Leptospiral Sphingomyelinases: Structure Prediction, Characterization and Expression Profile of these Virulence Factors in Pathogenic *Leptospira* spp.

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By

Narayanavari A Suneel

Reg. No. 07LAPH10

Department of Animal Sciences

School of Life Sciences

University of Hyderabad

Hyderabad - 500 046

India

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University of Hyderabad

(Central University established in 1974 by an act of Parliament)

Hyderabad - 500046, India

CERTIFICATE

This is to certify that **Mr. Narayanavari A Suneel** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis entitled **"Leptospiral Sphingomyelinases: Structure Prediction, Characterization and Expression Profile of these Virulence Factors in Pathogenic *Leptospira* spp."** for submission for the award of the degree of Doctor of Philosophy in Animal Sciences of this University.

Prof. Manjula Sritharan
Ph.D. Supervisor _____

Head
Department of Animal Sciences _____

Dean
School of Life Sciences _____

University of Hyderabad

(Central University established in 1974 by an act of Parliament)

Hyderabad - 500046, India

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Leptospiral Sphingomyelinases: Structure Prediction, Characterization and Expression Profile of these Virulence Factors in Pathogenic *Leptospira* spp."** is the result of the investigation carried out by me in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad and has not been submitted to any other University for the award of any degree or diploma.

Narayanavari A Suneel

Research scholar

Prof. Manjula Sritharan

Ph.D. Supervisor

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BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
CDS	: Coding sequences
BC SMase	: *B. cereus* sphingomyelinase
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylene diamio tetra - acetic acid
EDDA	: Ethylene diamino N - N’ diacetic acid
EMJH	: Ellinghausen – McCullough – Johnson - Harris
FepA	: Ferric - enterobactin receptor
Fur	: Ferric - uptake regulator
H	: Hour
Ig	: Immunoglobulin
kb	: Kilobase pair
kDa	: Kilodalton
LPS	: Lipopolysaccharide
MAT	: Microscopic agglutination test
MAFFT	: Multiple alignment program for fourier transform
MEGA	: Molecular Evolutionary Genetics Analysis
NCBI	: National Center for Biotechnology Information
OMP	: Outer membrane protein
OMV	: Outer membrane vesicle
O/N	: Over night
PAGE	: Poly acrylamide gel electrophporesis
PCR	: Polymerase chain reaction
RNA	: Ribonucleic acid
SDS	: Sodium dodecyl sulphate
Sph	: Sphingomyelinase
SMase	: Sphingomyelinase
Tris	: Tris - (Hydroxymethyl) aminoethane
TAE	: Tris – Acetate EDTA buffer
µM	: Micromolar
mg	: Milligram

mL	: Milliliter
mM	: Millimolar
nm	: Nanometers

Abstract

Leptospirosis is an emerging zoonotic disease of global importance. Frequent outbreaks of the disease following heavy rainfall and severe flooding have been reported worldwide. The causative organisms are the pathogenic members of the genus *Leptospira*. The disease is usually self - limiting in humans but can progress to a severe fatal form known as Weil's disease. The pathology includes the involvement of lungs, liver and kidneys with signs of pulmonary hemorrhage, jaundice, acute vasculitis, renal tubular interstitial edema and epithelial necrosis. The underlying mechanism involved in pathogenesis is still unclear.

Recent advances in genome sequencing of pathogenic and non – pathogenic *Leptospira* have yielded some insights into pathogenesis. Potential virulence factors such as lipopolysaccharide, hemolysins, immune modulators and adhesins have been suggested to be involved in the pathogenesis. Both pathogenic and the non - pathogenic *Leptospira* spp. have phospholipase genes, while the sphingomyelinase genes are seen only in the former, implicating their role in pathogenesis. Hemorrhagic complications associated with leptospirosis have been attributed to sphingomyelinases. The expression of these molecules is probably influenced by several environmental factors as these pathogens, thriving successfully in diverse habitats must adapt to their immediate environment by switching on / off several genes. The dynamic interplay between the survival mechanisms of pathogen and host determines the outcome of any infection. *Leptospira*, upon entering the mammalian host encounter various conditions like iron and nutrient limitation, serum, high osmolarity, elevated temperature and oxidative stress. Very little information is available on the environmental signals and the regulation of expression of these virulence genes in response to the encountered stimuli. Earlier reports from our lab showed for the first time that pathogenic *Leptospira* express sphingomyelinase under iron - limiting conditions.

There are five sphingomyelinase genes encoding Sph1, Sph2, Sph3, Sph4 and SphH in *L. interrogans* serovar Lai, with all of them, with the exception of Sph4 possessing the exo – endo phosphatase domain. The focus of the current study includes a) structure prediction and functional characterisation of these proteins and b) factors influencing the expression of these virulence factors. Using Insight II Modeler, we demonstrated similar three – dimensional folding of the leptospiral

sphingomyelinases as the template molecules SmcL and SMase from *Listeria ivanovii* and *Bacillus cereus* respectively. Unlike the template sequences, all the leptospiral sphingomyelinases possessed a C - terminal extension of approximately 186 amino acids and lacked the characteristic β - hairpin loop. The latter, rich in hydrophobic aromatic acids was proven to be essential for the anchoring of these molecules onto the host cell membrane. *In silico* analysis of the leptospiral sphingomyelinases identified a notable number of cell - surface exposed aromatic amino acids (varying from 8 – 12 residues) that led us to hypothesise that these molecules form hydrophobic clusters thereby enabling attachment of these molecules to the host cell membrane. Analysis of the amino acids at the active site of modeled leptospiral sphingomyelinases identified Sph2 as the only sphingomyelinase possessing conserved residues including the Mg^{2+} - binding Glu53 residue in the metal - binding site and the two His residues (His151 and His286) in the catalytic site. Functional characterisation with the recombinant protein proved the Mg^{2+} - dependent hemolytic and enzymatic activity of Sph2.

The influence of different environmental factors on the expression of leptospiral sphingomyelinases was studied as pathogenic *Leptospira* are likely to encounter various *in vivo* - like conditions within the mammalian host such as exposure to serum, high osmolarity, iron limitation, and increase in temperature. We studied the response of these pathogens to iron - limiting conditions coupled with shift in temperature,120 mM NaCl (osmolarity), 10% rat serum and hydrogen peroxide (oxidative stress). Iron limitation resulted in up - regulation of SphH both at the transcript and protein levels. The latter was observed as a processed protein of approximate molecular mass of 42 kDa in the outer membrane vesicles released into the spent growth medium. Salt and serum up - regulated the transcription of *sph2* with marked increase upon simultaneous addition of both these components. The up – regulation of *sph1* though not marked as *sph2* showed a similar trend. Sph2 was detected in whole cell lysates and spent growth medium; it was seen as an 89 kDa protein in serovar Manilae and 95 kDa protein in Pomona. While oxidative stress induced by the addition of hydrogen peroxide resulted in the increase of *sphH* mRNA at transcript level, the corresponding effect at the protein level in the whole cell sonicates was not seen. Biological activity was demonstrated in the above experiments. The serovar Pomona differed not only with size of the Sph2 but

possibly was also regulated in a different manner, as Sph2 levels were high even in the absence of salt. The influence of presence of 330 nucleotide insertion sequence in the upstream region of *sph2*$_{Pomona}$ on the expression of the protein in the serovar was ruled out as inserted region did not alter the expression. Further analysis and the search for a possible regulator is required to understand the differential levels of Sph2 expression in different serovars.

CHAPTER 1

REVIEW OF LITERATURE

1. Introduction

Leptospirosis is the most widespread zoonotic disease in the world and has emerged as an important public health problem affecting both humans and animals (Levett, 2001). It is frequently encountered in tropical countries where moist conditions favour environmental survival of the pathogen. Leptospirosis is primarily an occupational hazard affecting farmers and others who work in close contact with animals. Over the last decade, increasing incidence of leptospirosis associated with recreational sports and adventure tourism has been witnessed conspicuously (Narita *et al.*, 2005). The disease is under - reported due to lack of awareness and poor diagnostics resulting in increased burden on impoverished populations from developing countries and tropical regions (McBride *et al.*, 2005). The causative organisms are the pathogenic members of the genus *Leptospira* belonging to the family *Leptospiraceae*. The disease is usually self - limiting in humans, but can manifest itself into the severe form called the Weil's disease which is characterized by the involvement of the liver, kidney and lungs, either alone or in combination (Bharti *et al.*, 2003; Levett, 2001; Plank & Dean, 2000). Leptospires migrate into different tissues from the systemic circulation after gaining entry into the host via the skin through small cuts or abrasions (Bharti *et al.*, 2003; Plank & Dean, 2000).The basic underlying mechanisms of pathogenesis are not clear.

Frequent outbreaks of leptospirosis following heavy rainfall and severe flooding have been reported globally as reflected in the reports (Levett, 2001) by several groups. In recent decades, considerable efforts have been put forth in identifying novel diagnostic markers, unravelling the pathogenesis and understanding the epidemiology & pathology. In addition, rapid expansion in the area of molecular biology has led to the development of novel genetic tools that are used for identifying potential virulence factors. However, considerable efforts are needed in developing diagnostic kits and novel efficient vaccines which may be possible by focused research on characterising the large pool of hypothetical proteins that have putative roles in pathogenesis.

1.1. History of leptospirosis

Alfred Weil was the first to report the clinical manifestations of the icteric form of leptospirosis in 1886 (Weil, 1886). Since then, Weil's syndrome is synonymous with leptospirosis. Leptospires were first demonstrated in renal tubules of autopsy

specimens collected from a patient thought to have died of yellow fever using Levadeti staining technique (Stimson, 1907). Stimson described these organisms as *Spirocheta interrogans* as they possessed hooks at the ends resembling question mark. However, this observation was unnoticed till the saprophytic forms of these organisms were found in fresh water and were called as *Spirocheta biflexa* (Wolbach & Binger, 1914). The causative agent of the disease was identified in 1916 as spirochetal bacteria (Inada *et al.*, 1916). The same group also illustrated the zoonotic nature of the disease and rats as vectors. Subsequently several wild and domestic animals were identified as carriers (Faine, 1994).

Leptospirosis is observed worldwide and has been given different names in different parts of the world: '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 *et al.*, 1999). In early 1980's, seasonal outbreaks of enigmatic febrile illness were reported from Andaman and Nicobar Islands region which is known as Andaman hemorrhagic fever (AHF) whose etiology remained unknown for a long time. The mystery of AHF was unravelled in 1995 when evidence of leptospiral etiology was obtained (Sehgal *et al.*, 1995). Much of the basic knowledge about leptospires and leptospirosis was understood within a decade after the discovery of leptospires, during which period several serovars were identified (Kmety & Dikken, 1988; Kmety & Dikken, 1993).

1.2. Biology of *Leptospira*

1.2.1. General features

The name *Leptospira* was derived from Greek - *leptos* (thin) and Latin - *spira* (coiled) (Levett & Haake, 2010). Leptospires are thin (0.1 µm), long (6 – 20 µm) spirally coiled and hooked at the ends. They stain poorly and can be visualised by dark - field or phase contrast microscopy due to the characteristic cork – screw movement of these bacteria. The genus *Leptospira* includes both pathogenic and saprophytic members; the former are usually shorter.

1.2.2. Growth and metabolism

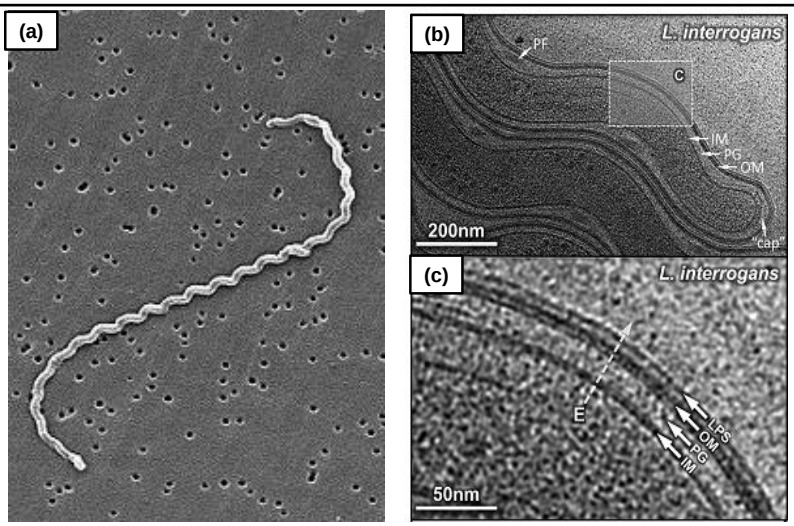
Leptospires are obligate aerobes growing at an optimum temperature of 28°C to 30°C with a doubling time of 6 – 8h. They have simple but unique nutritional needs requiring vitamins (vitamins B_2 and B_{12} are growth factors), long - chain fatty acids and ammonium salts (Johnson & Faine, 1984). They cannot utilise glucose as a

source of carbon (Baseman & Cox, 1969) and depend on β - oxidation of fatty acids to meet their carbon and energy needs *in vitro* (Henneberry & Cox, 1970). Leptospires are commonly grown in Ellinghausen – McCullough – Johnson - Harris (EMJH) medium (Ellinghausen & McCullough, 1965a; Ellinghausen & McCullough, 1965b) containing 1% bovine serum albumin and Tween 80 as source of long - chain fatty acids. Commercial preparations of EMJH medium are currently available of late. Often serum is required for their growth (Ellis & Michno, 1976) and serum containing media include Korthof's media (peptone, NaCl, $NaHCO_3$, KCl, $CaCl_2$, KH_2PO_4, Na_2HPO_4) and Fletcher's media (peptone, beef extract, NaCl, and agar) (Faine *et al.*, 1999). Antibiotics like 5 - fluorouracil or neomycin sulphate are added to the medium to avoid growth of contaminating bacteria (Ellis & Michno, 1976). Liquid media is often used for growth and proliferation of leptospires while semi – solid medium containing 0.1 – 0.2% agar is used for long - term maintenance, in which the organisms grow sub - surface and appear as an opaque mass called 'Dinger's ring'. Non – pathogenic *L. biflexa* can be differentiated from pathogenic *L. interrogans*; the former can grow at 13°C and in the presence of 8 - azaguanine (225 mg / mL) (Levett, 2001).

1.2.3. Leptospiral membrane architecture

Leptospira possess features of both Gram - positive and Gram - negative bacteria. The presence of inner and outer membrane architecture resembles the features of Gram - negative bacteria. The attachment of peptidoglycan to the cytoplasmic membrane resembles Gram - positive bacteria while in Gram - negative bacteria, it is located close to the outer membrane (Fig. 1). Electron microscopy studies revealed that *Leptospira* possess an outer envelope (OE), two periplasmic flagella (PF) or axial filaments and protoplasmic cylinder (PC) (Hovind-Hougen, 1976; Swain, 1957). The protoplasmic cylinder represents cylindrical cell body wound helically around the axis encompassing flagella, nuclear material, ribosomes and occasionally mesosomes. The outer envelope consists of lipopolysaccharide (LPS) attached to the outer membrane. LPS is highly antigenic and variability in its structure and composition is responsible for the immense diversity among the approximately 230 leptospiral serovars (Bulach *et al.*, 2000). Flagella located in the periplasmic space with polar insertions is responsible for fastidious motility exhibiting translational and non – translational movements (Berg *et al.*, 1978). The morphology and motility of

leptospires depends on the medium in which they are grown. They tend to grow as clumps and appear bent or hooked at ends when grown *in vitro* using EMJH liquid medium (Ellis *et al.*, 1983). It is hard to distinguish leptospires belonging to different serovars based on morphology. Morphology of individual isolates varies with sub - culture *in vitro* which can be re - established by passage in hamsters (Ellis *et al.*, 1983).



(phil.cdc.gov/) (Raddi *et al.*, 2012)

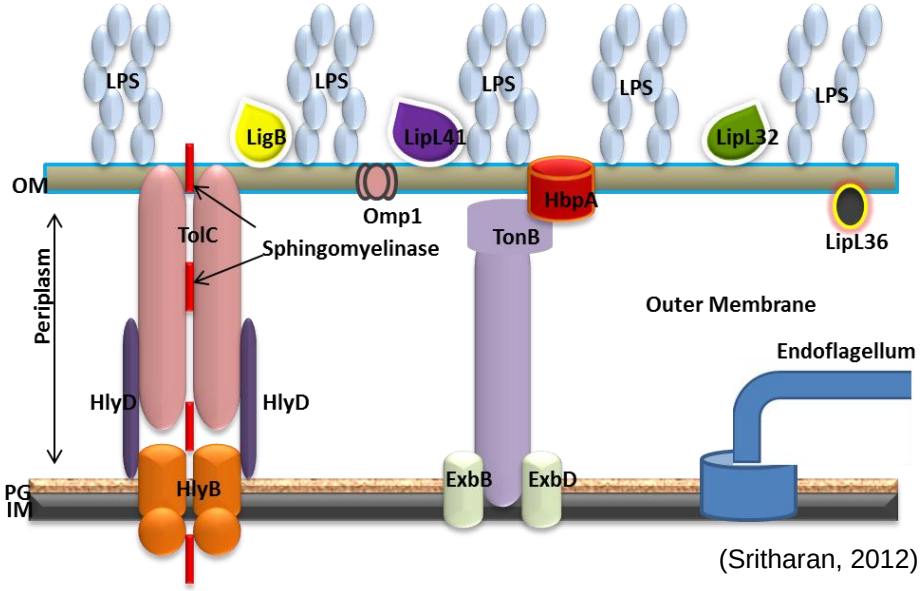
Fig. 1. Morphology of *Leptospira*

Panel (a) shows the scanning electron micrograph of *L. interrogans*; courtesy of Rob Weyant, Public Health Image Library, Center for Disease Control and Prevention (PHIL #1220). Panel (b) represents the ultrastructure of intact *L. interrogans* from 3 - D reconstruction by cryo – electron tomography showing periplasmic flagella (PF) and "cap" at the cell end. Panel (c) represents the zoom - in view of the marked region in panel (b). Structural components of cell envelope of *L. interrogans* such as lipopolysaccharide (LPS), outer membrane (OM), peptidoglycan (PG), inner membrane (IM) and periplasmic flagella (PF) can be distinguished from the picture.

