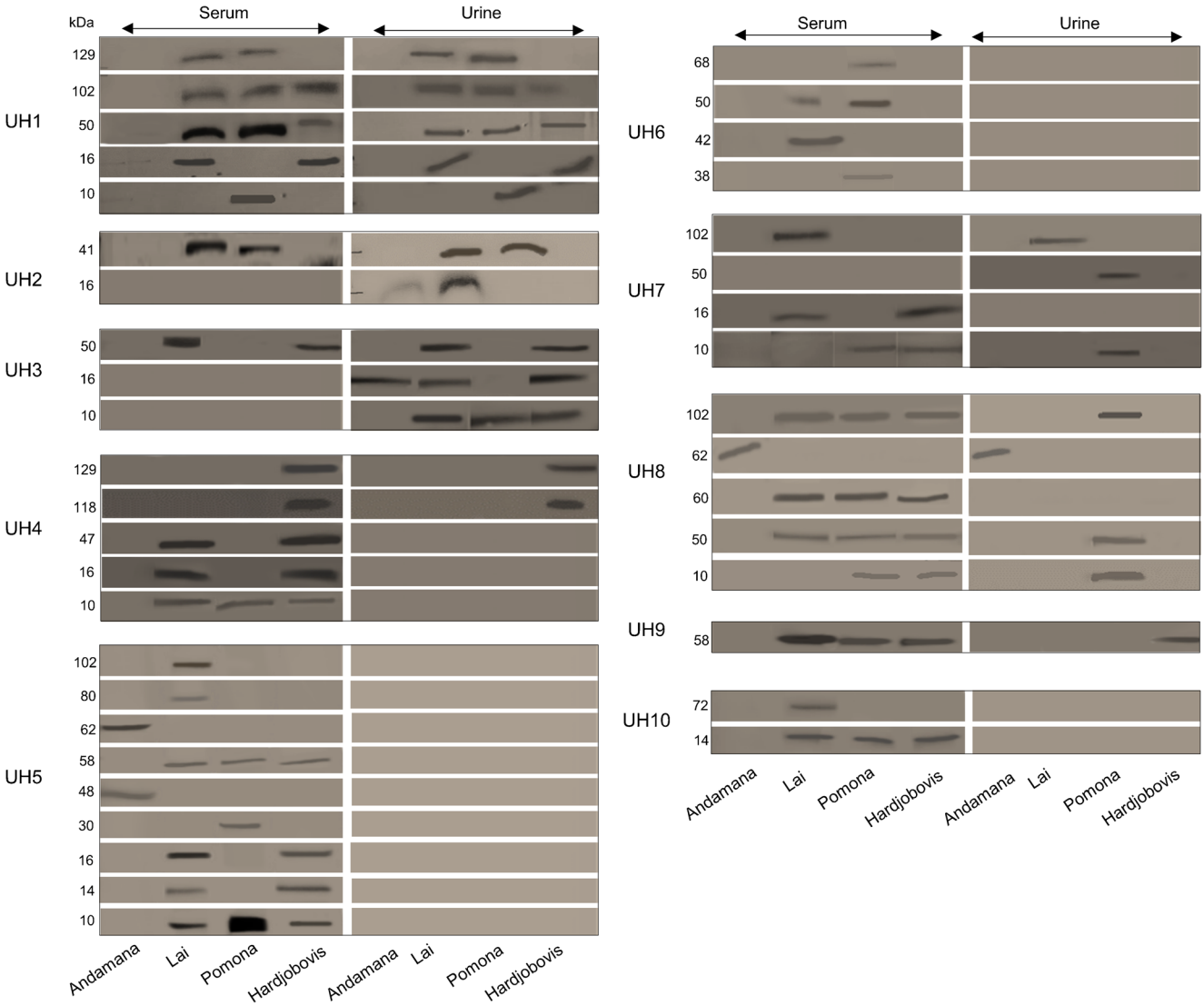

Fig. 4 Immunoreactivity of serum and urinary antibodies with leptospiral proteins from pathogenic *Leptospira*. Whole cell sonicates of the serovars Lai, Pomona, Hardjo-bovis and Andamana were subjected to SDS-PAGE and upon transfer to nitrocellulose membrane subjected to immunoblotting with both serum and urine from ten patients in Group I (UH1–UH10), who showed high levels of serum anti-leptospiral antibodies

leptospiral antibodies that were considered as baseline values and used for comparison of the titres in Groups I and II. Though the small increase in the circulating anti-leptospiral antibody levels in the two PUO cases was not suggestive of leptospiral infection in the light of MAT being negative, this ambiguity was cleared upon detecting leptospiral antigens in their urine samples. Leptospiral aetiology was further strengthened by demonstrating the presence of antibodies against the pathogen- and leptospiral-specific antigens HbpA and LipL41 (Fig. S2 in the supplementary material) in these two patients. Though MAT is a specific test for leptospirosis, it is serovar-specific and omission of a serovar in the reference panel can result in concluding a sample as negative. Due to other disadvantages of MAT, including the need for live serovars, failure to detect non-agglutinating antibodies [30] and the need to perform the test with a second sample after 48 h to demonstrate rise in titre in patients from endemic regions for disease confirmation [30, 31], there is a need for the development of alternative tests for the diagnosis of leptospirosis. Antigen detection reflects active infection and can be effectively done using specific antibodies against defined antigens with established diagnostic potential.