1.2.4. Leptospiral membrane proteins

Membrane proteins are involved in maintaining cell integrity, anchorage to various substrates and uptake of nutrients (Fig. 2). Leptospiral membrane proteins include outer membrane proteins (OMPs), periplasmic proteins and inner membrane proteins. A 31 kDa OmpL1 is the first leptospiral OMP to be characterised; it consists of ten amphipathic β - strand transmembrane domains forming a barrel or pore similar to the structure described for *E. coli* OMPs (Haake *et al.*, 1993; Shang *et al.*, 1995). In contrast, many OMPs are lipoproteins which associate with membrane via N - terminal lipid moiety (Pinne & Haake, 2009). Lipoproteins represent the most

abundant group of membrane proteins which include LipL32, Loa22, HbpA, LipL36, LipL41, LipL21, LipL45 and LipL48 (Haake & Matsunaga, 2010).



(Sritharan, 2012)

Fig. 2. Leptospiral membrane architecture

The outer membrane (OM) with the associated lipopolysaccharide (LPS) and outer membrane proteins, including the lipoproteins LipL32, LipL41 and LigB protein are in contact with the immediate environment (surface - exposed Loa22, leptospiral endostatin - like protein A (LenA), LenD, LigA and LigC proteins are not shown but are also known to be present at the surface of leptospires). The HbpA is an example of a TonB - dependent outer membrane receptor that mediates the transport of hemin via the TonB protein located in the inner membrane (IM) along with ExbB and ExbD. TolC, in association with HlyC and HlyD forms a channel from the IM to the OM that possibly mediates the export of sphingomyelinase from the cytoplasm to the outside. The peptidoglycan (PG) is associated with IM.

LipL32 and LipL41 are highly conserved and unique to pathogenic *Leptospira* spp. which are expressed during infection. LipL32 is the most abundant outer membrane lipoprotein which is studied extensively because of its surface location, dominance in the host immune response and conservation among pathogenic species. It also known as hemolysis - associated protein - 1 (HAP-1) as it is linked with hemolysis and has the ability to bind Ca^{2+}, laminin, collagen type IV and fibrinogen (Hauk *et al.*, 2009; Hoke *et al.*, 2008). Loa22 is the second most abundant outer membrane protein conserved in non - pathogenic and pathogenic *Leptospira.* Deletion of *loa22* in pathogenic *Leptospira* resulted in attenuation of virulence (Koizumi & Watanabe, 2003; Ristow *et al.*, 2007). Pathogenic *Leptospira* express the iron - regulated TonB dependent hemin binding outer membrane protein A (HbpA)

on the cell surface that plays a role in iron acquisition via uptake of hemin (Asuthkar *et al.*, 2007; Sritharan *et al.*, 2005). The multi - functional leptospiral immunoglobulin - like protein family (LigA, LigB, LigC) is present in the outer membranes in pathogenic leptospires; they are thought to mediate adhesin and immune evasion activities (Matsunaga *et al.*, 2005).

The periplasm of *Leptospira* contains the flagella composed of a core surrounded by a sheath of four FlaB and two FlaA proteins. The configuration of the subunits within the leptospiral flagellum and their role in motility has not yet been determined.

The peptidoglycan - associated inner membrane contains lipoproteins such as FeoA – FeoB type iron transporter (Louvel *et al.*, 2006), LipL31 (Haake & Matsunaga, 2002), ImpL63 (Haake & Matsunaga, 2002), penicillin - binding proteins (Brenot *et al.*, 2001) and signal peptidase (Trueba *et al.*, 1995).

1.3. Classification

Leptospires belong to the Division - Gracillicutes, Class - Scotobacteria, Order – Spirochaetales, Family – Leptospiraceae and Genus – *Leptospira* (Paster & Dewhirst, 2000). Genus *Leptospira* was earlier classified based on antigen - relatedness (serological) and is now classified based on DNA – relatedness.

1.3.1. Serological classification

Traditionally, the genus *Leptospira* is categorized into two species, *L. interrogans* and *L. biflexa* comprising of pathogenic and non - pathogenic strains respectively (Johnson & Faine, 1984). Both *L. interrogans* and *L. biflexa* are divided into several serovars using the cross - agglutination absorption test (CAAT) (Dikken & Kmety, 1978; Terpstra, 1992). If more than 10% of the homologous titre remains in atleast one of the two antisera on repeated testing, two strains are said to belong to different serovars. More than 250 serovars of pathogenic and 60 serovars of non - pathogenic leptospires have been described; because of the large number of serovars, antigenically - related serovars were grouped into serogroups for convenience in serologic testing (Levett, 2001). Serological classification of leptospires is important for clinical or epidemiological investigations, as identification of serovars and serogroups provides clues on the host reservoirs involved in transmission. However, serotyping is possible in only few reference laboratories worldwide as it requires serological reagents, sophisticated laboratory setup and skilled technical personnel

for the growth, maintenance of *Leptospira* and the performance of the serological testing (Ko *et al.*, 2009).

1.3.2. Genotypic classification

The serological classification of leptospires has been replaced by genotypic classification based on the DNA - relatedness. Molecular approaches like restriction endonuclease analysis (Ellis *et al.*, 1991), pulsed - field gel electrophoresis (PFGE) (Herrmann *et al.*, 1991; Herrmann *et al.*, 1992), ribotyping (Perolat *et al.*, 1993), mapped restriction site polymorphism (MRSP) and arbitrarily primed PCR have been applied which allowed characterization of the leptospires at the species and sub - species level. The term genomospecies is used to indicate a species determined on the basis of DNA relatedness (Brenner *et al.*, 1993). The genus *Leptospira* is divided into 17 species, defined as being atleast 70% DNA - related and whose related DNA sequences contain atleast 5% unpaired bases (divergence) (Brenner *et al.*, 1999): They include *L. interrogans* (91 strains), *L. santarosai* (65 strains), *L. borgpetersenii* (49 strains), *L. kirschneri* (29 strains), *L. noguchii* (20 strains), *L. weilii* (15 strains), *L. inadai* (10 strains), *L. meyeri* (5 strains), *Leptonema illini* (3 strains), *L. biflexa* (2 strains), *L. wolbachii* (2 strains), *L. parva* (1 strain), *Leptospira* genomospecies 1 (2 strains), *Leptospira* genomospecies 2 (6 strains), *Leptospira* genomospecies 3 (1 strain), *Leptospira* genomospecies 4 (1 strain) and *Leptospira* genomospecies 5 (1 strain). The advantages of genetic classification include the identification of distinct subtypes, as seen with the serovar Hardjo. For example, Hardjoprajitno and Hardjobovis, grouped earlier under serovar Hardjo now belong to *L. interrogans* and *L. borgpetersenii* respectively (Ellis *et al.*, 1988; LeFebvre *et al.*, 1987; Thiermann *et al.*, 1986). But genotypic characterisation is not possible in all research laboratories as it requires sophisticated laboratory setup with good infrastructure and skilled personnel. The genotypic classification is also difficult for the clinical microbiologists, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years. Until simpler DNA - based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the future (Levett, 2001).

1.4. Epidemiology

Leptospirosis, one of the most wide spread disease in the world is more prevalent in tropical countries where the moist and humid climate has favoured the survival of the pathogen. Disease out - breaks have been observed globally in United States (CDC, 1994; Katz *et al.*, 1997), Korea (Park *et al.*, 1989), Nicaragua (Zaki & Shieh, 1996), El Salvador and Brazil (Ko *et al.*, 1999), India (Vijayachari *et al.*, 2008a) and Mexico (Vado-Solis *et al.*, 2002). The incidence of human infections is rampant in tropical regions with high rainfall where human population get exposed to water contaminated with the urine of infected animals (Everard & Everard, 1993; Ratnam, 1994). Populations living in developing countries with poor sanitary conditions have high chances of getting infected.

1.4.1. Leptospirosis outbreaks in India

Leptospirosis is widespread in several states of India and frequent outbreaks from coastal regions like Gujarat (Clerke *et al.*, 2002), Mumbai (Karande *et al.*, 2002), Kerala (Kuriakose *et al.*, 2008), Chennai (Ratnam *et al.*, 1993) and the Andaman Islands (Gamage *et al.*, 2012; Sehgal *et al.*, 1995) are reported. The first report on leptospirosis appeared back in 1931 (Taylor & Goyle, 1931) from Andaman & Nicobar Island region. Several others have confirmed the prevalence of leptospirosis by isolating leptospires from humans (Gupta & Chopra, 1937; Gupta, 1938; Lahiri, 1941). In 1960, serological evidence of *L. icterohaemorrhagiae* and *canicola* antigen was found in five cases of jaundice (Dalal, 1960). In 1966, leptospiral etiology was reported from the suspected cases of pyrexia of unknown origin (Joseph & Kalra, 1966). In 1967, in Bombay, out of 150 sera from infective hepatitis cases, one was found to be positive for *Leptospira* infection due to *L. pyrogenes* (Bhatnagar *et al.*, 1967). During 1984 to 1985, acute renal failure due to leptospirosis in 19 human patients was reported in Madras (Muthusethupathi & Shivakumar, 1987). In 1988, 33 out of 40 patients suspected with leptospirosis had leptospiral antibodies as revealed by MAT (Venkataraman *et al.*, 1991) in Madras region and couple of other cases were reported in subsequent years from 1990 to 1993 (Muthusethupathi *et al.*, 1995; Ratnam *et al.*, 1993). In 1993, in north Andaman region - an outbreak of febrile illness with pulmonary hemorrhagic complications was reported (Sehgal *et al.*, 1995). In 1994, individuals with uveitis associated with leptospirosis was reported from Aravind Eye hospital, Madurai after an epidemic of leptospirosis in South India; the

epidemic followed severe flooding of the Tamil Nadu district in the autumn of 1993; 37 / 46 patients (80%) had *Leptospira* DNA and 33 / 46 patients (72%) had positive serology (Chu *et al.*, 1998). Subsequently, in 1995, a seroprevalence rate of 12% leptospirosis was found among febrile and jaundice patients in Pondicherry (Prabhakar *et al.*, 1995). Of the thirty - eight acute renal failure cases with clinical suspicion of leptospirosis, 27 (71%) were seropositive as diagnosed by MAT (Saravanan *et al.*, 1995).

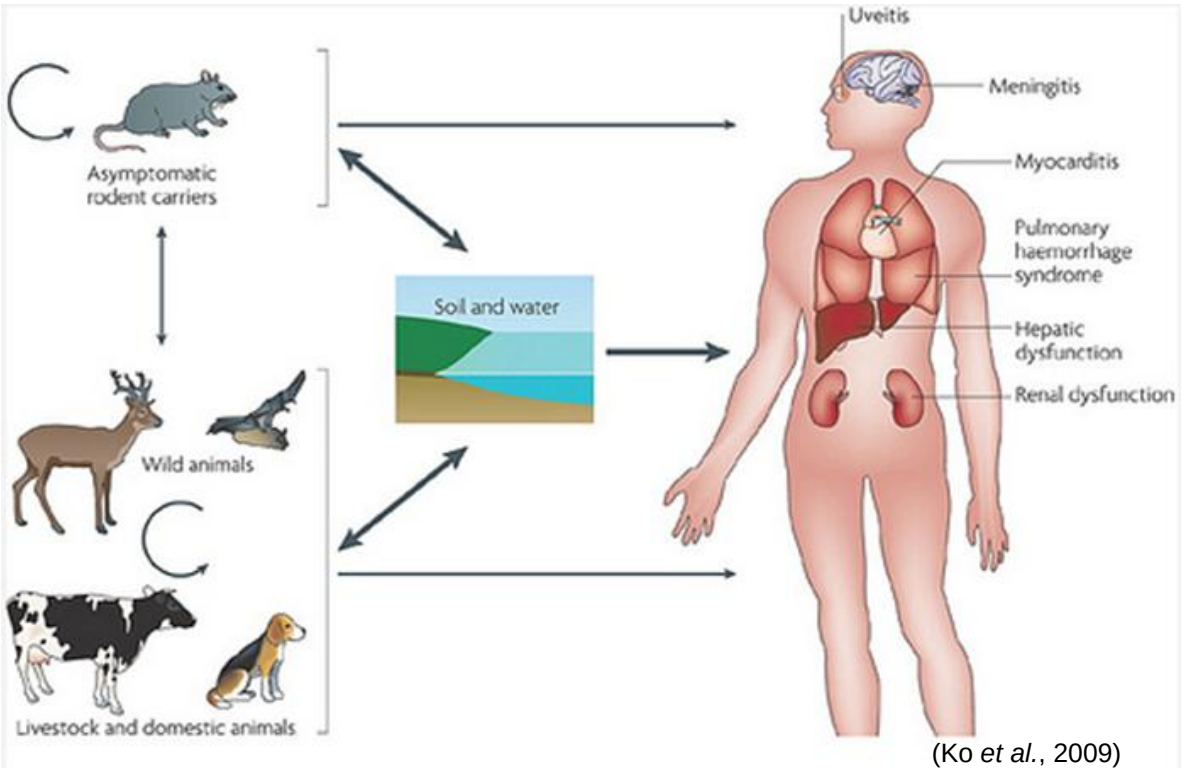
In 2000 and 2005, following super cyclone and flood, outbreaks were reported in Mumbai (Sehgal, 2006). The outbreak during the 2005 flooding in Mumbai clearly demonstrates the need for proper surveillance and control measures during such times of need. Around 310 cases of leptospirosis, with 27 deaths were reported, giving an incidence of 7.85 per 0.1 million population and a case fatality rate of 8.7%. In contrast, during the corresponding period the year before when no flooding had occurred, the incidence of leptospirosis and case fatality rates were 2.1 per 0.1 million and 7.3%, respectively (Kshirsagar *et al.*, 2006). In Orissa, following the super - cyclone that hit the coastal villages, nearly 14% of the studied subjects had febrile illness and serological evidence of leptospiral infection (Faine, 1994).

In the state of Andhra Pradesh, the disease remains largely under - reported. In 2007, a retrospective hospital - based study on human leptospirosis in Hyderabad region has been reported (Sritharan, 2012; Velineni *et al.*, 2007) in which, among 55 human sera tested by MAT, IgM ELISA and LeptoTek Dri - dot, *L. interrogans* serovar Lai (68%) emerged as a predominant serovar followed by Australis (22%), Autumnalis (8%) and Javanica (2%). Incidence rates are often underestimated due to lack of awareness of the disease and timely diagnosis (Bharti *et al.*, 2003; Levett, 2001; Sritharan, 2012).

1.4.2. Modes of transmission

Leptospirosis is a zoonotic disease and affects both humans and animals. Humans are accidental hosts whereas wild and domestic animals serve as reservoir hosts with rodents playing a major role in disease transmission. The infected animals remain symptom – free and shed leptospires via urine into the surrounding environment (Faine *et al.*, 1999; Leonard *et al.*, 1992; Thiermann, 1981). Humans acquire the infection through direct or indirect contact with urine or tissues of infected animals (Fig. 3).

Leptospirosis is maintained in nature by chronic infection of the renal tubules of maintenance hosts (Babudieri, 1958). The usual portal of entry into the host is through abrasions or cuts in the skin or via the conjunctiva (Levett, 2001). Direct contact is important in transmission to veterinarians, workers in milking sheds on dairy farms, abattoir workers, butchers, hunters, and animal handlers (Tangkanakul *et al.*, 2000; Terry *et al.*, 2000). Indirect contact is more common, and is responsible for disease following exposure to contaminated environment including soil or water. The great majority of cases are acquired by this route in the tropics, either through occupational exposure to water, as in rice or taro farming, flooding after heavy rains, or exposure to damp soil and water during vocational activity (Bharti *et al.*, 2003). Recreational exposures have become relatively more important resulting in outbreaks (Sejvar *et al.*, 2003).



(Ko *et al.*, 2009)

Fig. 3. The cycle of leptospiral infection

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

1.5. Clinical manifestations of leptospirosis

Clinical presentation of the disease includes flu - like symptoms such as fever, headache, chills and severe myalgia. The disease is clinically difficult to diagnose as the symptoms overlap with other diseases like flu (influenza), malaria and dengue. The disease is usually self – limiting with 90% - 95% of the cases recovering from the disease. But 5 – 10% of the cases develop the severe form of the disease known as Weil's disease which can become fatal if not diagnosed in the early stages. Weil's disease represents the icteric or hemorrhagic form of leptospirosis involving several organs including the liver, lungs and kidneys (Levett, 2001). Jaundice associated with liver involvement is attributed to the failure in the secretion of bilirubin into bile canaliculi (Bharti *et al.*, 2003). Oliguria with impaired excretory functions and uraemic symptoms are associated with acute renal failure associated with the disease. In patients with the classical AHF (Andaman Hemorrhagic Fever), pulmonary hemorrhage involving lungs has been the predominant complication, with hemoptysis as the common symptom.