The screening of urine samples for leptospiral antigens was done because urinary shedding of leptospires is well established [32–34]. All Group I leptospirosis patients excreted LipL32, LipL41, HbpA and sphingomyelinase in the urine. These pathogen-specific antigens, expressed in large amounts in one of the patients (UH3) with the severe form of the disease, clearly indicated active infection. The specificity of detection in patients with leptospirosis and not with dengue

reflected the potential of these proteins in the differential diagnosis of the two diseases. LipL32, an abundantly expressed outer membrane protein by pathogenic *Leptospira* spp. [35], is expressed in vivo [36, 37] and has been demonstrated in tissues isolated from experimentally infected animals [36, 38–40]. Though the functional role of the protein remains a mystery despite its abundance [35], its potential as a diagnostic antigen is well known. Several reports on the usefulness of screening for anti-LipL32 antibodies in serum [12, 41, 42], including preparation of better antigenic peptides of the protein [12], highlight the potential of this protein in the serodiagnosis of leptospirosis. Complementing its diagnostic potential, this study highlights the detection of the protein in the urine of leptospirosis patients both by ELISA and Western blotting, the latter providing evidence of a clear, defined band of 32 kDa. LipL32 antigen detection, being more confirmatory for disease diagnosis than demonstrating anti-LipL32 antibodies in serum, is a good alternative to MAT.

The detection of sphingomyelinases in the urine of leptospirosis patients is an important finding in this study. They are virulence factors produced by pathogenic *Leptospira* spp. Historically, haemolytic activity was reported in leptospiral culture filtrates in 1956 [43]. They are implicated in host tissue damage, mainly the lungs, liver and kidneys, with manifestations including haemorrhage, jaundice and renal failure [29, 40]. They act either by virtue of their enzymatic activity or pore-forming activity [29]. Whole-genome sequencing, first reported in *L. interrogans* serovar Lai [27], revealed the presence of five sphingomyelinase (*sph*) genes. While it is not established as to why a single serovar should harbour several *sph* genes, a plausible explanation is that they are differentially regulated by changes in the immediate environment. In addition, different species differ in the total number of *sph* genes; five in serovars Lai and Copenhaginii, three in *L. borgpetersenii* [26] and seven in *L. santarosai* [28], with none detected in the non-pathogenic *L. biflexa* [44]. We reported Sph2 of serovar Lai to be a true sphingomyelinase based on its structural features and biological activity [29, 45, 46]. In this study, urinary sphingomyelinases were detected using antibodies against the common exo-endophosphatase domain ($SphCD_{210}$) and against Sph2 ($Sph2_{163}$). The performance of anti-$SphCD_{210}$ antibodies was superior to anti-$Sph2_{163}$ in the detection of sphingomyelinase by ELISA, possibly due to the recognition of the enzymatic domain presented in all functional sphingomyelinases. This also explains the negligible titres seen with antibodies against the sphingomyelinase Sph4 that lacks the enzymatic domain. In addition to the clinical significance of detecting these virulence factors only in leptospirosis patients, their release into the urine even within the first few days after the onset of clinical symptoms is noteworthy. Thus, the screening of urine for its presence can assist the clinician not only in diagnosis but also in assessing the severity of the disease.

The identification of sphingomyelinase with two different molecular masses warrants attention. With immunoblotting being less sensitive than ELISA, only three samples tested positive, with the 58-kDa band seen in two samples and a 42-kDa sphingomyelinase in patient UH3, who presented with severe symptoms and excreted notably high levels of all the antigens. The 42-kDa sphingomyelinase is highly likely to be regulated by iron levels, as inferred from our earlier finding of the 42-kDa sphingomyelinase protein in vesicles isolated from iron-limited *L. interrogans* serovar Lai [47]. Since iron-limiting conditions inside in the mammalian host and the regulatory role of iron on the expression of bacterial toxins [48] are well-studied phenomena, it seems highly likely that this sphingomyelinase is the consequence of the pathogen responding to the iron-limiting conditions. It remains to be seen if this 42-kDa protein is the cleaved product of a bigger molecule and, if so, what triggers the cleavage? While iron levels appear to be one of the contributing factors, factor(s) influencing the expression and release of the 58-kDa sphingomyelinase warrants further analysis.

HbpA is an iron-regulated protein expressed in vivo and our earlier reports highlight the clinical significance and the sero-diagnostic potential of detecting high levels of circulating anti-HbpA antibodies [17, 18] in patients with leptospirosis. Here, HbpA in urine not only confirmed the iron-limiting conditions faced by the pathogen, but also confirmed leptospiral aetiology, as none of the dengue patients showed HbpA in their urine. ELISA detected HbpA in the urine of all of the Group I leptospirosis patients, with a 73-kDa HbpA band seen in one sample (UH22) in Western blotting. Since it is smaller than the predicted size of 81 kDa in serovar Lai, it is possible that this band represents HbpA from a different infecting serovar. LipL41, also a hemin-binding protein, is an antigen of diagnostic potential [49, 50]. Analysis of a larger sample size for both these hemin-binding proteins will help us to understand their diagnostic potential; here, though we demonstrated LipL41 in urine by ELISA, we could not perform Western blotting due to unavailability of the urine samples.

As detailed above, we highlighted the usefulness of screening for leptospiral proteins in the urine of human patients. It may be mentioned that all these cases were examined within a week of developing clinical symptoms of fever and myalgia. Detailed studies are needed to assess the window period for antigen detection upon infection and assess the severity of the disease based on antigen levels, specifically sphingomyelinase. Specific and timely diagnosis of leptospirosis will contribute to timely and effective control measures that can prevent the progression of the disease to the severe fatal form. Renal damage is well known in leptospirosis and, in this study, albumin and antibodies were seen in the urine of some of these patients, with amounts ranging from traces to highly positive, even within the first week of developing symptoms. The possibility of renal damage appears likely, since not only albumin but also

the larger antibodies were detected. These observations, coupled with sphingomyelinase expression, can be extended to a larger population to develop better control measures, particularly for early diagnosis.

Our earlier studies [24] and this study showed anti-leptospiral antibodies in the urine of patients with leptospirosis. Here, we demonstrated that these antibodies recognised several antigens from pathogenic *Leptospira*, with an identical pattern of reactivity as serum antibodies. Among several proteins recognised by these antibodies from leptospirosis patients, six strongly reacting antigens, identified by MALDI-TOF MS/MS analysis, were found to be enzymes/proteins involved in metabolic pathways, including a 16-kDa ferritin-like protein. While the functional significance of these proteins under in vivo conditions warrants further studies, immunoblotting must be done with in vivo-derived leptospiral cultures, as it is likely that some of the in vivo-expressed antigens were not detected in this study because protein extracts from laboratory-grown cultures were used.

In conclusion, the study highlights the diagnostic potential of leptospiral proteins in the urine of leptospirosis patients. Diagnosis of the disease and differentiation from other febrile illnesses may be achieved by a combination of tests, including the evaluation of serum/urinary anti-leptospiral antibodies using ELISA and testing of urine for leptospiral antigens. This combination will ensure that all cases will be detected, irrespective of when the patient comes to the hospital after the appearance of clinical symptoms. This will help to identify cases without performing the cumbersome MAT, as seen with the two cases of undiagnosed fever. The findings also provide scope for the development of point-of-care devices for leptospiral antigen detection in urine. The sensitivity of detection must be increased to establish effective diagnosis.

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Author contributions MS conceptualised the study, designed the experiments and prepared the manuscript; RC performed the experiments and compiled the data; KCT coordinated the processing of the clinical samples and biochemical analyses; CKE, BJZ and RP were the clinical partners and coordinated patient selection, sample collection and collection of clinical data.

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Compliance with ethical standards

Conflict of interest All authors report no conflict of interest with reference to this article.

Ethical approval Ethical clearance and informed consent from the study subjects were obtained from MOSC Medical College Hospital. Clearance from both the Institutional Ethical Committee and Institute Biosafety Committee from the University of Hyderabad were obtained for the study.

Informed consent Informed consent from the study subjects were obtained as per the guidelines of the Ethical Clearance Committee of MOSC Medical College Hospital.

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Original Research Article

Proteinuria in early detection of human leptospirosis

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ABSTRACT

Background: Leptospirosis is an infectious disease caused by spirochetes bacteria Leptospira spp. and is reported from all over the world. As the clinical signs and symptoms of Leptospirosis often are nonspecific and the disease is early mistaken for other major infectious febrile illness, laboratory test to confirm the clinical diagnosis thus is essential for optimal treatment and patient management.
Methods: Serum and urine samples were collected from patients clinically suspected cases of Leptospirosis. Preparations of urine concentrate by precipitation and centrifugation.
Results: It was interesting to note that immunoglobulins are present in the urine protein concentrate of patients with Leptospirosis on the day of admission in the hospital, with urine albumin reports either positive or negative. By ELISA test it was noted that antibodies present in urine and serum were of both IgM and IgG class against the Leptospiral antigens from three pathogenic serovars and one non-pathogenicserovars. In the immunospot test which was done and compared with standard ELISA test for serum antibodies using same antigen showed that antibodies present in urine protein concentrate, which was collected on the day of admission when patients come with suspecting symptoms of Leptospirosis.
Conclusions: Proteinuria is the most frequent abnormality noted in all patients at some stage of illness. This is the first report on the presence of immunoglobulins in urine samples, which were found to be of IgM and IgG classes. These findings are of significant diagnostic potential as a simple immune-spot test can be done for detecting anti-leptospiral antibodies in urine samples of suspected cases. The present attempt was aimed at developing an immunospot test, a simple and rapid diagnostic test to detect Leptospirosis using urine samples of clinically suspected patients of the infection at the earliest. It was found to be in good correlation with standard ELISA method which is being used to detect serum antibodies in Leptospira infected patients using the same antigen.

Keywords: Immunospot, Leptospirosis, Urine IgM and IgG

INTRODUCTION

Leptospirosis can be endemic in countries with wet and warm climates. It is an important infectious disease of humans and animals worldwide, caused by the pathogenic serovars of genus Leptospira. The symptoms and severity of Leptospirosis vary greatly from mild flu-like illness to the often fatal hemorrhagic form, called Weil's disease characterized by Jaundice, pulmonary haemorrhage, renal damage due to involvement of vital

organs such as liver, lung and kidney.[1] Early and accurate diagnosis of Leptospirosis is important for proper and prompt treatment, which is life-saving for patients with severe illness. Leptospirosis may be confused with Malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, meliosidosis and others.[2]

Human are accidental host and acquire the infection from contaminated environments. Rodents and farm animals contribute to the transmission of the disease by contaminating the environment by shedding the leptospires in their urine. Hence, the disease transmission is enhanced due to poor hygienic conditions and is an occupational. In humans, while the disease is often self-limiting, the pathogen may cause serious damage when it colonises in the kidneys, where it evades the host immune responce.[3] Due to the strong humoral immune response, several serological assays have been developed for detecting the rise of serum antibodies such as Flow-through, Ig dipstick immunoflurescence, latex agglutination, microcapsule agglutination test, the direct enzyme linked immune sorbent assay (ELISA) for immunoglobulin IgM antibodies, and dot ELISA for IgM.[4-11]

These methods are being questioned, thinking that appropriate immune response might not yet have been elicited by the time of specimen collection. Immunochromatography based methods for detection of Leptospiral lipopolysaccharide antigen in urine have also been developed.[12] The sensitivity and specificity of these methods are often debatable due to prior exposure of the individuals in endemic areas and due to the contaminating nonpathogenic lectospires in the environment.