In India, there are well documented evidences of ocular manifestations associated with leptospirosis resulting in uveitis (Rathinam *et al.*, 1997). Uveitis is an important late complication that can cause reversible or irreversible blindness in people and in horses (Barkay & Garzozi, 1984; Rathinam, 2002). Though it is thought to be an immune response, leptospiral etiology has been proved (Chu *et al.*, 1998; Sivakolundu *et al.*, 2012). Patients suffering from leptospiral uveitis possess symptoms including conjunctival suffusion, hypopyon (accumulation of pus in the anterior chamber of the eye), vasculitis, optic disc edema, membranous vitreous opacities and absence of choroiditis that can result in blindness (Rathinam, 2005; Sivakolundu *et al.*, 2012).

1.6. Pathogenesis and virulence

Leptospirosis is an invasive infection manifested by a broad spectrum of symptoms that are often mistaken for other infections. The disease is usually self - limiting but can progress to the severe form characterized by renal failure, hemorrhagic diathesis and jaundice. Another occasional complication is hemolytic anaemia (Feigin & Anderson, 1975). Although several putative virulence factors have been suggested, their role in pathogenesis still remains unclear. Potential virulence factors such as

LPS, hemolysins, adhesins, surface proteins and immune mechanisms of the host may be involved in pathogenesis as described below

1.6.1. Leptospiral hemolysins

Hemolysins are toxins produced by Gram - positive and Gram – negative bacteria (Goebel *et al.*, 1988) which can be classified as enzymatic, pore forming and surfactant - based depending on the mechanism of action on the target cell membranes (Rowe & Welch, 1994). Some of the examples of bacteria expressing hemolysins include *T. denticola* (Chu *et al.*, 1995) *V. cholerae* (Stoebner & Payne, 1988), *S. marcescens* (Poole & Braun, 1988)*, P. gingivalis* (Chu *et al.*, 1991), *A. caviae* (Karunakaran & Devi, 1994) and *E. tarda* (Janda & Abbott, 1993).

Leptospiral hemolysins are postulated to be involved in the cell lysis, tissue damage due to sphingomyelinase, phospholipase, or pore - forming activities (Bernheimer & Bey, 1986; Lee *et al.*, 2002). Through their action on host cell membranes, leptospiral sphingomyelinases are potentially involved in aspects of pathogenesis, including tissue invasion, endothelial damage, immune evasion and nutrient acquisition (Narayanavari *et al.*, 2012). Pulmonary hemorrhage is a feared complication caused by damage to the endothelial lining of blood vessels (Dolhnikoff *et al.*, 2007), possibly caused by a toxin as leptospires are often not detected at the site of the lesion (Miller *et al.*, 1974).

Spent growth medium of leptospiral pathogens have long been known to hemolyse erythrocytes. The hemolytic activity associated with *Leptospira* was first reported way back in 1956 (Alexander *et al.*, 1956). Sphingomyelinase activity was first detected in *Leptospira* cultures in the 1960’s (Kasarov & Addamiano, 1969), yet cloning of a sphingomyelinase gene was not reported until 1989 (del Real *et al.*, 1989), when a genomic expression library of *L. borgpetersenii* serovar Hardjo was screened for hemolytic activity. Hemolytic and sphingomyelinase activities were expressed from a single gene that was later designated *sphA* (del Real *et al.*, 1989; Segers *et al.*, 1992). The sphingomyelinase encoded by *sphA* shared significant similarity to those found in *S. aureus* and *B. subtilis* (Segers *et al.*, 1990). Multiple sphingomyelinase sequences were detected in pathogenic members of *Leptospira* by low stringency southern hybridization using *L. borgpetersenii sphA* as a probe (Segers *et al.*, 1992).

SphH, one of the sphingomyelinase homologs in the genome of serovar Lai, was identified from a genomic library using *sphA* as the probe (Lee *et al.*, 2000). The protein showed 75% similarity to SphA. However, the clone failed to express sphingomyelinase (or phospholipase) activity, although the partially purified recombinant protein lysed sheep erythrocytes (Lee *et al.*, 2000; Lee *et al.*, 2002). The hemolytic activity of SphH was neutralized with rabbit antiserum raised against SphH, eliminating the possibility that hemolysis was due to the cryptic hemolysin of *E. coli*. Transmission electron microscopy of sheep erythrocytes incubated with the SphH preparation revealed pores in the membrane, suggesting that the hemolytic activity of SphH was due to pore - forming ability (Lee *et al.*, 2002). However another group was unable to confirm the hemolytic activity of a purified preparation of rSphH (Carvalho *et al.*, 2010), possibly due to improper refolding of the insoluble recombinant protein.

Genome sequencing studies revealed that pathogenic *Leptospira* possessed multiple hemolysin genes. Pathogenic *L. interrogans* serovar Lai possessed nine hemolysin genes consisting of four sphingomyelinases, one pore - forming hemolysin and four non - sphingomyelinase hemolysins as shown in Table 1 (Ren *et al.*, 2003). Among the group of leptospiral hemolysins, sphingomyelinases and the pore – forming hemolysins have been implicated in the pathogenesis of leptospirosis (Alexander *et al.*, 1956; Klopfleisch *et al.*, 2010; Miller *et al.*, 1974; Thompson & Manktelow, 1989). The serovar Lai and the serovars Copenhageni, Manilae and Pomona belonging to *L. interrogans* species possess five sphingomyelinase genes annotated as *sph1*, *sph2*, *sph3*, *sph4* and *sphH.* In contrast, the genomes of two *L. borgpetersenii* strains harboured only three sphingomyelinase genes namely *sphA*, *sphB* and *sph4* (Bulach *et al.*, 2006b) and it lacks the corresponding *sph1* and *sphH* orthologs. It is fascinating to see that the newly sequenced genomes of pathogenic *L. santarosai* (Chou *et al.*, 2012) and intermediate – pathogenic *L. licerasiae* (Ricaldi *et al.*, 2012) possess seven sphingomyelinase genes. Sphingomyelinases are considered as putative virulence factors due to their absence in non – pathogenic *L. biflexa* (Picardeau *et al.*, 2008).

Sphingomyelinases are enzymes that catalyse the hydrolysis of sphingomyelin into ceramide and phosphocholine. Biochemically, sphingomyelinases are classified as acidic, neutral or alkaline, depending on their pH optimum for activation. Most of the neutral sphingomyelinases of bacteria and

mammals form a family defined by a set of conserved catalytic core residues and overall sequence relatedness (Clarke *et al.*, 2011). Mammalian members of the neutral sphingomyelinase family are membrane - associated whereas the bacterial members are secreted. Mammalian sphingomyelinases act on the sphingomyelin present on the membranes and release ceramide, which controls cellular functions by acting as a signaling molecule and by altering the biophysical properties of the membrane (Hannun & Obeid, 2008). Ceramide is also the central hub of the sphingolipid signaling network, which includes other bioactive sphingolipids such as sphingosine and sphingosine – 1 - phosphate. The levels of ceramide and other sphingolipids are therefore tightly controlled (Breslow & Weissman, 2010) and their dysregulation contributes to the patho - biology of numerous infectious and non - infectious disease processes (Zeidan & Hannun, 2007). For example, cellular infection by diverse pathogens, including *Neisseria gonorrhea*, rhinovirus and *Cryptosporidium parvum* involves activation of the host acid sphingomyelinase by translocation of the enzyme from the endolysosoemal compartment to the plasma membrane (Grassme *et al.*, 2005; Zeidan & Hannun, 2007). Hydrolysis of sphingomyelin in the plasma membrane by acid sphingomyelinase leads to assembly of ceramide - enriched membrane platforms, which may be necessary to concentrate receptors to facilitate intracellular signal transduction and microbial internalization (Lafont & van der Goot, 2005).

Table 1. Hemolysin candidate genes of different *Leptospira* spp.

Type of hemolysin	**Gene**	***L. interrogans* serovar Lai**	***L. interrogans* serovar Copenhageni**	***L. biflexa* serovar Patoc**
Sphingomyelinases	*sph1*	LA1027	LIC12632	-
	sph2	LA1029	LIC12631	-
	sph3	LA4004	LIC13198	-
	sph4	LA3050	LIC11040	-
Pore - forming hemolysin	*sphH*	LA3540	LIC13198	-
Non – sphingomyelinase hemolysins	*tlyA*	LA0327	LIC10284	LEPBIa0082
	hlyX	LA0378	LIC10325	LEPBIa2375
	hlpA	LA1650	LIC12134	LEPBIa2015
	hlyC	LA3937	LIC13143	LEPBIa0717

Sphingomyelinases produced by *Bacillus cereus*, *Staphylococcus aureus* and *Listeria ivanovii* are the best characterized among the bacterial sphingomyelinases. As most bacteria do not synthesize sphingomyelin, bacterial sphingomyelinases probably target the sphingomyelin in the external leaflet of the host cell plasma membrane. Their inactivation in *S. aureus* and *L. ivanovii* diminished their infectivity in animal models (Bramley *et al.*, 1989; Gonzalez-Zorn *et al.*, 1999). Sphingomyelinase from *L. ivanovii* enables the intracellular pathogen to escape from phagocytic vacuoles in epithelial cells by rupturing the membrane of the vacuole (Gonzalez-Zorn *et al.*, 1999). The sphingomyelinase activity of *S. aureus* β - toxin promotes excessive inflammation and vascular leakage in the lungs by inducing shedding of the ectodomain of the proteoglycan syndecan - 1 in a mouse model of pneumonia (Hayashida *et al.*, 2009). The response does not occur when the catalytic residues of β - toxin are altered, highlighting the importance of the enzymatic activity of the toxin in triggering uncontrolled inflammation.

1.6.2. Lipopolysaccharide

Humans are susceptible to leptospiral infection due to poor recognition of leptospiral LPS by the innate immune system (de Souza & Koury, 1992; Werts *et al.*, 2001). Human Toll – like receptor - 4 (TLR - 4), involved in recognition of Gram - negative LPS even at extremely low concentrations failed to identify and bind leptospiral LPS (Nahori *et al.*, 2005; Werts *et al.*, 2001). The presence of unique methylated phosphate residue of lipid A in leptospiral LPS may be responsible for loss of recognition by TLR - 4 (Que-Gewirth *et al.*, 2004).

1.6.3. Adhesins and other surface proteins

Leptospiral adhesins play a role in the attachment of these pathogens to eukaryotic cell surfaces and extracellular matrix (ECM). They are essential for penetration, dissemination and pathogenesis (Haake, 2000). It has been shown that *L. interrogans* is capable of, binding to host ECM and variety of cell lines (including fibroblasts, monocytes/macrophages, endothelial cells and kidney epithelial cells grown *in vitro*) (Breiner *et al.*, 2009). Surface lipoproteins present at the interface have been implicated to play roles as adhesins and notable are the leptospiral immunoglobulin - like proteins (Lig proteins). LigA, LigB and LigC are induced under high osmolarity conditions. They contain immunoglobulin - like domains and are involved in interaction with fibronectin, fibrinogen, and other extracellular matrix

factors (Choy *et al.*, 2007). Loa22, the first genetically described virulence factor in *Leptospira* is up – regulated during acute leptospiral infection (Nally *et al.*, 2007; Ristow *et al.*, 2007). A number of *L. interrogans* proteins have been shown to bind the ECM component laminin which includes Lsa21 (Atzingen *et al.*, 2008), Lsa27 (Longhi *et al.*, 2009), Lsa63 (Vieira *et al.*, 2010), LfhH or LenA (Barbosa *et al.*, 2006; Stevenson *et al.*, 2007) and a 36 - kDa membrane protein (Merien *et al.*, 2000). LfhH or LenA, member of the leptospiral endostatin - like protein (Len) family has also been shown to bind complement factor H, fibrinogen and fibronectin (Barbosa *et al.*, 2006; Stevenson *et al.*, 2007). Other proteins including LenB, C, D, E and F belonging to the same group were also shown to bind fibronectin (Stevenson *et al.*, 2007). In summary, much remains to be established in the cellular and molecular mechanisms underlying the clinical manifestations of leptospirosis.

1.6.4. Immune mechanisms of the host

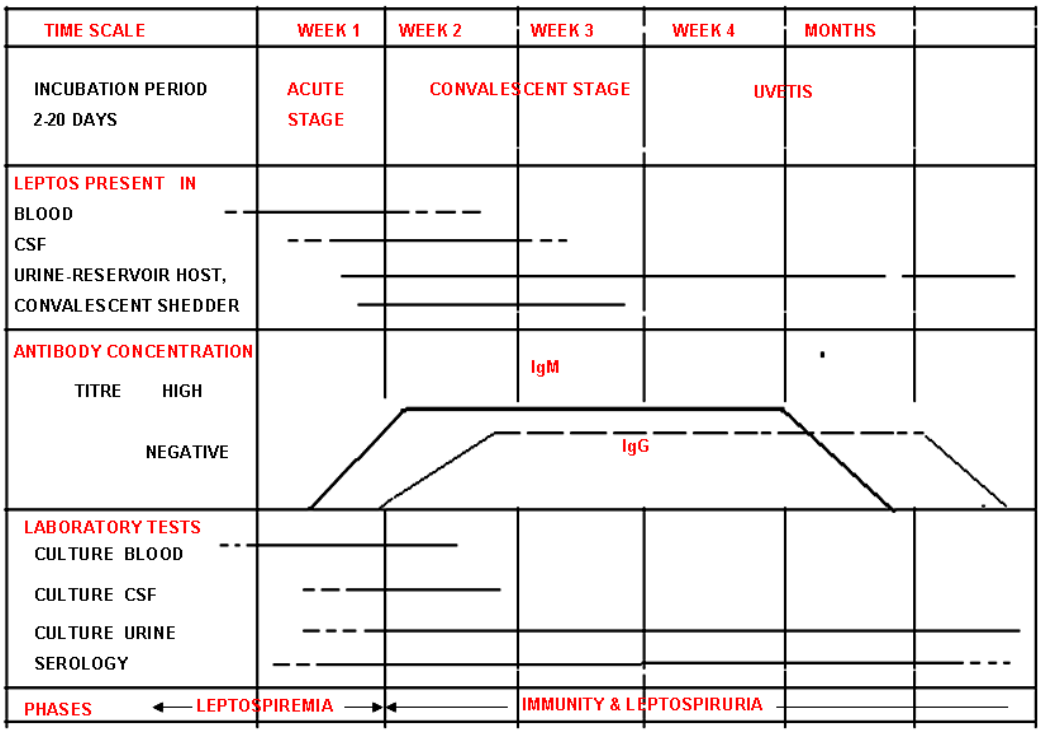
Immune - mediated mechanisms have been suggested to play a role in disease manifestation (Abdulkader *et al.*, 2002). Human leukocyte antigen (HLA) DQ6 has been reported as an independent risk factor for leptospirosis. Leptospires express superantigen that can cause nonspecific T - cell activation in susceptible individuals (Lingappa *et al.*, 2004). Other immune mechanisms, including circulating immune complexes, anti - cardiolipin antibodies, and anti - platelet antibodies, have been proposed but their significance is not known (Levett, 2001).

1.7. Immune response

The clinical presentation of leptospirosis is biphasic (Fig. 4), with the leptospiremic phase lasting about a week, followed by the leptospiruric phase or immune phase (Edwards & Domm, 1960). After successful invasion of *Leptospira* into the host, they appear in blood circulation for about 7 days which is known as leptospiremic phasic. During this phase, diagnosis by PCR or blood culture is possible. This phase is followed by the leptospiruric phase during which the host mounts a strong humoral immune response characterised by production of IgM antibodies. With a rise in the level of circulating anti – leptospiral antibodies, the leptospires migrate to different organs and ultimately colonise in the renal tubules from where they are shed in the urine. Diagnosis during this stage is usually done serologically by the detection of anti - leptospiral antibodies; culture of urine samples is also possible due to the shedding of leptospires in the urine. The leptospires tend to lodge in the renal

tubules in order to avoid attack by the host immune system that contributes to high - grade persistence of the leptospires, particularly in animals that serve as carriers. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase (Ko *et al.*, 2009). The production of immune complexes leading to inflammation in the central nervous system has also been postulated (Tong *et al.*, 1971).

Immunity against leptospires is largely humoral and is serovar - specific. Seroconversion occurs during the early stage of the disease wherein IgM antibodies appear first and reaches detectable levels within one week or as early as on third or fourth day of illness. They reach peak levels during the third or fourth week and then decline slowly over months and become undetectable within six months (Fig. 4). IgM may persist for long periods after infection. IgG antibodies appear later than IgM and reach peak level after a few weeks of illness. IgG antibody may persist at low level for years (Fig. 4). Compared to IgG, higher levels of IgM titres are observed during the first two months of the disease.



(Levett, 2001)

Fig. 4. Biphasic nature of leptospirosis.

During the first week of leptospiral infection, leptospires multiply in the blood and spread to different organs (leptospiremic phase). During second week, leptospires are cleared from the bloodstream due to rise in serum agglutinating antibody titres. Leptospires ultimately migrate to kidneys where they colonise and shed via urine (leptospiruric phase).

Other complications associated due to the host immune response include uveitis - the production of antibodies against a leptospiral antigen which cross - reacts with ocular tissues (Lucchesi *et al.*, 2002; Parma *et al.*, 1987).

1.8. Diagnosis

The clinical presentation of leptospirosis necessitates opportunities for its diagnosis as discussed in Section 1.7. Diagnostic methods include detection of the pathogen by culture and molecular methods like PCR & qRT – PCR as well as by serological techniques for the detection of anti – leptospiral antibodies. The merits and demerits of both are discussed below.

1.8.1. Leptospiremic phase: staining, DFM, culture and PCR

After gaining entry into the mammalian host, leptospires can be detected in the blood for about a week. This period is known as leptospiremic phase which facilitates diagnosis by direct visualization of leptospires in clinical samples by staining, dark - field microscopy (DFM), culturing and by molecular methods such as PCR.