Leptospires are shed in the urine of patients with the disease. However, it has not been practical to detect them either by culture or molecular methods. Detection of urine leptospiral LPS, though the sensitive and specific, suffers from the disadvantage of laborious sample preparation before analysis. In this study we report for the first time the presence of antibodies of IgM and IgG classes that are specific for leptospiral antigens in the urine of patients with leptospirosis.[13] This has significant diagnostic implications as a simple, rapid, cost effective test for early diagnosis by detection of anti-leptospiral antibodies in urine of patients with the disease.

METHODS

The sample collection was coordinated by department of Biochemistry in collaboration with Department of Medicine. The antigen preparation, ELISA test and MAT analysis were shared by Microbiology of MOSC Medical College, Kolenchery and Department of Animal Biology, University of Hydrabad, India. Tests including immuno-electrophoresis and immune spot test were done in Biochemistry Department, MOSC Medical College. The study was approved by institutional ethics committee. Urine and serum samples were collected from patients admitted in our hospital with various febrile illnesses, with their consent. Preparation of antigen as per world health organization guide lines.[2,10]

Leptospiral serovars used in this study: *L. interrogans* serovars Lai and Pomona; *L.borgpetersenii* serovar Hardojobovis and the non-pathogenic *L.biflexa* serovar Andamana for the detection of IgM and IgG –specific antibodies in serum and urine samples. *L. biflexa* Patoc I for routine ELISA test for Diagnosis of Leptospira infection in patients was used for immune-spot test.

Preparation of whole cell sonicates of the leptospiral serovars

All the serovars were grown till log phase in EMJH-BSA liquid media. The cultures were induced with 120mM NaCl for 4h at 30^0C (this was to induce the expression of sphigo-myelinases).The cells were harvested and washed twice with 0.1M PBS. Whole cell sonicate of each serovars were prepared by sonication at 40Hz amplitude, 20s pulses for 5 min with cooling for 20s in Vibra Cell sonicator, (Sonics, Newtown, CT,USA).Protein was estimated by BCA method .

Clinical samples

Serum and urine samples were collected from clinically suspected cases of Leptospirosis.

Preparation of urine concentrate by precipitation and centrifugation-5ml urine collected from normal and clinically suspected cases of *Leptospira* infection, mixed with 5ml of saturated ammonium sulphate (AR) solution and kept for 10 min. The mixture was centrifuged at 12,000rpm for 10 min. The supernatant was decanted and the precipitate was re-suspended in 200µl distilled water. Protein concentration was estimated by Qubit Fluorometer along with normal urine protein concentrate.

Electrophoresis of Urine protein concentrate was done along with serum protein to detect the presence of gamma globulin fraction. Amido-black staining solution was used.[14] Immuno-electrophoresis for detecting the presence of anti leptospiral antibodies using Patolc antigen was done on Whatman paper - IV.[15]

Microscopic agglutination test (MAT)

The leptospiral reference strains used for the study were obtained from the Regional Medical Research Centre and WHO Collaborating Centre for Diagnosis, Reference, Research and Training in Leptospirosis (ICMR), Port Blair, Andaman and Nicobar Islands. The following *Leptospira* spp. were used: *L. Interrogans* (Australis strain Ballico; Bankinang strain B. Bankinang I; Canicola strain Hond Utrecht IV; Hebdomadis strain Hebdomadis; Lai strain 56601; Pomana strain Pomana; Hardjo strain

Hardjoprajitno), *L. weilii* (Celledoni strain Celledoni), *L. borgpetersenii* (Hardjo-bovis strain L550, Tarassovi strain Perepelicin), L.biflexa (Andamana strain CH11; Patoc strain Patoc I). The organisms were grown in liquid EMJH medium with 10% enrichment medium (Difco Laboratories, USA) and maintained at 30^0C.

MAT was done with the above live serovars on a total of 12 serum samples from patients and 5 normal controls. 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.

Enzyme-linked immunosorbent assay (ELISA)

This was done as reported earlier. Three pathogenic serovars, namely *L. interrogans* serovars Lai and Pomona, *L. borgpetersenii* serovar Hardojobovis and one non-pathogenic spp. *L. biflexa* serovar Andamana were used as antigens for the detection of IgM and IgG specific antibodies in serum and urine samples. The organisms were grown to a cell density of 1-2 $x10^8$ cells / mL in EMJH – enrichment medium and the incubated for 4 hr after adding 120 mM NaCl. The cells were harvested at 12,000 x g for 20 min, washed twice with 0.1M phosphate-buffered saline PBS, sonicated for 5 min (20 second pulses at 40 Hz in a Vibra Cell sonicator, USA) and centrifuged to obtain the cell-free whole cell sonicate, in which the total protein was estimated by the commercial BCA kit (Sigma Aldrich, St. Louis, MO, USA).

ELISA was done with the serum and urine samples using the above three antigens for the detection of IgM and IgG antibodies. Briefly, 500 ng antigens was used and serum and urine were added at 1:200 dilution and the secondary antibody-enzyme conjugate included goat anti-human IgG (Fc specific)-peroxidase conjugate and goat anti-human IgM (µ-chain specific)-peroxidase conjugate (1:5000 dilution) respectively for the detection of IgG and IgM-specific antibodies. The substrate was 3, 3', 5, 5'-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO, USA) and 3µl of H_2O_2. The colour was allowed to develop for 20 min, followed by addition of 1.25 M sulphuric acid as stop solution. The absorbance was measured at 450 nm with ELISA reader (Model 680XR, Bio-Rad, CA, USA). Experiments were performed in duplicate and repeated twice. The controls included antigen and antibody blanks, known positive and negative serum samples. Statistical analysis of the data was done with Graph Pad Prism software (Version 6, Graph Pad Software, Inc., San Diego, CA) and graph was generated using Sigma Plot 10 software.