1.8.1.1. Staining

Leptospires can be isolated from blood, peritoneal dialysate and CSF samples during the first 7 – 10 days of leptospiremic phase of illness and from urine during the second and third week of illness (Levett, 2003). Staining methods have been applied to increase the sensitivity of direct microscopic examination of pathogen from clinical samples. Various staining techniques like immunofluorescence staining, immunoperoxidase staining and silver staining have been employed. In addition, a variety of histo - pathological stains have been applied for the detection of leptospires in tissue samples. These methods are not widely used because of the lack of commercially available reagents and their relatively low sensitivity. However, silver staining is still used for the detection of these pathogens.

1.8.1.2. Dark - field microscopy (DFM)

Visualisation of leptospires by DFM is reported as a method of diagnosis. It suffers from disadvantages like low sensitivity (40.2%) & specificity (61.5%) when compared to other diagnostic tests like culture, microscopic agglutination test (MAT), IgM ELISA and Lepto - Dipstick (Vijayachari *et al.*, 2001). Another disadvantage of using DFM is that artefacts in biological samples are often mistaken for leptospires leading to false positivity.

1.8.1.3. Culture

Culture can be done by directly inoculating semi – solid media with few drops of blood in which addition of anticoagulant like citrate must be avoided as it inhibits the growth of organisms (Wolff, 1954). Media should be inoculated within 24h upon collection and incubated at 30°C for several weeks. Though culturing and isolation of leptospires from clinical samples is confirmative, it is time consuming, has low sensitivity and is not useful during epidemics.

1.8.1.4. PCR

PCR has been used as a diagnostic tool for the detection of leptospires in clinical samples. Amplification of *Leptospira* - specific DNA from clinical samples like serum, urine, aqueous humour and tissues of autopsy (Brown *et al.*, 2003) is done by polymerase chain reaction (PCR). Several targets, specific for pathogenic serovars have been used. One of them is the 16s rDNA from which a 631 bp product (Hookey, 1992) or a 330 bp product (Senthilkumar *et al.*, 2001) can be amplified. A real - time PCR method based on 16S rDNA was developed that could be used on samples without the need for prior isolation and culture (Smythe *et al.*, 2002). Another popularly used method (Gravekamp *et al.*, 1993) is based on 2 sets of PCR primers, namely G1 / G2 and B64 - I / B64 - II that amplify products of 285 bp and 563 bp respectively. The former detected *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri*, while the later identified *L. kirschneri*. Other variations included 'Magnetic Immuno PCR Assay' (MIPA) (Taylor *et al.*, 1997) that consists of the immune - magnetic separation of leptospires from inhibitors in frozen formalin - fixed bovine urine prior to PCR detection that resulted in a marked improvement in the detection of leptospires in urine samples. PCR based on *ompL1* (Reitstetter, 2006) detected serovars belonging to *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. santarosai*, *L. weilli* and *L. noguchii*. Recently *hbpA* was used as a target for PCR (Sridhar *et al.*, 2008) that detected all serovars belonging to *L. interrogans* from clinical isolates obtained from different geographical locations around the world.

1.8.2. Immune phase: serological assay of anti - leptospiral antibodies

As a result of strong humoral response mounted by the mammalian host, anti – leptospiral antibodies belonging to IgM class appear 7 – 10 days after infection (Fig. 4) (Fennestad & Borg-Petersen, 1957; Fennestad *et al.*, 1968; Ratnam *et al.*, 1983;

Ratnam *et al.*, 1993). Leptospires are cleared from the blood circulation by the antibody - complement mediated system. However, leptospires migrate to multiple organs including kidneys where they colonise in the convoluted tubules and are shed via urine. Hence detection of the pathogen in the urine samples by dark field microscopy, PCR or culture may be attempted. Shedding of leptospires may not be uniform and occurs randomly and intermittently, with periods of nil shedding resulting in negative results. The antibody response is classical, with peak IgM levels appearing first, followed by IgG antibodies (Fig. 4). The IgM antibodies however remain in circulation for considerably long periods, even up to 2 months. It has also been observed that anti - leptospiral antibodies can be detected even after several years of infection. This, coupled to the endemicity of the infection may result in relatively high levels of antibodies within a population. Thus, it is common to collect a second serum sample from a suspected case of leptospirosis, 3 - 4 days after the first sample. Seroconversion with a four - fold rise in titre in paired serum samples in the presence of clinical symptoms is an important criterion for the definitive diagnosis of leptospirosis. Anti – leptospiral antibodies can be detected by several methods, including the microscopic agglutination test (MAT), ELISA, lateral flow devices, latex bead agglutination tests etc.

1.8.2.1. Microscopic agglutination test (MAT)

Microscopic agglutination test (MAT) is considered as “gold standard” or “reference standard” for serological diagnosis of leptospirosis having high sensitivity and specificity (Cole *et al.*, 1973; Cumberland *et al.*, 1999). MAT is based on the agglutination of live organisms in the presence of serum containing anti - leptospiral antibodies. The agglutination results in the formation of highly refractive spheroids of various sizes and with time, when maximal degree of agglutination is seen and no free leptospires are visible due to the disintegration of the organisms.

In MAT, live antigens representing different serovars of leptospires are mixed with serum samples, incubated and then examined by dark - field microscopy for agglutination. Titres were determined based on the degree of agglutination, usually assessed in terms of the proportion of free leptospires. The endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are agglutinated. As per WHO guidelines, agglutination at dilution of 100 is considered positive for MAT. Antibodies in the serum of infected patients /

animals, predominantly against the surface - exposed lipopolysacharides are serovar - specific, although cross - reactivity may be recorded against other serovars within the same serogroup. It is thus necessary to include several serovars, including the prevalent local isolates for screening by this method. As mentioned earlier, paired samples are to be considered for diagnosis. However, a positive diagnosis can also be made with a titre of more than 800 with single samples (Ko *et al.*, 2009). A serologically confirmed case of leptospirosis is defined by a four - fold rise in MAT titre to one or more serovars between acute - phase and convalescent – phase serum samples run in parallel.

MAT has been used as the test of choice in outbreaks and sporadic cases. It has also been useful in retrospective studies in confirming leptospirosis cases and identifying the prevalent serovar during that period (Boqvist *et al.*, 2002; Ismail *et al.*, 2006; Velineni *et al.*, 2007). The major disadvantages of MAT are that it is labour intensive, time consuming, requires skilled personnel and difficulty in interpretation of results.

1.8.2.2. Macroscopic slide agglutination test (MSAT)

MSAT is done by mixing formalin - treated leptospires with serum from suspected patients on a slide and observed for agglutination. Neither MAT not MSAT can distinguish between IgM antibodies indicative of current infection and IgG antibodies indicative of past infection.

1.8.2.3. Enzyme linked immunosorbent assay (ELISA)

Anti – leptospiral IgM specific antibodies begin to appear in the circulation after the first week of illness. Diagnosis by the detection of IgM antibodies has been shown to be more sensitive than MAT (Cumberland *et al.*, 1999). Antigens used in ELISA include whole cell sonicate, formalin - extract of a culture of leptospires (Terpstra *et al.*, 1985) and even whole leptospires which were coated on polysterene microtitre plates (McBride *et al.*, 2007). Outer membrane proteins like rLipL32 (Fernandes *et al.*, 2007; Flannery *et al.*, 2001), rLipL41 (Flannery *et al.*, 2001; Mariya *et al.*, 2006), rLig proteins (Croda *et al.*, 2007; Srimanote *et al.*, 2008) and rHbpA (Sivakolundu *et al.*, 2011; Sivakolundu *et al.*, 2012; Sridhar *et al.*, 2008) have been used as antigens in ELISA.

Several commercial kits for sero - diagnosis of leptospirosis are available which includes PanBio IgM ELISA kit (Brisbane, Australia), Serion ELISA kit (Institut

Virion / Serion GmbH, Wurzburg, Germany), Lepto dipstick (Smits *et al.*, 1999; Vijayachari & Sehgal, 2006), LeptoTek Dri - dot (Organon Teknika in collaboration with the Royal Tropical Institute in Amsterdam) (Velineni *et al.*, 2007; Vijayachari *et al.*, 2002; Vijayachari & Sehgal, 2006), Lepto lateral flow (Vijayachari & Sehgal, 2006) and Latex agglutination test (Organon Teknika) (Smits *et al.*, 2000). These kits are expensive and there is a need for the development of economically viable indigenous kits.

1.9. Prevention and control

1.9.1. Treatment

Chemoprophylaxis with doxycycline has been shown to be effective for short - term prophylaxis in high - risk environments (Sehgal *et al.*, 2000). In acute cases, benzyl penicillin is given by injection in doses of five million units per day for five days (Watt *et al.*, 1988). Doxycycline 100 mg twice daily for 10 days is also recommended (Vijayachari *et al.*, 2008b). Delayed or inadequate treatment can lead to severe form of the disease thereby emphasising the need for timely diagnosis.

1.9.2. Prevention

Prevention of leptospirosis essentially is by identifying the source and interrupting the transmission. However, preventive measures that block transmission can be practiced by maintenance of hygiene and prevention of infection by using protective clothing and footwear (Faine, 1994). Implementation of rodent control measures is also important in limiting the extent of contamination.

1.10. Host - pathogen interactions

The successful establishment of an infection depends on the outcome of the host – pathogen interactions. While pathogenic *Leptospira* employ various strategies for invasion, colonisation and persistence within the mammalian host, the latter restricts pathogen - entry and survival by physical barriers, innate immunity, humoral immunity and cell - mediated immunity (Fig. 5). Upon entering the mammalian host, *Leptospira* will be exposed to host conditions such as temperature, pH, osmotic strength, oxygen availability, and nutrient limitations. Pathogenic *Leptospira* spp. responds to various environmental stimuli and other unknown cues in the body of the host by altering protein expression including virulence factors which allow them to colonise, survive, and grow within the host (Artiushin *et al.*, 2004; Palaniappan *et al.*,

2002). The occurrence of multiple two - component regulatory systems and extra – cytoplasmic function (ECF) sigma factors in the genomes of pathogenic *Leptospira* spp. permit them to respond to diverse array of environmental signals (Nascimento *et al.*, 2004; Ren *et al.*, 2003).

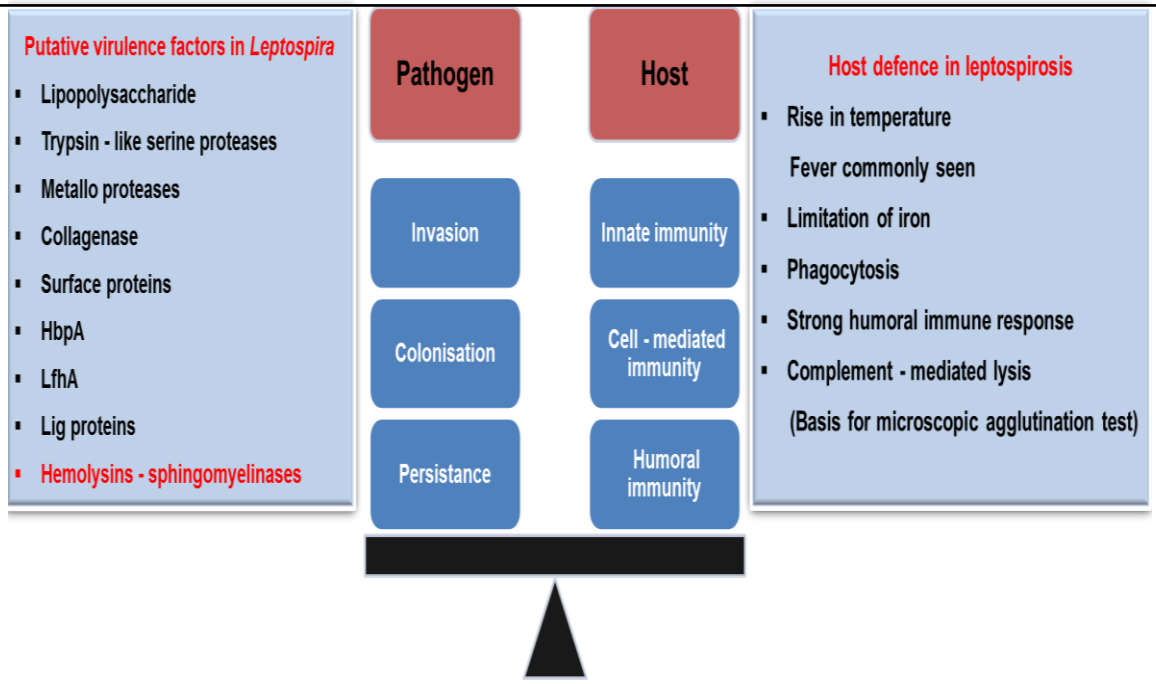


Fig. 5. Host – pathogen interactions in leptospirosis

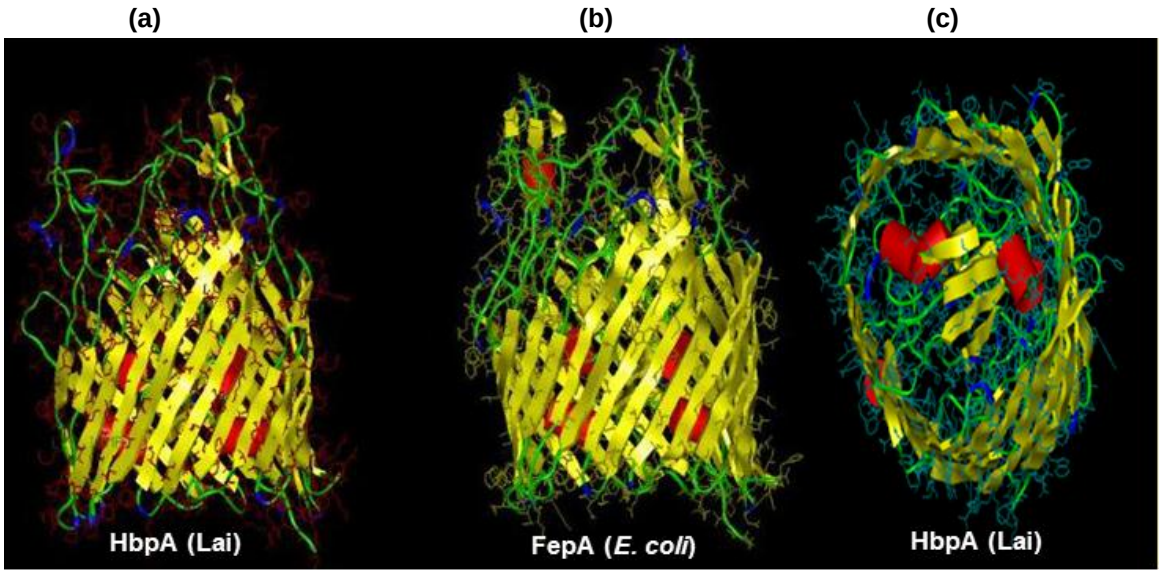
Inside the host, pathogenic *Leptospira* express various virulence factors for disease establishment whereas the host restricts the pathogen by defence mechanisms. The dynamic interplay between the survival mechanisms of pathogen and host determines the outcome of an infection. Disease is established if the balance tilts towards pathogen.

1.10.1. Iron limitation

Iron is an essential micronutrient that is required for metabolism of almost all bacteria except *Borrelia burgdorferi* and lactobacilli (Posey & Gherardini, 2000). Like other bacteria *Leptospira* is no exception in iron requirement for its survival (Faine, 1959; Sritharan *et al.*, 2006). Though iron is the second most abundant metal after aluminium and the fourth most abundant element in the earth's crust it is not biologically available (Sritharan *et al.*, 2006). Iron exists in Fe^{2+} (ferrous) form which is soluble and reactive while the Fe^{3+} (ferric) form, seen at biological pH is insoluble and less reactive (Boukhalfa & Crumbliss, 2002). Iron, by virtue of its wide redox potential is important in biological systems as it catalyses several biochemical reactions and is associated with the transport of reducing equivalents in the electron transport chain. However, its insolubility at biological pH makes it unavailable to

bacteria, as it exists as insoluble ferric hydroxides and oxyhydroxides. Nature has perhaps made iron highly insoluble, as excess iron is toxic, due to its catalytic role in the Fenton reaction, resulting in the formation of free radicals (Sritharan, 2000). At physiological pH 7.0, iron is present as $Fe(OH)_2^+$ that is sparingly soluble with a solubility of approximately 1.4 X 10^{-9} M which is too low to support the growth of microorganisms (Chipperfield & Ratledge, 2000). In addition, most of the iron is held by lactoferrin, transferrin, heme and hemoglobin within the mammalian host thereby curbing the iron availability further (Andrews *et al.*, 2003).

Bacteria have adapted to conditions of iron - limitation by elaborating novel iron acquisition machineries. The two well - studied mechanisms include siderophore - mediated iron acquisition and direct removal of the protein - bound iron via specific receptors for transferrin, lactoferrin, hemin and hemoglobin (Sritharan, 2000). *Leptospira* spp. does not synthesize and secrete siderophores; however, they could use exogenous siderophores of other microorganisms as an iron source (Louvel *et al.*, 2006). The first evidence on direct acquisition of iron by hemin - binding protein HbpA was published by our lab (Fig. 6) (Sritharan *et al.*, 2005). Using *in silico* tools we identified HbpA as the leptospiral homologue of the ferric enterobactin receptor FepA of *E. coli*. Structure prediction and bioinformatic analysis confirmed the protein to be a TonB - dependent protein with the TonB box in its N - terminal region (Sritharan *et al.*, 2005). HbpA is 81 kDa iron – regulated outer membrane protein (Asuthkar *et al.*, 2007) whose levels are up – regulated under the conditions of iron limitation and increase in temperature (Sridhar *et al.*, 2008). This observation is significant as these conditions are probably encountered by *Leptospira* inside the mammalian host. HbpA is involved in chelating hemin and internalising it using the TonB system, comprising of the cytoplasmically localised TonB, ExbB and ExbD proteins. It is unclear, if the entire hemin molecule is internalized or the iron is released at the cell surface. But pathogenic *Leptospira* are capable of extracting iron from hemin using heme oxygenase which metabolises the tetrapyrrole ring of the heme molecule, thereby releasing ferrous iron (Sritharan, 2012).



(Sritharan *et al.*, 2005)

Fig. 6. Structure of hemin - binding protein HbpA

Structure of hemin – binding protein HbpA of *L. interrogans* serovar Lai predicted using FepA of *E. coli* (c). Panels (a) and (c) represents the β – barrel structure and plug domain of HbpA respectively.

1.10.1.1. Molecular regulation of iron acquisition by ferric uptake regulator (Fur)

Expression of genes involved in iron acquisition machinery is regulated by intracellular iron levels (Sritharan, 2000) at the molecular level that is well studied in *E. coli*. In the latter, under iron - sufficient conditions, Fe^{2+} binds to the 17 kDa regulatory protein Fur (ferric uptake regulator encoded by *fur* gene) and this dimeric form of Fur - Fe^{2+} complex binds to Fur box / iron box with the consensus sequence 5'- GATAATGATAATCATTATC – 3' present upstream of the iron - regulated genes, there by blocking the transcription of the latter. Conversely, under low iron conditions, Fur - Fe^{2+} complex cannot be formed and the Fur protein can no longer bind to target genes; thus, under low iron conditions, the expression of the genes associated with iron acquisition is facilitated (Braun *et al.*, 1998). Iron must act via the Fur protein in *Leptospira* as they show the presence of Fur homologs and Fur box (Sritharan, 2012). *L. interrogans* serovar Lai possesses multiple *fur* genes (LB183, LA3094, LA1857 and LA2887) that however remain to be experimentally proved as iron regulators. Fur, encoded by LB183, is located in the neighbourhood of hemin – binding protein A (*hbpA;* LB191) and may act as iron regulator by binding to the Fur box (5' GATAATCATAATAATTT) located upstream of *hbpA* (Sritharan *et al.*, 2005).

1.10.1.2. Iron levels regulates the expression of virulence factors

The intracellular iron levels regulate not only the iron acquisition machinery but also the expression of virulence factors / toxins in several bacterial systems. This was first demonstrated in the expression of the diphtheria toxin by *Corynebacterium diphtheriae* under low iron conditions. Other examples include expression of hemolysin by *E. coli*, Shiga toxin by *Shigella dysenteriae*, vero - cytotoxin by *E. coli* and exotoxin A by *Pseudomonas aeruginosa*. It is clear that pathogens employ these molecules for gaining access to host nutrients, by effecting host cell lysis (Sritharan, 2006).

The ability of pathogens to utilise heme is particularly important as heme is one of the most abundant forms of organic iron in animals. Acquisition of iron from heme or hemoglobin may be facilitated by the production of hemolysins or cytotoxins which lyse host cells and release the intracellular iron. Cytotoxin production coupled with the capability to utilise heme and/or hemoglobin could serve as an effective iron acquisition strategy during the progression of infection. Chemotaxis for hemoglobin has been demonstrated in leptospires (Yuri *et al.*, 1993). It is well known that leptospires cause localized damage to the endothelium of the small blood vessels that leads to severe damage in tissues like kidneys, liver and lungs. The necrosis in the renal tubules, hepatocellular and pulmonary hemorrhage is irreversible and is often fatal. The leptospiral sphingomyelinases are implicated in this damage. Our lab is the first to show the direct evidence that *Leptospira* express sphingomyelinase under iron - limitating conditions (Velineni *et al.*, 2009).

Microarray studies have shown that 43 genes were up - regulated and 49 genes were down - regulated upon iron limitation in *L. interrogans* serovar Manilae (Lo *et al.*, 2010). Genes encoding proteins with predicted involvement in inorganic ion transport and metabolism (including TonB - dependent proteins and outer membrane transport proteins) were over - represented in the up - regulated list, while 54% of differentially expressed genes had no known function. There were 16 up - regulated genes of unknown function which are absent from the saprophyte *L. biflexa* and which therefore may encode virulence - associated factors. Putative virulence factors that were down - regulated include LipL36, pL50 and pL24. Virulence factors that were up – regulated includes LruB, LenA, LenB and LenD (Lo *et al.*, 2010).

1.10.2. Osmolarity

Transmission of pathogenic *Leptospira* between mammalian hosts usually involves dissemination via soil or water as reservoir hosts like *Rattus norvegicus* exhibit constant renal tubular carriage and urinary shedding of pathogenic leptospires into the surrounding environment. Pathogenic *Leptospira* can survive for prolonged periods of time in the water / environment (Trueba *et al.*, 2004) before encountering a mammalian host. Invasion of host tissues by *Leptospira interrogans* involves transition from the aqueous moist environment outside the host to a higher physiologic osmolar environment within the host.

Expression of hemolysin like Sph2 and adhesins like LigA and LigB were strongly induced upon exposure to physiological osmolarity conditions found in mammalian host tissues. It was also demonstrated that LigA and Sph2 were released into the extracellular environment while LigB was shown to be present on the surface (Matsunaga *et al.*, 2005; Matsunaga *et al.*, 2007b). Whole - genome microarray approach was used to understand how *L. interrogans* serovar Copenhageni responds to conditions of osmolarity by overnight incubation in medium with physiological osmolarity conditions. It was observed that physiologic osmolarity significantly altered the transcript levels of 6% of *L. interrogans* genes. Genes that were up - regulated were mostly involved in signal transduction. Other genes that were strongly up – regulated includes *lipL53* and *lfhA*. *lipL53* codes for a lipoprotein that reacted with the antisera from patients with leptospirosis (Gamberini *et al.*, 2005). These findings suggest that increase in osmolarity experienced by pathogenic *Leptospira* spp. after entering a mammalian host from the freshwater environment could be a signal for pathogenic *Leptospira* spp. to induce the expression of virulence genes required during the early stages of infection of the host.

1.10.3. Temperature

Temperature is a key environmental factor known to affect leptospiral protein expression. Leptospires grow optimally at 30°C in axenic media and once inside the mammalian host there is a shift in temperature to 37°C and higher during the febrile stage of infection. Heat shock proteins (GroEL, DnaK, Hsp15), TolC (LA3927) and Qlp42 were up – regulated as anticipated (Ballard *et al.*, 1998; Nally *et al.*, 2001; Qin *et al.*, 2006; Stamm *et al.*, 1991). Various outer membrane proteins like LipL21,

LipL41 and LipL48 were unaffected by temperature while LipL36 and LruB were down - regulated at higher temperatures (Cullen *et al.*, 2002). It was also observed that LipL32 underwent cleavage to yield products of varying mass and pI at higher temperature (Cullen *et al.*, 2002). Microarray studies revealed that differential expression of genes were seen which are involved in chemotaxis and motility, signal transduction systems and outer membrane (Lo *et al.*, 2006; Qin *et al.*, 2006).

1.10.4. Exposure to serum

Leptospires, enter the circulation shortly after entering via skin and mucous membrane. They are exposed to different factors in blood / serum like complement system proteins, serum proteins and alterations in pH, osmolarity & electrolyte concentration. Leptospires can actively counteract the bactericidal effects of the complement system by capturing key components and regulators of the immune system. Johnson and colleagues described almost fifty years ago the resistance of pathogenic *Leptospira* strains to serum factors that killed non - pathogenic strains (Johnson & Muschel, 1966; Johnson & Harris, 1967). Pathogenic *Leptospira* express surface proteins like LfhA / LenA (Verma *et al.*, 2006), LigB (Castiblanco-Valencia *et al.*, 2012; Choy, 2012) and LcpA (Barbosa *et al.*, 2010) to confer complement evasion. Mechanisms include the binding of LfhA to factor H (the major inhibitor of the alternative pathway of complement activation) and binding of LcpA to C4BP (the inhibitor of the classical and lectin pathways of complement activation). Recently it has been shown that LigB can inhibit both alternative and classical pathways of complement system (Choy, 2012).

Leptospira responds to these integrated and complex stimuli by regulating gene expression. Indeed microarray studies using *L. interrogans* serovar Copenhageni have shown that 55 genes were up – regulated including *hemO*, *ligB* and *fliS* while 113 genes were down - regulated. Most of the regulated genes were involved in transcriptional regulation, signal transduction systems, membrane biogenesis and metabolic pathways. Serum represented distinct pattern of gene expression in comparison to those of single - stimulus microarray studies on the effect of temperature and osmolarity upshift (Patarakul *et al.*, 2010).

1.10.5. Oxidative stress

Previous studies indicate that *Leptospira* experiences oxidative stress conditions during the course of infection (Eshghi *et al.*, 2012). Hepatitis is one of the severe

complication associated with Weil's disease characterised by microscopic alterations in the liver and enlargement of liver macrophages i.e., Kupffer cells (KCs) (Arean, 1962). Due to host immune response, leptospires are phagocytised by KCs. Activated KCs can non – specifically damage the liver by generating inflammatory cytokines (Marangoni *et al.*, 2000; Marangoni *et al.*, 2004). During this process KCs trigger respiratory burst, which results in the production of reactive oxygen species (ROS) and nitric oxide (NO) that can be toxic to *Leptospira* (Kawahara *et al.*, 2004; Marangoni *et al.*, 2006). The formation of ROS has also been demonstrated in cattle diagnosed with leptospirosis displaying elevated levels of serum oxidative stress biomarkers like uric acid, albumin, reduced glutathione and malondialdehyde (Erdogan *et al.*, 2008; Kalaiselvi & Panneerselvam, 1998).

Pathogenic *Leptospira* must evade oxidative killing mediated by host cells including macrophages. Pathogenic bacteria circumvent the ROS toxicity using enzymes like catalase and peroxidase which degrade hydrogen peroxide and superoxide dismutase which detoxify superoxide anions. It was observed that *L. interrogans* can sustain higher concentrations of H_2O_2 (50 - fold higher) than those tolerated by *L. biflexa* (Corin & Cox, 1980; Murgia *et al.*, 2002). Though both *L. interrogans* and *L. biflexa* possess *katE* encoding catalase, the former exhibits catalase activity while the latter exhibits predominantly peroxidadic activity (Corin *et al.*, 1978). Recently, it has been shown that catalase (KatE) was up – regulated upon iron limitation (Eshghi *et al.*, 2009), elevated temperature (Lo *et al.*, 2006) and exposure to serum (Patarakul *et al.*, 2010; Xue *et al.*, 2010). Insertional inactivation of *katE* in pathogenic *Leptospira* drastically diminished leptospiral viability in the presence of extracellular H_2O_2 and reduced virulence in an acute infection model (Eshghi *et al.*, 2012).

1.10.6. Identification of potential virulence factors using microarray and mutagenesis studies

Previous microarray studies were helpful in identifying potential genes required for host adaptation and virulence factors (Adler *et al.*, 2011). Fourteen genes (LA0594, LA0802, LA0816, LA1433, LA1456, LA1879, LA2014, LA2117, LA2778, LA3867, LA4299, LB102, LB186 and LB187) were shown to be differentially expressed under conditions of osmolarity, iron limitation and temperature shift as revealed by microarrays. Among them six genes (LA0802, LA1456, LA2117, LA3778, LA3867

and LB102) are absent in *L. biflexa* and therefore represent potential virulence factors required for host adaptation and survival (Adler *et al.*, 2011). Genes encoding heme oxygenase (LB186), putative heme permease (LB187) and LigB (LA3778) were up - regulated across three different experiments (physiological osmolarity, presence of serum and iron limitation).

Mutants of some of these genes have been tested for virulence in animal models (Adler *et al.*, 2011). Five defined *L. interrogans* mutants have been identified with varying degrees of attenuation which includes *loa22* (Ristow *et al.*, 2007), *fliY* (Liao *et al.*, 2009), *hemO* (Murray *et al.*, 2009b) and two LPS biosynthesis genes (Murray *et al.*, 2010). It is surprising that mutants of conserved unique leptospiral proteins bearing the hallmarks of a virulence factor such as LipL32 (Murray *et al.*, 2009c), LigB (Croda *et al.*, 2008), LenB & LenE (Murray *et al.*, 2009a) and PerR (Lo *et al.*, 2010; Murray *et al.*, 2009a) did not show attenuation. However before making conclusions a number of parameters responsible must be considered which includes functional redundancy of virulence factors, choice of animal models and route of infection as these variations may be responsible for the observed discrepancy.

1.11. Comparative genomics of pathogenic and saprophytic *Leptospira*

Whole genome sequencing of *L. interrogans* serovar Lai strain 56601 (Ren *et al.*, 2003), *L. interrogans* serovar Copenhageni strain Fiocruz L1 - 130 (Nascimento *et al.*, 2004), *L. borgpetersenii* serovar Hardjo strains L550, JB197 (Bulach *et al.*, 2006b), and *L. biflexa* serovar Patoc strain Patoc 1 (Ames strain) (Picardeau *et al.*, 2008) are available; re – annotation confirmed CDS to be 3613 and 3530 respectively in both the serovars Lai and Copenhageni (Bulach *et al.*, 2006b). Genome of *L. interrogans* is 4.6 Mb, bigger than *L. borgpetersenii* and *L. biflexa* both of which are 3.9 Mb in size. *L. interrogans* and *L. borgpetersenii* possess two chromosomes and *L. biflexa* contains an additional 74 Kb plasmid. Genome of non – pathogenic *Leptospira* is more stable compared to pathogenic *Leptospira* which display genome plasticity due to insertion sequence-mediated rearrangements (Picardeau *et al.*, 2008). By comparison, pseudogenes and transposases make up only 2% and 1%, respectively of the *L. interrogans* and *L. biflexa* genomes (Picardeau *et al.*, 2008).

Both pathogenic and non – pathogenic *Leptospira* encode 2,052 genes associated with housekeeping functions such as DNA and RNA metabolism, protein

processing and secretion, maintenance of cell structure and cellular processes, and energy metabolism (Picardeau *et al.*, 2008). *L. biflexa* possess 1,348 unique genes that are involved in environmental sensing and nutrient acquisition & metabolism. Both *L. interrogans* and *L. borgpetersenii* share 2,708 genes between them demonstrating common virulence mechanisms. *L. interrogans* and *L. borgpetersenii* possess 627 and 265 unique genes respectively. *L. interrogans* retained more number of genes when compared to *L. borgpetersenii* which can be related to their survival abilities in the external environment. Additionally, the unique genes of known function include LPS biosynthesis genes required for the expression of each unique LPS, those encoding the known lipoproteins LipL32, LipL36, LipL41 and LipL45, Lig and Len proteins. The absence of sphingomyelinases in *L. biflexa* reflects their role in pathogenesis rather than environmental nutritional scavenging. Recently the genome sequence of pathogenic *L. santarosai* serovar Shermani (Chou *et al.*, 2012) and intermediate – pathogenic species like *L. licerasiae* strains VAR010, MMD0835 (Ricaldi *et al.*, 2012) are available.

The *L. interrogans* and *L. borgpetersenii* genomes contain approximately 3400 and 2800 predicted coding regions (excluding transposases and pseudogenes) respectively, of which 656 are unique to pathogenic *Leptospira.* Approximately 40% of leptospiral genes encode proteins of unknown function. However, of the 655 proteins unique to *L. interrogans*, 78% have no known function. Likewise, 58% of the 308 unique *L. borgpetersenii* proteins have no defined function (Fig. 7).

In addition to the larger genome size and higher genome coding capacity, the genome of *L. interrogans* possesses genes needed for survival in non – host environments for longer periods of time before encountering a mammalian host. In contrast the genome of *L. borgpetersenii* is 16% (~700 Kb) smaller and has lower coding capacity than *L. interrogans*; essentially due to insertion mediated genome reduction. *L. borgpetersenii* lacks genes that are necessary for environmental sensing, metabolite transport and utilisation as a result of which it has lost the capacity to sense and survive in non – host environments and depends on host to host transmission (Bulach *et al.*, 2006b).The major challenge will be the characterization of the large number of genes of unknown function identified by comparative genomics and microarray analyses. Accordingly, comparative genomic analysis needs to be combined with other approaches to identify the determinants of virulence for this enigmatic pathogen.