The results were analysed by Mann Whitney test considered significant when $P<0.05$. The cut-off value for IgM and IgG ELISA was calculated by adding two times standard deviation (SD) to the mean value of normal endemic controls (2SD + Mean).

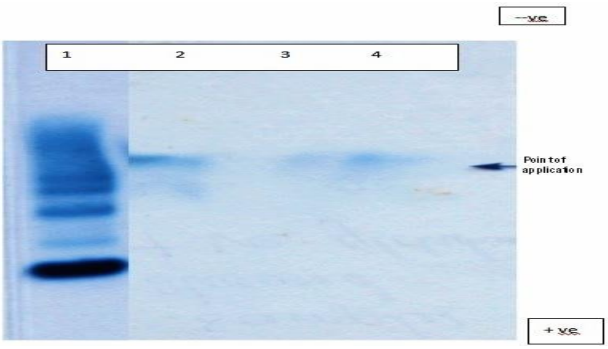
Immunospot test

According to the above observations tried to develop Immunospot test. Two sets of clinical specimens were used in this study. In the first set, 192 urine samples were collected from patients with different febrile illness with history of 3-8 days of fever. WHO criteria used for the diagnosis of Leptospirosis and compared with the Enzyme linked immune-sorbent assay for IgG antibodies, which is the diagnostic test performed in our Microbiology lab. In second set, serum and urine samples were collected on the first day of admission and after 5 or 6 days hospitalization from clinically suspected cases of Leptospirosis. In blood samples, the presence IgM antibodies were detected using ELISA assay. In urine samples, protein was concentrated by precipitation and centrifugation. 3µl of antigen (*L.biflexa* Patoc I) spoted on Whatman paper IV and allowed to dry for 3 min. Applied 5µl of urine concentrate on the same spot and kept 3min. for antigen-antibody reaction, the excess protein concentrate was removed by washing with distilled water, stained with diluted (1:9 with distilled water) amido-black protein stain for 3min. and destained with destaining solution[15] for 10 min. Deeply stained spot could be observed in samples with *Leptospira* infection compared to less or no staining for normal and differently infected samples. Healthy inhabitants of the same geographical area were considered as normal.

RESULTS

Detection of anti-leptospiral antibodies in the urine of leptospirosis patients

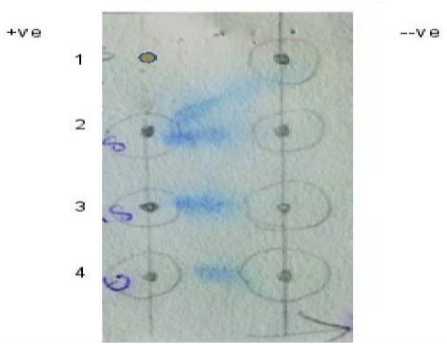
The preliminary study of urine protein concentrate showed that the protein concentration was increased in infected samples compared to normal samples. The electrophoresis of urine protein concentrate along with serum showed the presence of Immunoglobulins in infected samples with urine albumin positive or negative (Figure 1).



Sample no.1-serumsample; 2, 3, and 4-Urine protein concentrate of Leptospira infected patients

Figure 1: Agar gel-electrophoresis.

The immune-electrophoresis showed the presence of immuno-precipitin formation, indicated that the antibodies of *Leptospira* is present in the urine concentrate. There is an interesting finding that Leptospiral antibodies are moving towards the nearby antigen spot when the corresponding antigen spot is kept as blank (Figure 2).



Sample: 1- Blank; 2, 3, 4 Urine protein Concentrate, Leptospiral antigen move towards anode and form precipitin line with antibodies. Antigen opposite to blank move towards sample no.2

Figure 2: Immunoelectrophoresis on Whatman IV, paper.

Anti-leptospiral antibodies are of IgM and IgG classes

A selective panel of 12 serum and urine samples were tested by MAT and ELISA to identify if the antibodies were against the leptospiral antigens. The serovar Lai was identified as the predominant serovar by MAT analysis. MAT identified 10 of the 12 serum samples as positive, with titres ranging from 100 to 3200, with mixed infection also seen. Serovar Lai was predominant (41%), with the others including Hardjobovis (18%), Pomona and Celledoni (6%) (Table 1). All the healthy controls and two patients' samples tested negative by MAT.

IgM and IgG ELISA done with were serum and urine samples are represented in Figure 3 and 4. The two MAT negative samples were not included for analysis. The serum showed high titres of both IgG and IgM classes were observed against the pathogenic serovars as compared to the non-pathogenic serovar Andamana.

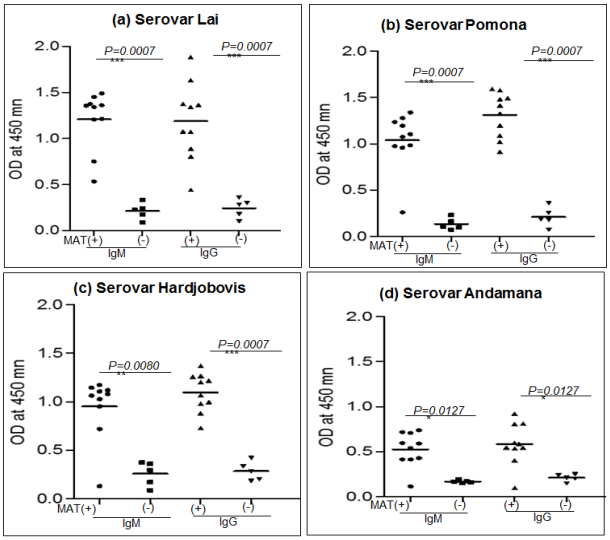
Interestingly, the urine samples showed antibodies of the IgG class. Notable is the unusually high titre (with $OD_{450\ nm}$ being 1.953) in one of the samples and it must also be noted that antibodies were seen against the non-pathogenic serovar Andamana. As this reflects cross-reacting antigens, pathogen-specific antigens may be preferred.