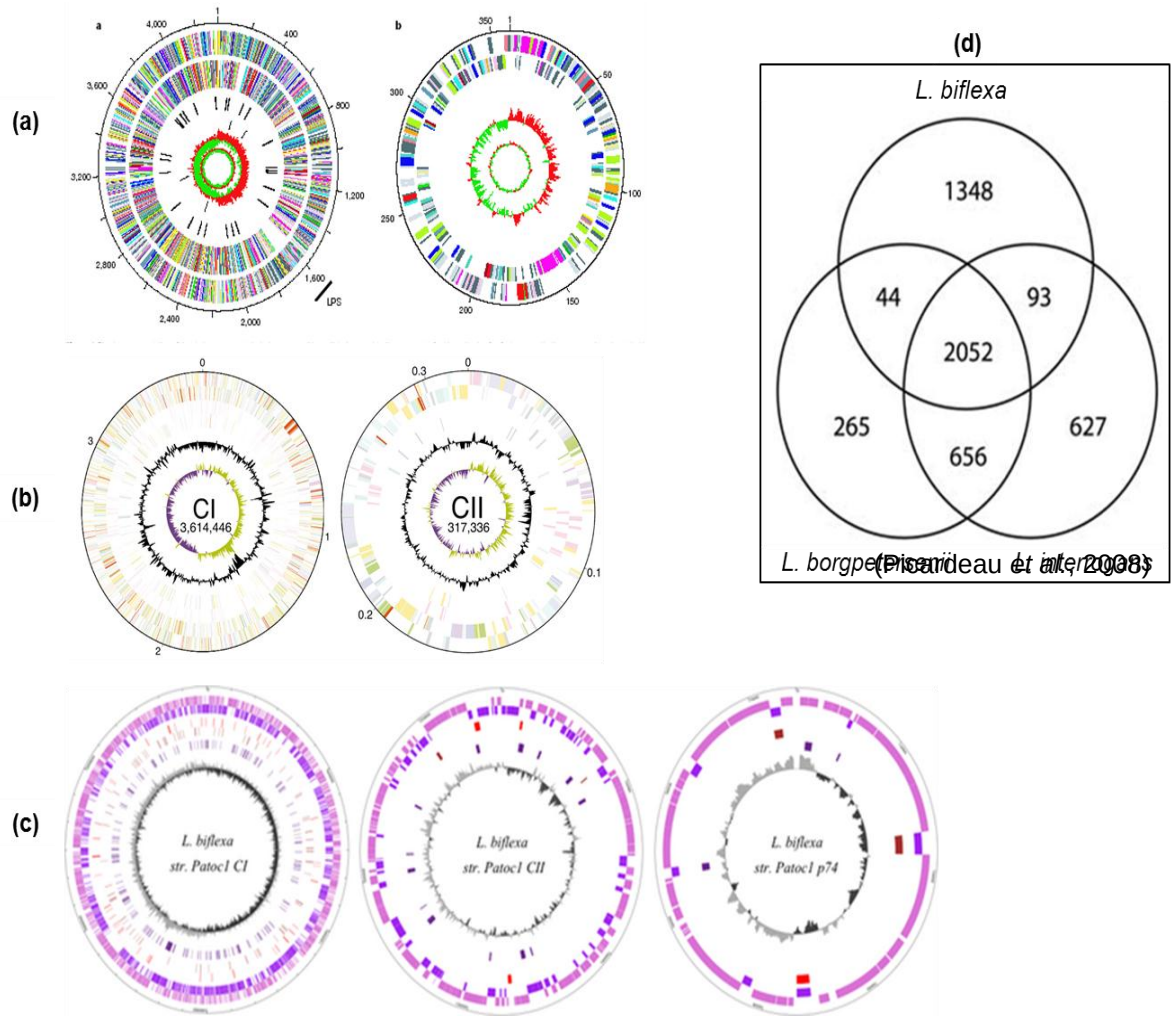


Fig. 7. Comparative genomics of pathogenic and non – pathogenic *Leptospira*

Circular representation of genomes of *L. interrogans* serovar Lai (a), *L. borgpetersenii* serovar Hardjo strain L550 (b) and *L. biflexa* serovar Patoc (c). The number of unique and shared genes among *L. interrogans, L. borgpetersenii* and *L. biflexa* are shown in the Venn diagram (d).

Studies in our lab focuses on the role of iron in pathogenic *Leptospira*. We demonstrated that iron limitation resulted in the expression of HbpA whose role is to acquire iron from the host via the hemin molecule. Further, iron limitation also resulted in the expression of sphingomyelinase and the release of the cleaved product of sphingomyelinase in outer membrane vesicles. In order to gain further insights into structural and functional aspects of leptospiral sphingomyelinases, their expression and regulation under diverse environmental conditions, this study was conducted with the following objectives.

Objectives of the study

I. Structure prediction of the leptospiral sphingomyelinases from *L. interrogans* serovar Lai.

II. Characterisation of the sphingomyelinase Sph2.

III. Influence of the environmental conditions on the expression of leptospiral sphingomyelinases.

IV. Molecular basis for the elevated expression of Sph2 in *L. interrogans* serovar Pomona.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and methods

2.1. Source of chemicals and reagents

Commonly used chemicals were purchased from Qualigens. List of special chemicals are listed in Table 2.

Table 2. Chemicals and reagents used for the study

Name of the chemical	Name of the supplier
EMJH base	Becton and Dickinson, USA
EMJH enrichment	
Noble agar	
Bovine serum albumin (BSA) fraction V	Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	
Ethylene diamine tetra-acetic acid (EDTA)	
Ethylene diamine N - N' diacetic acid (EDDA)	
Acrylamide	
Agarose	
Ethidium bromide	
NaCl (Molecular biology grade)	
Coomassie Brilliant blue R - 250	
Bicinchoninic acid (BCA) protein estimation kit	
3, 3' - diaminobenzidine (DAB)	
Sphingomyelinase C from *Staphylococcus aureus*	
Ceramide from bovine spinal cord	
Sphingomyelin from bovine brain	
Plasmid DNA isolation kit	
EZview Red protein A affinity gel	
Hydrogen peroxide	
Reaction clean up kit	Qiagen, USA
QIAquick PCR purification kit	
RNeasy Mini kit	
Protein molecular weight marker	MBI Fermentas, USA
DNA molecular weight marker	

Isopropyl β – D – 1 - thiogalactopyranoside (IPTG)	
Turbo DNase	Ambion, USA
SUPERase.In	
Luria Bertani agar	Himedia, India
Oligo nucleotide primers and sequencing	Eurofins, India; Invitrogen, USA
5 – bromo – 4 – chloro – 3 - indolyl phosphate / nitroblue tetrazolium (BCIP – NBT)	Bangalore Genei, India
Bug Buster Ni – NTA His - bind purification kit	Novagen, USA
Rat serum	Rockland Immunochemicals, USA
Donkey anti - rabbit IgG horse radish peroxidise (HRP)	Amersham Biosciences, USA

2.2. *In silico* analysis of leptospiral sphingomyelinases

2.2.1. Sequence alignment and identification of conserved domains

The five sphingomyelinase protein sequences from *L. interrogans* serovar Lai namely Sph1 (NP 711208.1), Sph2 (NP 711210.1), Sph3 (NP 714184.1), SphH (NP 7137120.1) and Sph4 (NP 713230.1) were retrieved from NCBI. The template sequences included the sphingomyelinases from *L. ivanovii* (SmcL, Accession No. Q9RLV9), *B. cereus* (BC SMase, Accession No. CAA31333), *S. aureus* (Accession No ZP_05693550.1) and *Pseudomonas* spp. strain TK4 (Accession No. BAB69072.1). Sequence alignment was performed using MAFFT tool (Version v6.713b) (Katoh & Toh, 2008) and the phylogenetic tree was drawn using MEGA 4.0 software (Tamura *et al.*, 2007) utilising the neighbour - joining method. The robustness of the tree was determined using bootstrapping with 500 replicates. The presence of signal peptide and location of signal peptide cleavage site in protein sequences were predicted using SignalP 3.0 server (Bendtsen *et al.*, 2004) utilising a combination of several artificial neural networks and hidden Markov models. The presence of conserved domains were predicted using NCBI conserved domain data base (Marchler-Bauer *et al.*, 2011) utilising reverse position specific BLAST algorithm and SMART tool (EMBL) (Letunic *et al.*, 2009). Repetitive sequences within the proteins were analysed using rapid automatic detection and alignment of repeats tool (RADAR) (Heger & Holm, 2000). Amino acid residues associated with

metal binding and catalytic activity in the template sequences were used to identify the corresponding amino acid residues in the query molecules.

2.2.2. Structure prediction using Insight II modeller

Homology modeling of Sph1, Sph2, Sph3 and SphH was done by Insight II Modeler (Version 2000, Accelrys Inc.) using the templates obtained from Protein Data Bank which included SmcL from *L. ivanovii* (PDB: 1ZWX) and BC SMase from *B. cereus* (PDB: 2DDR). 3D - PSSM (Kelley *et al.*, 2000) and 3D - Phyre (Kelley & Sternberg, 2009) were used to determine the fold similarity of Sph1, Sph2, Sph3 and SphH. Sequence alignment was done using the pair - wise alignment algorithm of the homology module in the software followed by manual adjustments to decrease the number of gaps. Care was taken not to insert gaps in regions that were part of the regular secondary structural motifs. Taking the coordinates of the respective 1.90 Å and 1.40 Å resolution structures of the templates 1ZWX and 2DDR, the 3D structures of the query sequences were predicted and superimposed on their respective template structure using Chimera Alpha Version 1.30 (build 2577) to study the fold deviation among them. Validation of the modeled structures was done by Ramachandran plot using Rampage (Lovell *et al.*, 2003).

2.3. Growth and maintenance of leptospiral cultures

2.3.1. List of leptospiral strains

Leptospira interrogans serovars Lai (strain 56601), Manilae (strain L495), Copenhageni (strain Fiocruz L1-130), Pomona (strain Pomona) and *Leptospira biflexa* serovar Patoc (strain Patoc - I) used in the study were obtained from the National Repository at the Regional Medical Research Center, ICMR, Port Blair, Andaman and Nicobar Islands, India & WHO / FAO Collaborating Center for Reference and Research on Leptospirosis, Royal Tropical Institute, Amsterdam.

2.3.2. Preparation of Ellinghausen – McCullough – Johnson - Harris (EMJH) medium

2.3.2.1. EMJH - enrichment medium

0.23 g of commercial EMJH base was dissolved in 90 mL of autoclaved triple distilled water in a 250 mL conical flask and autoclaved at 15 lbs / inch^2 pressure for 30 min. The flask was allowed to cool to room temperature and then 10 mL of EMJH enrichment (Difco) was added to it and mixed well by swirling. The EMJH semi -

solid medium was prepared by the addition of 0.18 g of noble agar to EMJH base before autoclaving. Media were stored at 4°C for future use.

2.3.2.2. EMJH - BSA medium

EMJH - BSA medium was prepared as per standard procedure published elsewhere (Ellis & Thiermann, 1986).

EMJH basal medium was prepared by dissolving 1.0 g of Na_2HPO_4, 0.3 g of KH_2PO_4, 1.0 g of NaCl, 1.0 mL of 10% glycerol and 1.0 mL of 25% ammonium chloride in sterile double distilled water. The pH of the medium was adjusted to 7.4, volume made up to 1 L and then sterilised by autoclaving at 15 lbs / $inch^2$ pressure for 30 min.

Supplement was prepared by dissolving 100 g of Bovine Serum Albumin (BSA) Fraction V in 500 mL of distilled water in a 2 L beaker. The solution was stirred slowly to avoid foaming and kept at 4°C overnight. $ZnSO_4.7H_2O$ (0.2 g / 50 mL), $CaCl_2.2H_2O$ (0.5 g / 50 mL), $MgCl_2.6H_2O$ (0.5 g / 50 mL), thiamine chloride (0.25 g / 50 mL), Vitamin B_{12} (0.01 g / 50 mL), $MnSO_4.4H_2O$ (0.3 g / 100 mL), $FeSO_4.7H_2O$ (0.6 g / 120 mL) and 10% v / v Tween 80 were prepared separately and sterilized by filtration. 10 mL each of thiamine chloride, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, $ZnSO_4.7H_2O$, Vitamin B_{12}, 1 mL of $MnSO_4.4H_2O$, 100 mL of $FeSO_4.7H_2O$ and 125 mL of 10% Tween 80 were added to the BSA solution and slowly stirred for 1 h at room temperature. The pH of the solution was adjusted to 7.4 with 10 M NaOH and the volume was adjusted to 1 L using sterile double distilled water. The solution was filter sterilised, dispensed aseptically as 100 mL aliquots and stored at -80°C.

To 100 mL of supplement 1.0 g of lactalbumin hydrolysate, 100 µL superoxide dismutase (from 10 mg / mL stock), 0.04 g sodium pyruvate, 100 µg / mL 5-fluorouracil were added and allowed to dissolve by incubating at room temperature for 10 min. This was added with stirring to 890 mL of EMJH base, filter sterilised and stored at 4°C. 1% rabbit serum was included for the growth of *L. interrogans* serovar Copenhageni strain Fiocruz (serum was collected from rabbit free of anti - leptospiral antibodies, heat inactivated by incubating at 56°C for 30 min and filter sterilised).

2.3.2.3. Iron free EMJH – BSA medium

All glassware was made iron free by soaking in 2% methanolic KOH overnight followed by overnight soaking in 8N HNO_3 and subsequent washings with glass double distilled water.

Iron - free EMJH – BSA medium was prepared similar to that of EMJH - BSA medium as mentioned in Section 2.3.2.2 except that $FeSO_4.7H_2O$ was omitted during the preparation of supplement.

2.3.3. Growth of *Leptospira*

2.3.3.1. Semi - solid EMJH – enrichment medium

Leptospiral cultures were maintained in semi - solid EMJH - enrichment medium in screw cap test tubes at 30°C. Growth was monitored regularly by viewing under the dark - field microscope (Nikon Eclipse E600).

2.3.3.2. Liquid EMJH medium

They were grown in EMJH – enrichment / EMJH – BSA medium at 30°C. Experiments were performed with virulent, low - passage forms of *L. interrogans* serovars Lai, Manilae, Copenhageni, Pomona and *L. biflexa* serovar Patoc obtained by infection and re - isolation from Golden Syrian hamsters. Densities of the cultures were determined by directly counting the number of organisms by dark - field microscopy. Cultures were grown to a density of 3×10^8 cells / mL. Growth under iron – regulated conditions is discussed in Section 2.10.1.4.

2.3.4. Harvesting of leptospiral cultures

Cultures were harvested by centrifugation at 8,000 rpm for 20 min at 4°C (Sorvel high speed centrifuge, USA). The cell pellets and the spent growth medium were stored in -80°C. For preparation of RNA, the cultures were transferred into an Erlenmeyer flask, chilled for 7 s in a dry ice - ethanol bath and centrifuged at 8,000 rpm for 20 min at 4°C.

2.3.5. Preparation of leptospiral cell lysates

The cell pellet was washed with 1X phosphate - buffered saline (1.06 mM KH_2PO_4, 155.17 mM NaCl and 2.97 mM $Na_2HPO_4.7H_2O$), pH 7.4 containing 5 mM $MgCl_2$ and re - suspended in 100 µL of SDS - PAGE sample loading buffer. Samples were re - suspended by pipetting and boiled for 5 min in a boiling water bath. 10 µL volume was used for separation by SDS – PAGE equivalent 1×10^8 cells.

2.4. Estimation of protein concentration by bicinchoninic acid (BCA) method

Protein concentration was estimated by BCA protein assay reagent kit as per manufacturer's instructions.

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

Proteins were fractionated by SDS - PAGE as per published protocol **(Laemmli, 1970)** either on 10% PAGEr Gold precast Tris - Glycine gels (Lonza, USA) or on manually cast gels.

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

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

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

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

5) Sample buffer (2X): 0.125 M Tris - HCl, pH 6.8 containing 4% SDS, 20% glycerol and 0.002% bromophenol blue. In some experiments the following sample buffer containing 50 mM Tris - HCl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.25 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.1% bromophenol blue was used.

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

7) Coomassie stain: 0.25 g Coomassie Brilliant Blue R - 250 was dissolved in methanol and made upto 100 mL using methanol : glacial acetic acid : water (60 : 10 : 30).

8) Destaining solution: This was prepared by adding methanol, glacial acetic acid and water in the ratio of 1:1:8

2.5.1. Preparation of gels

Glass plates with spacers were sealed with agar. The resolving gel mix was prepared either as 5 – 20% gradient gel or 10% gel using the recipe in Table 3 & Table 4. Gradient gel was prepared using a gradient maker (Bangalore Genei). The resolving gel was allowed to polymerize and then the stacking gel was poured over it after appropriately positioning the comb.

2.5.2. Protocol for SDS – PAGE

About 30 µg of protein was loaded onto mini gels and 75 µg of protein was used for gradient standard gels. Equal volumes of the protein samples and 1X sample buffer were mixed, boiled for 10 min and centrifuged for 10 min at 10,000 rpm to remove any insoluble material. The clear supernatant was loaded onto the gel and

electrophoresis was carried out at 25 mA constant current using Hoefer electrophoresis unit (SE600 series). The electrophoresis was allowed to run until the tracking dye was run out of the gel, followed by electrophoresis for an additional 15 min. The gel was stained with Coomassie Blue for 2 - 4 h and then destained overnight.

Table 3. Composition of resolving gel

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

Table 4. Composition of 5% stacking gel

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

2.6. Western blot analysis

2.6.1. Preparation of stock solutions

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

2) 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 1: 10 dilution.

3) Phosphate buffered saline – Tween solution (PBS - T): 81 mM Na_2HPO_4, 24.6 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20. The pH of the solution was adjusted to 7.5.

2.6.2. Procedure for transfer of proteins on to membrane

Proteins fractionated on polyacrylamide gels were electrophoretically transferred on to nitrocellulose membrane at 30 V constant voltage overnight or 60 V for 2½ h using 1X transblot buffer (Towbin *et al.*, 1979) in Broviga transfer apparatus (Balaji Scientific Services, India). For some experiments, the proteins were transferred using semi – dry transfer method, in which polyvinylidene fluoride (PVDF) membrane (Millipore) and the filter papers (two blotting pads) (BioRad) were trimmed according to the dimensions of the gel and allowed to wet by slowly sliding it at 45° angle into transfer buffer for 45 min. The gel was equilibrated in transfer buffer for about 5 min. Proteins were transferred from gel on to PVDF membrane using Trans - blot SD Semi - Dry transfer Cell system (BioRad) (10V for 45 min).

2.6.3. Development of blot

After the transfer, the membrane was removed and stained with Ponceau S stain to check the protein transfer efficiency and at the same time protein molecular weight marker was marked with pencil or pen. The membrane was incubated in blocking solution (5% skim milk, in PBS - T) for 30 min and then incubated with primary antibody for 30 min and washed three times (5 min each) with PBS - T. The membrane was then incubated with secondary antibody or protein A - horseradish peroxidase conjugate for immunoprecipitation experiments at 1:3,000 dilution (Amersham Biosciences, USA) in 5% blocking solution for 30 min and again washed three times in PBS-T and protein signals were detected.

Blots were developed either by 3, 3' - diaminobenzidine (in a 15 mL falcon tube add a pinch of DAB, 70 µL of H_2O_2 to 10 mL of 1x PBS) or by ECL Western blot detection system (Pierce, USA) and the bands were visualized with Hyperfilm (Amersham, Biosciences, USA) as per manufacturer's instructions.

2.7. Analysis of secretory - excretory proteins from the spent growth medium

2.7.1. Immunoprecipitation with specific antibody

Immunoprecipitation was done as per published protocol (Matsunaga *et al.*, 2005). The spent growth medium (1750 µL) was mixed with 5 µL of antibodies and left at 4°C overnight. A 25 µL volume of EZview Red protein A affinity gel was then added,

and the mixture was placed on an orbital mixer at 4°C for 2 h. The immune complex bound to the protein A was recovered by centrifugation for 7 s, washed twice with 800 µL of 10 mM Tris - HCl, pH 8.0 containing 0.4 M NaCl, and finally washed once with 800 µL of 10 mM Tris - HCl, pH 8.0. The pellet was re - suspended in 50 µL of SDS - PAGE sample loading buffer. Samples were boiled for 5 min and centrifuged for 7 s. 10 µL was used for immunoblot analysis.

2.7.2. Isolation of outer membrane vesicles (OMVs)

OMVs were isolated as per published protocol (Balsalobre *et al.*, 2006). The spent growth medium was transferred into ultracentrifuge tubes and subjected to ultracentrifugation (Beckman Ultracentrifuge, Ti70 rotor) at 40,000 rpm for 3 h at 4°C. The supernatant was carefully removed and the pellet containing the OMVs was re - suspended in 20 mM Tris - HCl buffer, pH 8.0. 50 µg was used for SDS – PAGE and immunoblot blot analysis.

2.8. Cloning and expression of recombinant leptospiral proteins

2.8.1. *E. coli* strains and vectors

E. coli strains included DH5α, BL21 (DE3), BL21 (DE3) Rosetta, BL21 pLysS, BLR (DE3) pLysS. pET 28a (+), pET 28b (+) were used in the study.

2.8.2. Preparation of Luria Bertani (LB) medium and growth of *E. coli*

1 g of tryptone, 0.5 g of yeast extract and 1 g of NaCl were dissolved in 75 mL double distilled water and the pH was adjusted to 7.0 with 1N NaOH. The final volume was made up to 100 mL using double distilled water. For the preparation of LB Agar, 1.5% of agar was added to the liquid medium. The media was sterilised by autoclaving at 15 psi pressure for 20 min. For the preparation of LB agar antibiotic plates, the media was allowed to cool to 50°C before adding the respective antibiotics (30 µg / mL chloramphenicol and 30 µg / mL kanamycin). About 25 mL of media was poured into 90 mm petri plates, allowed to solidify and stored at 4°C for future use.

A single colony of *E. coli* was inoculated into 5 mL of LB medium with appropriate antibiotics and allowed to grow at 37°C overnight. Glycerol stocks were prepared by adding equal volumes of sterile 40% glycerol and stored at -80°C.

2.8.3. Preparation of genomic DNA from *Leptospira*

Leptospiral genomic DNA was isolated as per published protocol (Marmur, 1961; Sambrook et al., 1989). The bacterial cell pellet from 10 mL culture was washed and

re - suspended in 400 µL of Tris - EDTA buffer (TE; 10 mM Tris and 1 mM EDTA, pH 7.6). It was incubated with 50 µL of lysozyme (10 mg / mL; Bangalore Genei, India) for 1 h at 37°C, followed by incubation at 50°C after the addition of 70 µL of 10% SDS and 60 µL of Proteinase K (1 mg / mL; Bangalore Genei, India). 100 µL of 5 M NaCl was added, mixed gently and the contents transferred to Phase lock gel tube (Eppendorf, Germany). 750 µL of chloroform: isoamyl alcohol mixture (24:1 v / v) was added, centrifuged at 13,000 rpm for 5 min and the upper aqueous layer was transferred into a fresh tube. The genomic DNA was precipitated by the addition of an equal volume of isopropanol, allowed to stand for 30 min at room temperature, followed by centrifugation at 12,000 rpm for 15 min. The DNA pellet was washed with 1 mL of 70% ethanol, dried and re - suspended in DNase free water. The concentration of the genomic DNA was determined using the Nano Drop spectrophotometer (Nano Drop technologies, Inc, USA) and stored at -20°C till use.

2.8.4. PCR amplification of *sph2* and *sph4*

sph2 (1872 bp) and *sph4* (420 bp) were amplified using sequence - specific primers (Table 5). PCR amplification reactions were performed in a 25 µL volume containing 2.5 µL of 10X Dream Taq buffer containing 2 mM $MgCl_2$ (Fermentas, USA), 200 µM dNTP mix (Fermentas, USA), 10 pmol each of forward and reverse primers, 1.0 Unit of Dream Taq DNA Polymerase (Fermentas, USA) and 100 - 200 ng of leptospiral genomic DNA. Amplifications were carried out in the thermal cycler (Eppendorf Master Cycler gradient. USA), using an initial denaturation for 15 sec (95°C) followed by 34 cycles of amplification (3 min at 68°C & 15 sec at 95°C) and a final extension for 3 min at 72°C. Annealing and extension were both done at 68°C. Negative control without template DNA was included. The PCR products were purified using Qiagen PCR purification kit as per manufacturer's instructions.

Table 5. Sequence specific primers for amplification of sph2 and sph4

Gene	Primer[#]	Sequence (5'to 3')	Restriction site
sph2	FP	GCGGCCGCATATGATAAACAAAATAACAAAACC	NdeI
	RP	ATAGGACTCGAGTTAGCGATAAATAAGATCCGC	XhoI
sph4	FP	GCTAGCCGACCTGACGGATGGTTAAG	NheI
	RP	CTCGAGATTCCTCAGGGCCTTCATTC	XhoI

[#] FP and RP represent forward and reverse primers respectively.

2.8.5. Restriction digestion

For cloning *sph2*, 1 µg of pET 28a(+) plasmid DNA and 1 µg of the *sph2* amplicon were individually subjected to double digestion. The reactions were setup in a final volume of 20 µL containing 10 units each of NdeI and XhoI, with the appropriate buffer given by the manufacturer. The tubes were incubated at 37°C for 5 h followed by purification of the fragment with Qiagen reaction clean - up kit as per manufacturer's instructions.

For cloning *sph4*, 1 µg of pET 28a(+) plasmid DNA and 1 µg of the *sph4* amplicon were individually subjected to double digestion. Reactions were setup in in a final volume of 20 µL containing 10 units each of NheI and XhoI, with the appropriate buffer given by the manufacturer. The tubes were incubated at 37°C for 5 h followed by purification of the fragment with Qiagen reaction clean - up kit as per manufacturer's instructions.

2.8.6. Ligation

The concentration of vector and insert were estimated and ligated in a ratio of 1:3 (vector: insert) in a sterile eppendorf tube as per the following formula.

$$\text{Concentration of the insert (ng)} = \frac{\text{ng of the vector} \times \text{Size of the insert (bp)}}{\text{Size of the vector (bp)}} \times 3$$

The ligation reaction was carried out in a total reaction volume of 20 µL containing vector DNA (100 ng), insert DNA (104 ng of *sph2*; 23 ng of *sph4*), 1 µL of T4 DNA ligase (5 U / µL), 2 µL of 10X ligation buffer and milli - Q water. Ligation mixture was incubated at 16°C for 16 h. 10 µL of ligation mixture was taken to transform *E. coli*.

2.8.7. Transformation

2.8.7.1. Preparation of ultra - competent cells

Ultra - competent cells were prepared as per standard procedure (Inoue *et al.*, 1990). 1% overnight *E. coli* culture was inoculated into 250 mL of LB broth in 1 L flask and allowed to grow at 18°C. The culture was allowed to grow till the cell density showed $OD_{600\ nm}$ of 0.4 - 0.5. The culture was kept on ice for 10 min and harvested by centrifuging at 2,500 g for 10 min at 4° C. The cell pellet was suspended in 80 mL of ice - cold Inoue transformation buffer (55 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 10 mM PIPES pH 6.7) and centrifuged at 2,500xg for 10 min at 4°C. The pellet was then re - suspended into 20 mL of ice cold Inoue

buffer and incubated on ice for 10 min after adding 1.5 mL of DMSO. The cells were quickly dispensed into sterile microfuge tubes as 100 µL aliquots and then frozen in a bath of liquid nitrogen. The tubes were stored at -80°C until further use.

2.8.7.2. Preparation of transformants

10 µL of ligation mixture of *sph2* / *sph4* was added to 100 µL of competent *E. coli* cells and incubated on ice for 30 min. After 30 min, the cells were subjected to heat shock at 42°C for 90 s and immediately chilled on ice for 2 min. 800 µL of LB broth was added and incubated at 37°C for 45 min. The cells were plated on LB agar plates containing kanamycin (30 µg / mL). The plates were incubated at 37°C for overnight and examined for the growth of transformed colonies.

2.8.7.3. Screening of transformants

Six to ten transformed colonies were picked and inoculated each into 5 mL of LB medium containing kanamycin (30 µg / mL) and incubated overnight at 37°C with shaking. Plasmids were isolated as described below and subjected to restriction digestion. The digested fragments were analysed by agarose gel electrophoresis. The recombinant plasmids pMS510 (pET28a containing *sph2*) and pMS511 (pET28a containing *sph4*) were verified by sequence analysis (Eurofins Genomics India Pvt Ltd).

2.8.8. Isolation of plasmid DNA by alkaline lysis SDS: Miniprep method

Plasmid DNA was isolated as per the published protocol (Sambrook *et al.*, 1989). A single colony was inoculated into 5 mL of LB medium containing 30 µg / mL kanamycin and was incubated overnight at 37°C with shaking. Cells were harvested by centrifuging at 10,000 rpm for 5 min. Supernatant was discarded and pellet was centrifuged again to remove any residual liquid. The cell pellet was re - suspended in 100 µL of ice cold solution I (50 mM glucose; 25 mM Tris - Cl, pH 8.0; 10 mM EDTA, pH 8.0). 200 µL of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed and stored on ice for 3 min. 150 µL of solution III (5M potassium acetate pH 5.2, glacial acetic acid and double distilled water in ratio of 60:11.5:28.5 (v / v)) was added and the eppendorf tube was inverted 5 - 6 times and stored on ice for 3 - 5 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was transferred to a fresh eppendorf tube containing DNase - free RNase (20 µg / mL) and incubated at 37°C for 40 min followed by extraction with equal volumes of phenol:chloroform (1:1). The two phases were separated by centrifugation at 12,000xg for 20 min at 4°C. The aqueous phase containing plasmid DNA was

transferred to a fresh tube, 2 volumes of isopropanol was added and allowed to stand at room temperature for 20 min. The plasmid DNA was pelleted by centrifugation at 12,000xg for 30 min at 4°C. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol. The pellet was allowed to dry and dissolved in 20 µL of autoclaved milli - Q water. The concentration of plasmid DNA was estimated using NanoDrop spectrophotometer (Nano Drop Technologies Inc, USA).

2.8.9. Agarose gel electrophoresis

Plasmid DNA and PCR products were resolved on 1% agarose gels prepared in 1X TAE buffer (0.04 M Tris - acetate and 0.001 M EDTA pH 8). The samples were subjected to electrophoresis (at 70 V) in 1X TAE buffer after diluting with 6X loading dye (0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA).

2.8.10. Expression of Sph2 and Sph4

The recombinant plasmids were transformed into expression host *E. coli* BL21 (DE3). The recombinant clones carrying *sph2* / *sph4* were induced for optimal production of the recombinant proteins. A single colony was inoculated into LB medium containing 30 µg / mL kanamycin at 37°C in an orbital shaker, allowed to reach mid – log phase ($OD_{600\ nm}$ of 0.5). Both Sph2 and Sph4 were induced with 1 mM IPTG for 3h. Sph2 was maintained at 27°C and Sph4 at 37°C. An aliquot removed before induction was used as control. Both un - induced and induced cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C and subjected to sonication for 2 min (20 sec pulse in Vibra Cell sonicator, USA). The pellet and supernatant separated by centrifugation at 10,000 rpm for 20 min were analysed on 10% gel by SDS - PAGE.

2.8.10.1. Optimisation of expression of Sph2

The following strategies were employed for soluble expression of Sph2 protein.

2.8.10.2. Induction with varying concentrations of IPTG

E. coli BL21 (DE3) pLysE expressing Sph2 were induced separately with 0.3 mM, 0.7 mM and 1 mM IPTG respectively for 3 h at 37°C. The cultures were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C and subjected to sonication for 2 min (20 sec pulse in Vibra Cell sonicator). The pellet and supernatant were separated by centrifugation at 10,000 rpm for 20 min and the presence of Sph2 protein in the

supernatant was assessed by separating them on 10% SDS - PAGE. Cultures were analysed as before.

2.8.10.3. Solubilisation with lysis buffers

The induced cell pellets were solubilised using the following lysis buffers.

a) 50 mM Tris - HCl, pH 7.8
b) 50 mM Tris - HCl, pH 7.8 with 200 mM NaCl
c) 50 mM Tris - HCl, pH 7.8 with 200 mM NaCl & 5% Glycerol
d) 50 mM Tris - HCl, pH 7.8 with 200 mM NaCl & 0.1% Triton X-100
e) 50 mM Tris - HCl, pH 7.8 with 200 mM NaCl & 500 mM $(NH_4)_2 SO_4$
f) BugBuster protein extraction reagent

The re - suspended cultures were sonicated and processed as mentioned earlier. Cell pellet re - suspended with BugBuster protein extraction reagent was not subjected for sonication but instead incubated for 20 min at room temperature and processed as mentioned earlier.

2.8.10.4. Co - expression of Sph2 with chaperones

Co - expression of Sph2 with chaperones (DnaK, DnaJ, GrpE, GroES and GroEL) involved two steps: transformation of *E. coli* BL21 (DE3) with a chaperone plasmid (pG - KJE8) followed by transformation with pMS510. The transformant containing pMS510 and pG - KJE8 was grown in LB medium containing 30 µg / mL kanamycin, 20 µg / mL chloramphenicol, 10 ng / mL tetracycline, and 0.5 mg / mL L - arabinose at 37°C till mid - log phase of $OD_{600\ nm}$ 0.5. Cultures were induced with 1 mM IPTG and allowed to grow at 37°C for 3 h. Cultures were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C and subjected to sonication for 2 min (20 sec pulse in Vibra Cell sonicator, USA). Cultures were processed as before.

2.8.11. Purification of Sph2 and Sph4 from inclusion bodies

2.8.11.1. Preparation of inclusion bodies

E. coli cultures expressing Sph2 or Sph4 were pelleted by centrifuging at 10,000xg for 10 min at 4°C. Cells were lysed by vortexing in BugBuster protein extraction reagent containing rLysozyme (1KU / mL of BugBuster reagent) and Benzonase Nuclease (25 Units / mL of BugBuster reagent) (Novagen). 5 mL of BugBuster protein extraction reagent was used for 1 g wet weight of cell pellet, incubated at room temperature for 20 min and centrifuged at 16,000xg for 20 min at 4°C. The supernatant was discarded and the pellet containing inclusion bodies were washed and processed as per manufacturer's instructions.

2.8.11.2. Electrophoretic separation and gel elution

The insoluble inclusion bodies containing rSph2 / rSph4 protein were gel electrophoresed on 10% gel. The respective induced bands were excised, crushed in a syringe and eluted with elution buffer (0.25 M Tris - HCl, pH 8.8 containing 0.5% SDS) by subsequent incubations at 37°C. The eluted protein was subjected to SDS - PAGE to assess the purity of the recombinant protein.

2.8.11.3. Affinity chromatography on Ni – NTA column

The pellet containing inclusion bodies was solubilised in 50 mM Tris - HCl, pH 8.0 containing 8 M urea and incubated at room temperature for 30 min. This solution was incubated with 1 mL of Ni - NTA His Bind Resin pre - equilibrated with the above buffer for 30 min at room temperature. The column was tightly sealed with parafilm and incubated with shaking for 30 min at room temperature. The column was removed and washed extensively with wash buffer (50 mM Tris - HCl, pH 8.0 containing 10 mM imidazole & 8 M urea). The bound protein was then eluted with elution buffer (50 mM Tris - HCl, pH 8.0 containing 200 mM imidazole & 8 M urea) and collected as 1 mL fractions. The different fractions were analysed by SDS - PAGE and immunoblotting with HRP - conjugated anti - His antibodies. Fractions containing the pure recombinant Sph2 and Sph4 were pooled and concentrated.