Table 1: Serotyping of patient's serum samples by gold standard MAT.

Sample ID	MAT Result	Titre
NS	P	Lai and Ponona (1:3200), Hardjobovis (1:400), Tarassovi (1:200), Celledoni (1:100)
JH	P	Lai (1:3200), Ponona (1:1600), Hardjobovis (1:400), Celledoni (1:200)
Sample 1	P	Lai (1:3200), Hardjobovis (1:1600), Tarassovi (1:200), Pomona (1:100)
Sample 2	P	Hardjobovis (1:3200), Pomona (1:400), Bankinang (1:200), Lai (1:100)
Sample 3	P	Hardjobovis (1:800), Lai (1:400), Pomona (1:200), Celledoni and Tarassovi (1:100)
Sample 4	N	MAT negative
Sample 5	P	Pomona (1:1600), Lai (1:800), Hardjobovis (1:400), Tarassovi (1:100)
Sample 6	N	MAT negative
Sample 7	P	Lai (1:800), Pomona, Hardjobovis and Tarassovi (1:100)
Sample 8	P	Hardjobovis (1:800), Pomona and Celledoni (1:400), Lai (1:200)
Sample 9	P	Lai (1:800), Pomona, Hardjobois and Celledoni (1:100)
Sample 10	P	Celledoni (1:3200), Lai (1:200), Pomona, Hardjobovis and Tarassovi (1:100)
HC1*	N	MAT negative
HC2	N	MAT negative
HC3	N	MAT negative
HC4	N	MAT negative
HC5	N	MAT negative

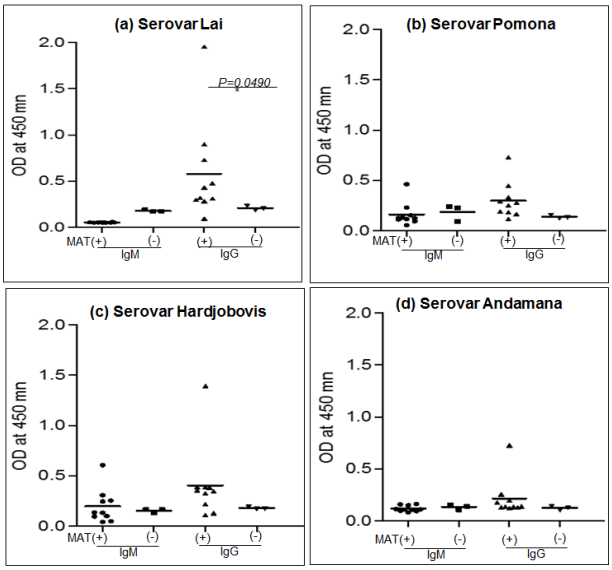
*HC refers to healthy controls.

The IgM class of antibodies was low in the urine, with only one sample showing the presence of antibodies against the serovars Pomona and Hardjobovis. It is highly likely the larger size of this class does not leak through unless the renal damage is severe. Urine, usually free of any proteins in normal healthy individuals showed the presence of protein, especially immunoglobulins under disease conditions may be indicating the involvement of the kidneys.



All the MAT positive (+) represent the patient samples with the MAT (-) representing the controls. The figure was generated using Graph Pad Prism. The values are the mean of four values obtained from duplicates done from two independent experiments.

Figure 3: IgG and IgM-specific anti-leptospiral antibodies in the serum of leptospirosis patients. Panels (a), (b) and (c) represent the serum antibodies against the cell-free whole cell sonicates of pathogenic serovars Lai, Pomona and Hardjobovis and the panel (d) represents the non-pathogenic serovar Andamana.

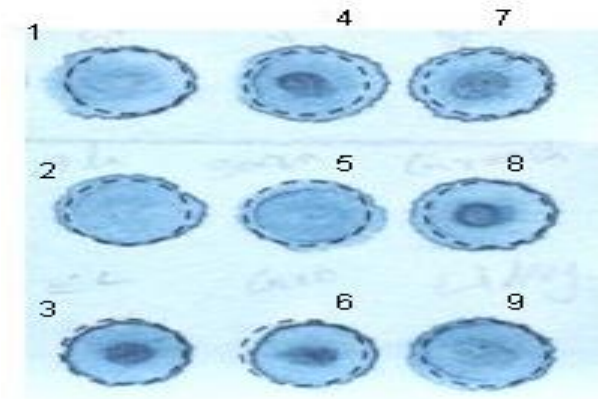


All the MAT positive (+) represent the patient samples with the MAT (-) representing the controls. The figure was generated using Graph Pad Prism. The values are the mean of four values obtained from duplicates done from two independent experiments

Figure 4: Urinary immunoglobulins: IgM and IgG-specific anti-leptospiiral antibodies in patients with leptospirosis. Panels (a) to (d) represent the levels of IgM and an IgG-specific antibody against whole cell sonicates of serovars Lai, Pomona, Hardjobovis and Andamana.

Potential of the immune spot test for the diagnosis of Leptospirosis

The immune-spot test was read in comparison with standard ELISA test using the same antigen (Figure 5). In the first set of the study it was observed that out of 192 samples 130 (68%) showed immunospot positive for Leptospira antibodies in urine. Among this 130 samples, 60% were positive for both ELISA and immunospot. 30% of immunospot positive samples have not been requested for ELISA. The 10% immune spot positive showed IgM titre in ELISA is <20, below the cut off value (<80). It can be noted that even at low titre value of serum antibodies, if the clinical findings are supportive, urine concentrate can give a positive result with immunospot test.



Samples 1 and 2 are Negative controls, Sample 3 positive control; Samples 4, 5, 6,7,8,9, are test samples. 4, 6, 8 were found to be positive and 5, 7, 9 found to be negative.

Figure 5: Immunospot test was done with urine samples of clinically suspected Leptospira infection.

In the second set, out of 21 samples collected from clinically suspected cases of Leptospirosis, 19 urine samples collected on the day of hospitalization and its corresponding serum samples collected during 5-8 days of hospitalization for ELISA test (WHO guide lines) showed significant correlation. Sensitivity found to be100% and specificity 25% (Table 2) Though antibody titre was less in serum samples collected on the day of hospitalisation it was found to be raised after 5-8 days. The second urine samples which collected during 5-8 days of hospitalization also showed positive correlation with first urine samples and the corresponding serum samples.

DISCUSSION

The infectious disease Leptospirosis is found to be life threatening due to its increase incidence in developing countries, increased severity of the disease, delayed diagnosis and/or misdiagnosis. Leptospirosis is not readily distinguishable based on the clinical presentation and epidemiological background of the patients from other infectious fevers which share the same geographical

areas of endemicity. Laboratory confirmation plays an important supplementary role to the clinical findings and helps in sorting out the early differential diagnosis. There are existing methodologies for Leptospirosis like MAT assay, which are laborious and time consuming, Flow through IgM dipstick, Immuno-florescence and latex agglutination, which are with low sensitivity and specificity during early stage of infection.[7]

Table 2: Comparative study of Leptospira infected urine samples using Immuno-spot test and ELISA Test.

ELISA IgM Titre 1st sample	ELISA IgM Titre 2nd sample	Immuno spot for 1st sample	Immuno spot for 2nd sample	Febrile days prior to hospitalisation
80	320	+ ve	+ve	5
20	20	+ ve	+ve	2-3
20	20	+ ve	+ve	3
20	640	+ ve	+ve	3
80	320	+ ve	+ve	5
20	80	+ ve	+ve	2
20	20	+ ve	+ve	2
80	80	+ ve	+ve	4
160	320	+ ve	+ve	3
160	160	+ ve	+ve	3
20	20	-ve	-ve	7
20	20	-ve	-ve	2-3
40	80	+ ve	+ve	4
20	20	+ ve	+ve	2
20	20	+ ve	+ve	4-5
20	80	+ ve	+ve	3
160	640	+ ve	+ve	5
40	320	+ ve	+ve	3
80	40	+ ve	+ve	4
80	80	+ ve	+ve	5
20	160	+ ve	+ve	2 weeks

PCR which is expensive and development of immune-chromatography for urine Leptospiral antigens needed to have laborious pre-treatment of samples. In this situation there is in need to develop a simple, early, rapid and cost effective method to diagnose Leptospirosis in endemic areas.

The preliminary studies showed the presence of Leptospiral antibodies in urine of patients when they come with the clinical symptoms including 3-5 days of fever.[19] This is also been supported that Leptospires colonize host renal tubules, and L*eptospira* persists despite active immune process, such as interstitial nephritis characterized by lymphosite infiltration, anti-leptospiral immunoglobulin production, MHC-II expression and TLR (toll-like receptors) activation.[16,17]

In this study presence of proteins in the urine of patients with renal damage indicating pathology. In leptospiral infected renal injury, albumin is often analyzed in urine samples for assessing renal damage. This is found to be first global report that reports the presence of immunoglobulins in the urine, immediately upon clinical diagnosis. Antibodies, predominantly of the IgG class are seen in the urine, compared to high levels of IgM and IgG in serum samples. The larger IgM antibodies were seen only in one patient urine sample. Though the titre of antibodies against the pathogenic serovars in urine is much lower than that seen in serum, the presence of these antibodies reflects the involvement of the kidney associated with leakage of the serum proteins in to the urine. The high levels of antibodies against the antigens from pathogenic serovars are of clinical relevance.

In the immunospot test, performed well when compared with serum ELISA with same antigen and with clinical symptoms. The test showed the presence of antibodies in the urine that was collected on the day of admission when patients come with suspecting symptoms of the disease. The sero conversion with increase in serum titres and the correlation of the results with urine samples collected on the subsequent days is further evidence of the usefulness of this test. The false positive results in comparison to ELISA Test may be due to the renal involvement during Leptospirosis.[18] Screening of a large sample size and inclusion of other cases with fever, including viral flu,

dengue and malaria will help to validate this test as a useful diagnostic tool.

CONCLUSION

Anti-leptospiral antibodies are present in the urine of leptospirosis patients. The immune-spot assay that is developed in this study can be used for the early, rapid, cost effective diagnosis of the disease in resource poor areas, where leptospirosis is usually endemic.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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Urban leptospirosis: A report of two cases

Dear Editor,

We report two cases of leptospirosis from urban areas of Hyderabad where the reporting is scarce.[1] A 16-year-old girl student (case 1) and a 27-year-old male, a cattle farm owner (case 2) reported to our institute during June–August 2013. The predominant clinical findings being icterus with or without subconjunctival haemorrhage [Figure 1] along with related non-specific signs and symptoms. General physical and systemic examination were, however, unremarkable. Information about exposure to potential sources of *Leptospira spp*. in contaminated water (case 1) and direct contact or possible infection from contaminated environment (case 2), narrowed the differential diagnosis.

Both the cases were investigated after obtaining written informed consent. Hepatorenal parameters indicated deranged hepatic function (case 1) and involvement of kidneys manifested by acute renal failure, non-oliguric (case 2). Malaria, enteric fever, viral hepatitis and others were ruled out considering leptospirosis as the possibility in the differential diagnosis of acute febrile illness [Table 1]. The etiology was considered definite when: (1) serum tested positive for leptospiral specific IgM antibodies (Leptocheck) (2) presence of anti-leptospiral antibodies with the predominant serogroup being Tarassovi 1:1600 (case 1) and Autumnalis 1:3200 (case 2). This was confirmed by gold standard MAT (microscopic agglutination test)[2] using a panel of 19 live leptospiral serovars (3) positive anti-HbpA IgG antibodies, a test validated on several samples of leptospirosis earlier.[3] Culture confirmation of leptospirosis from blood and urine samples was unsuccessful. Over the next 5-7 days, clinical response (with remission of fever,

Table 1: Laboratory investigations

Laboratory investigations	Case 1	Case 2
a) Hepatorenal parameters (with normal values in parenthesis)		
Serum bilirubin (0.2-1.2 mg/dL)	5.8	14.0
Direct bilirubin (0-0.2 mg/dL)	3.2	12.8
Indirect bilirubin (0.2-0.8 mg/dL)	2.6	1.2
Serum alkaline phosphatase (40-120 IU/L)	160	-
SGPT (≤40 IU/L)	185	-
SGOT (≤40 IU/L)	28	-
Serum urea (15-40 mg/dL)	18	100
Serum creatinine (0.2-1.1 mg/dL)	0.6	2.7
Serum electrolytes		
Sodium (120-145 mEq/L)	152.8	148.2
Potassium (3.5-5.5 mEq/L)	4.53	4.51
b) Leptospira-specific investigations		
Microscopic agglutination test (MAT)	Titre of 1600; *Leptospira borgpetersenii* serovar Tarassovi	Titre of 3200; *Leptospira interrogans* serovar Autumnalis
Leptocheck IgM	Pos[$]	Pos
HbpA-IgG ELISA	Pos	Pos
c) Other investigations		
Brucella SAT	Neg[#]	Neg
Widal tube test	Neg	Neg
HBsAG	Neg	Neg
Anti-HCV antibody	Neg	Neg
Dengue IgM	Neg	Neg
HIV1/HIV2/HIV1 and 2	ND*	Neg
Peripheral blood smear for malaria parasite	Neg	Neg
Blood culture for *Salmonella* spp.	Neg	Neg
Urine culture	Neg	Neg

[$]: Positive, [#]: Negative, *: Not done, SGPT: Serum glutamic pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase, Standard agglutination test: SAT; ELISA: Enzyme-linked immunosorbent assay, HCV: Hepatitis C virus, HIV: Human immunodeficiency virus

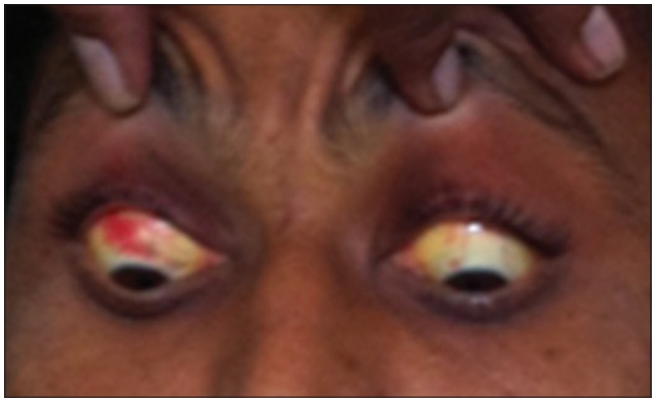

Figure 1: Photograph of a patient (case 2) showing icterus and sub-conjunctival hemorrhage

normal hepatic and renal parameters) to 10-day course of doxycycline 100 mg/twice/day substantiated the clinical diagnosis.

Icterus is the predominant clinical feature of the severity of illness. Hence, detection of systemic leptospirosis by a rapid dipstick assay Leptocheck, with a high sensitivity of 86.8% and specificity of 92.7%[4] plays a pivotal role in early diagnosis. The global criterion for laboratory confirmation of current leptospiral infection is defined as seroconversion or a four-fold rise in titre in paired serum samples, or a single MAT titre ≥1:400 in the presence of clinical signs and history of animal contact.[5] High titre of anti-leptospiral antibodies and the identification of the serovar in both the cases proved that the patients were definite cases of leptospirosis. MAT suffers several disadvantages as routine clinical testing is expensive, requiring technical expertise and maintenance of strains for preparation of live antigens. The knowledge of prevalent serovars in a particular geographic area is required, and a reasonable number of serovars must be included in the panel for effective screening, as MAT-negative sample could be due to the omission of the particular serovar in the test panel.

There are now increasing reports of the use of ELISA-based techniques to replace MAT.[3] Indigenous kits,

such as HbpA–IgG ELISA has shown considerable potential in the identification of positive cases of leptospirosis.[3] HbpA is an iron-regulated hemin-binding protein expressed only by pathogenic *Leptospira* spp. Its absence in the free-living non-pathogenic species and the expression of the protein *in vivo*, specifically under conditions of iron limitation (prevailing in the mammalian host in any bacterial infection), reflects active infection. It is presumed to contribute to the specificity of the test and prevent false positive results. As culture confirmation has not been easy to achieve, as seen here, screening for anti-leptospiral antibodies, using specific antigens such as HbpA would be useful.

In conclusion, timely diagnosis of leptospirosis by Leptocheck IgM, MAT and HbpA-IgG ELISA enabled confirmation and prompt treatment of the two patients effectively without further complications. There is a need for the development of a quick, economical and easy-to-do test, for which HbpA-IgG ELISA shows good promise. The availability of appropriate and affordable tests in routine laboratories will help in identification of more cases of leptospirosis.

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by Reetika Chaurasia

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