2.8.11.4. Raising antibodies against Sph2 and Sph4

Polyclonal antibodies were raised against Sph2 and Sph4 proteins separately in New Zealand white rabbits. Pre – immune sera were obtained by collecting 10 mL blood. Gel slices containing 100 µg of the respective protein was crushed using syringe and emulsified with Freund’s complete adjuvant (Bangalore Genii, India). The rabbits were immunised subcutaneously at multiple sites. Three weeks after the first immunization, the first booster was administered intramuscularly with 100 µg of the antigen emulsified in incomplete Freund’s adjuvant. One week later, blood samples were collected and the serum was analysed for antibodies against the injected proteins by dot – blot analysis. Boosters (second and third) were administered and analysed at an interval of three weeks following same immunisation process. Blood was collected from the ear vein and serum was prepared. All the protocols were approved by Institutional Animal Ethical committee.

2.9. Biological activity of Sph2

Despite attempts to express rSph2 in the soluble form, most of the protein was obtained as insoluble protein in inclusion bodies with small amounts of soluble protein in the supernatant. Hence whole cell sonicates of induced clones were used for assaying biological activities.

2.9.1. Hemolytic assay

The cell free whole - cell sonicate of the induced clone was prepared by sonicating the washed and re - suspended (in 0.9% saline) cell pellet for 5 min (30 s pulses at 12 Hz in a Vibra Cell sonicator, USA), followed by centrifugation at 10,000 rpm for 20 min. 300 µg of total protein in a volume of 100 µL was used for the hemolytic assay. The assay was performed in a 96 well round - bottomed microtitre plate. The reaction was set up in duplicate in a final volume of 200 µL as follows: a) cell - free sonicate alone, (b) cell - free sonicate with 10 mM EDTA, (c) cell - free sonicate with 10 mM magnesium chloride, (d) cell - free sonicate with 5 mM manganese chloride, (e) cell - free sonicate with 10 mM calcium chloride, (f) cell - free sonicate with 10 mM magnesium chloride and 10 mM calcium chloride, (g) cell - free sonicate with 10 mM manganese chloride and 10 mM calcium chloride. Commercial sphingomyelinase C from *Staphylococcus aureus* (Sigma; 0.8 µg in 100 µL saline with10 mM magnesium chloride) and whole cell sonicates of *E. coli* BL21 (DE3) containing empty vector (pET28a) and *E. coli* BL21 (DE3) containing pET 28a - *hupB* (expressing non - hemolytic histone - like DNA - binding protein from *M. tuberculosis*) were included in the test. 40 µL of a 10% suspension of washed sheep erythrocytes in normal saline was added to all the above wells, incubated for 2½ h at 37°C followed by incubation for 30 min at 4°C. The microtitre plate was then subjected to centrifugation (Kubota, High speed refrigerated centrifuge, Model 6500, Tokyo, Japan) at 2,500 rpm for 10 min. 100 µL of the supernatant was transferred to another flat - bottomed microtitre plate and the absorbance was measured in a Microplate Reader (BioRad, Microplate reader Model 680XR, USA) at 570 nm using 0.9% saline as blank. Hemolysis in the respective test sample was calculated using the following formula

$$\% \text{ Hemolysis} = \frac{(OD_{570\ nm} \text{ of test}) - (OD_{570\ nm} \text{ of negative control})}{(OD_{570\ nm} \text{ of positive control})} \times 100$$

where negative and positive controls show zero and complete lysis of the erythrocytes when incubated in normal saline and double distilled water respectively. All experiments were performed atleast three times in duplicates.

2.9.2. Enzymatic assay

Sphingomyelinase activity was assessed by either of the two methods that detect the products obtained upon enzymatic activity.

2.9.2.1. Thin layer chromatography (TLC)

This was done as per published protocol (Segers *et al.*, 1990) with slight modifications. The reaction was set up in 2 mL microfuge in a final reaction volume of 1 mL consisting of ether:methanol (9:1 v / v), 150 µg of cell free extracts containing rSph2, 25 mM $MgCl_2$, 2 mg sphingomyelin and 0.9% saline. A reaction set up without cell free extract served as negative control. The mixture was vigorously shaken for 4 h at 37°C, the two phases separated and 20 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 upon exposure to iodine vapours. Commercial sphingomyelinase C from *S. aureus* & ceramide were used as substrate and product controls respectively.

2.9.2.2. Amplex Red Sphingomyelinase Assay Kit

Sphingomyelinase activity was analysed using Amplex Red Sphingomyelinase assay kit (Molecular probes, Invitrogen, USA) as per the manufacturer's instructions. The reactions were set up in 96 - well special optics flat clear bottom black polystyrene Microplate (Corning, product # 3720). The reaction mixture (200 µL) contained 100 µL test sample and 100 µL of 100 µM Amplex red reagent (containing 2U / mL HRP, 0.2 U / mL choline oxidase, 8 U / mL alkaline phosphatase and 0.5 mM sphingomyelin). The whole plate was incubated inside the Microplate reader at 37°C protected from light. Fluorescence was continuously measured at different time points at 30 min time intervals to follow the kinetics of the reactions. Positive controls included (a) 0.04 U / mL sphingomyelinase (from *B. cereus*) and (b) 10 µM H_2O_2. The fluorescence was measured at excitation and emission wavelengths of 485 / 20 and 590 / 35 respectively using Synergy2 Multi - Mode Microplate Reader (BioTek, USA). The background fluorescence was corrected by subtracting the negative control which lacked sphingomyelinase. All experiments were performed twice in triplicates each.

2.10. Influence of environmental conditions on the expression of leptospiral sphingomyelinases

2.10.1. Culture conditions

The effects of various environmental conditions likely to be encountered by the pathogenic Leptospira inside the mammalian host were studied on cultures grown in EMJH - BSA medium at 30°C. The leptospiral serovars used in the study includes *Leptospira interrogans* serovars Lai (strain 56601), Manilae (strain L495), Copenhageni (strain Fiocruz L1 - 130), Pomona (strain Pomona) and *Leptospira biflexa* serovar Patoc (strain Patoc - I). After reaching a density of 3 - 4 x 10^8 cells / mL cultures were subjected to the conditions of osmolarity, serum, iron limitation and oxidative stress as mentioned below and processed as mentioned in 2.3.4. The experiments were performed in triplicate.

2.10.1.1. Effect of 120 mM NaCl

Serovars Pomona, Lai, Copenhageni and Manilae, grown to a cell density of 3 – 4 x 10^8 cells / mL were supplemented with 120 mM NaCl and allowed to incubate for 4 h.

2.10.1.2. Effect of rat serum

Serovars Pomona, Lai, Copenhageni and Manilae, grown to a cell density of 3 – 4 x 10^8 cells / mL were supplemented with 10% rat serum and allowed to incubate for 4 h. Dose - dependent effect of rat serum was studied further by the addition of serum to a final concentration ranging between 5 – 40%. To assess the effect of heat - inactivated serum, serum was incubated at 56°C for 30 min before addition to culture medium.

2.10.1.3. Combined effect of salt and serum

Serovars Pomona, Lai, Copenhageni and Manilae, grown to a cell density of 3 – 4 x 10^8 cells / mL were supplemented with 120 mM NaCl and 10% serum simultaneously and allowed to incubate for 4 h.

2.10.1.4. Effect of iron limitation

10^7 cells from *L. interrogans* serovar Lai culture were incubated at 30°C with iron - free EMJH – BSA medium that was pre - incubated with 300 µM EDDA; iron – replete growth was achieved with EMJH – BSA medium to which 10 µg Fe / mL was added. For studying the effect of temperature, identical cultures were incubated at 37°C. Cultures were allowed to grow to a cell of density of 5 x 10^8 cells / mL before harvesting.

2.10.1.5. Addition of H_2O_2

Serovars Pomona and Manilae, grown to a cell density of 3 – 4 x 10^8 cells / mL were supplemented with H_2O_2 at 5 µM, 20 µM and 80 µM respectively and incubated for additional 4 h before harvesting.

2.10.2. Assays for the expression of leptospiral sphingomyelinases

Sph1, Sph2, Sph3 and SphH were assayed by qRT - PCR, immunoblot analysis with specific antibodies and biological activity.

2.10.2.1. Quantification of mRNA transcripts by qRT- PCR

2.10.2.1.1. Isolation of total RNA

RNA was isolated from leptospires from 25 mL culture as follows. 1 mL Trizol was added to the cell pellet, re - suspended thoroughly by pipetting followed by incubation at room temperature for 5 min. 200 µL of chloroform was added, mixed vigorously for 15 s followed by incubation at room temperature for 3 min. The tubes were centrifuged at 12,000 g for 15 min at 4°C and the upper aqueous layer pipetted into fresh tubes. RNA was precipitated by the addition of equal volumes of isopropanol, mixed and incubated for 10 min and subjected to centrifugation at 12,000 rpm for 15 min at 4°C. The pellet was washed with 1 mL of 75% ethanol, air dried and dissolved in 84 µL of RNase free water. The dissolved RNA was subjected to DNase treatment using 1µL of 'SUPERase. In', 10 µL of 10X Turbo DNase buffer and 5 µL of Turbo DNase followed by incubation in 37°C water bath for 2 h. The samples were subjected to RNA clean - up using RNeasy Mini kit as per manufacturer's instructions. The concentration of the RNA was determined using NanoVue spectrophotometer (GE Healthcare, USA) and the quality of RNA was assessed by loading 200 ng of RNA onto 1.2% agarose gel.

2.10.2.1.2. cDNA synthesis and qRT-PCR

The cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad) following manufacturer's protocol. The reaction was set up in a microfuge tube in a reaction volume of 20 µL containing 4 µL of 5X iScript reverse transcription supermix (containing iScript MMLV RNase H^+ reverse transcriptase, RNase inhibitor, dNTPs, oligo (dT), random primers, buffer, $MgCl_2$ and stabilisers), 1 µg RNA and nuclease - free water. The cDNA synthesis was carried out in the thermal cycler (Eppendorf Master Cycler gradient, USA) with an initial priming at 25°C for 5 min followed by reverse transcription at 42°C for 30 min and inactivation at 85°C for 5 min. A separate reaction without reverse transcriptase was set up as negative control. The

synthesised cDNA was diluted with RNase free water to obtain 10 ng / µL working concentration.

PCR was performed using gene specific primers and cDNA using HotStarTaq Master Mix Kit, USA), using an initial denaturation for 15 min (95°C) followed by 29 cycles of amplification (15 s at 95°C, 30 s at 58°C & 30 s at 72°C) and a final extension for 2 min at 72°C. Amplification products were analysed on 1.2% agarose gel electrophoresis

The quantification of cDNA was done using Real - time PCR (qPCR) using iQ^{TM} SYBR Green Supermix (BioRad) and iQ^{TM} 5 Multicolour Real-time PCR detection system (BioRad). Each reaction contained cDNA derived from 50 ng of RNA, 10 pmol of each forward and reverse primers, and 12.5 µL of 2X iQ^{TM} SYBR Green supermix in a total volume of 25 µL with RNase free water. Gene specific primers (Table 6) were designed using Primer3 program (Rozen & Skaletsky, 2000). The amplification protocol consisted of an initial denaturation for 12 min 30 s (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. Standard curve was constructed by 5 - fold serial dilutions (50 ng to 0.08 ng) of cDNA as template in triplicates. The resulting data were analysed using BioRad iQ^{TM} 5 2.0 standard edition optical system software. An E value in the range of 90% to 110% indicated the robustness of the assay as determined by the software. The C_T values were normalised using *lipL41* as normaliser and the expression levels were calculated using $2^{-\Delta C}{}_T$ method. The relative fold change was calculated by comparision with the medium control. The assay was performed using three independent samples and statistical analysis was performed using GraphPad InStat v3.0 (La Jolla, CA). The statistical tests used are described in described in the respective figure legends.

2.10.2.2. Immunoblot analysis of leptospiral proteins with antibodies against sphingomyelinases

2.10.2.2.1. Source of antibodies

Anti - $Sph2_{163}$ [raised against Sph2 N - terminal 27 to 190 amino acids in rabbits (Matsunaga *et al.*, 2007b)], anti - LipL41, anti - GroEL, and anti - LipL32 were from the lab collection of Prof. David A Haake (UCLA). Specific peptide for SphH (AERIGIDAQVSSEVDIK - C) was chemically synthesized (Pacific Immunology, San Diego, CA). Assistance from Pacific Immunology was received in selecting the

specific peptide and care was taken to avoid terminal cysteines. Synthesized peptides were coupled (~10 mg peptide) to keyhole Limpet Hemocyanin carrier protein via cysteine that has been added at the N or C - terminal end of the peptide (underlined) for immunizing New Zealand white rabbits.

2.10.2.2.2. Immunoreactivity with leptospiral whole cell lysates

Leptospiral whole cell lysates were prepared as mentioned in Section 2.3.5. Proteins were resolved on 10% PAGEr Gold precast Tris - Glycine gels and electrophoretically transferred onto PVDF membrane, probed as mentioned in Section 2.6.2 and developed using enhanced chemiluminescence western blot detection system as per manufacturer's instructions (Thermo Scientific).

2.10.2.3. Biological activities of leptospiral spent growth medium

2.10.2.3.1. Hemolytic activity

This was done essentially as described earlier in Section 2.9.1 except that sheep RBC (50%) were procured commercially from Quad Five (MT, USA) as Alsever - buffered solution (50:50 v / v).

2.10.2.3.2. Enzymatic assay using Amplex Red sphingomyelinase assay kit

Sphingomyelinase activity was analysed using Amplex Red Sphingomyelinase assay kit as described earlier in Section 2.9.2.2 except that 100 µL spent growth medium from different cultures were used as test.

2.11. Molecular basis for the elevated expression of Sph2 in *L. interrogans* serovar Pomona

2.11.1. Promoter prediction analysis

The upstream sequences of sph2 (338 bp in Copenhageni, 650 bp in Pomona, 336 bp in Lai and 348 bp in Manilae) were retrieved and analysed for the presence of putative promoter elements using BPROM (Softberry Inc, NY, USA) (http://linux1.softberry.com/berry.phtml). Sphingomyelinase sequences of L. interrogans serovars Manilae and Pomona were kindly provided by Dr. Ben Adler (Monash University).

Table 6. List of primers used for qRT - PCR

Gene	Primer name[#]	Sequence (5'to 3')	Remarks[##]
sph1	LIC12632-F	TGAAACCGTTTTGATTACGGGTGA	P, C, L
	LIC12632-R	CGGCAAGTGCGTTTGTTTTTGTAT	
	LIL495 01006-F	TCTTCAAATTCAAGCTCCGCAAAA	M
	LIL495 01006-R	GCCCCAACTTGAAAGATTCGTAGG	
sph2	LIC12631-11F	ATGTTGCCCACAAATCTTCCACG	P, C, L, M
	LIC12631-12R	CCACATCTGTTTGATACGGATATTCTTCT	
sph3	LIC13198-F	TTTCTTGCAGTCTGGGAAAATGGA	P, C, L
	LIC13198-R	TGCTCCAATCTCTTTCAGGTGAGG	
	LIL495 03485-F	CCGGTTTGGCAGAATTTAGCGTAT	M
	LIL495 03485-R	TGCCCTGATTTTGTAGGAGTGGAA	
sphH	LIC10657-F	AGCCGATCCTAAATCCGATACCAA	P, C, L, M
	LIC10657-R	CTCCGAGCCACCAATTCCAATAAT	
sph4	LIC11040-F	TTTAAAAGTCAGCGGCTGTTTGGA	P, C, L
	LIC11040-R	CTGCCTCCATCCCAATCTATGACA	
	LIL495 02742-F	ACGTTTTAAAGGTCAGCGGCTGTT	M
	LIL495 02742-R	GCCCATCCCAATCTATGACGAAAT	
lipL41	LipL41-7F	TCGGAAATCTGATTGGAGCGGAAGCA	P, C, L, M
	LipL41-8R	AGAAGCGGCGAAACCTGCCACT	
katE	LIC12032-F	GGGCCGGTTTTAATTCAGGATACA	L
	LIC12032-R	CCCAGCACCTTTTGCATGAACTAC	

F: Forward primer; R: Reverse primer; [##]Primers used for qRT - PCR quantification from cDNA of P - Pomona; C - Copenhageni; L - Lai; M - Manilae.

2.11.2. Prediction of transcriptional start site of *sph2*$_{Pomona}$ by 5'RACE

For predicting the transcriptional start site of *sph2*$_{Pomona,}$ *L. interrogans* serovar Pomona was grown in EMJH medium to a density of 3 x 10^8 cells / mL and then supplemented with 120 mM NaCl for 4 h. For comparison *L. interrogans* serovar Copenhageni (grown and supplemented with 120 mM NaCl for 4 h) was included as positive control whose genome sequence is available.

The transcriptional start site of *sph2*$_{Pomona}$ was determined using 5' rapid amplification of cDNA ends using second generation 5' / 3' RACE kit (Roche Diagnostics, USA) essentially as recommended by manufacturer. First strand cDNA

was synthesised in a 20 µL volume containing 4 µL of 5X cDNA synthesis buffer (250 mM Tris – HCl buffer, pH 8.5 containing 40 mM $MgCl_2$, 150 mM KCl and 5 mM dithiothreitol), 2 µL of deoxynucleotide mixture (10 mM each of dATP, dCTP, dGTP and dTTP in Tris - HCl, pH 7.5), 1.25 µL of 10 µM cDNA synthesis primer (5' CGTTTTGCTCTTTCATCGTGTC - 3'), 2 µg RNA, 1 µL of Transcriptor Reverse Transcriptase (Roche Diagnostics, USA) and nuclease - free water. Reaction mixture was incubated at 55°C for 1 h followed by incubation at 85°C for 5 min and the cDNA synthesised was purified using a commercial kit (Qiagen, USA). Homopolymeric A - tail was added at the 3' end of first strand cDNA using recombinant Terminal Transferase and dATP. PCR amplification of dA - tailed first strand cDNA was done with oligo dT - anchor primer (5' - GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV - 3') and the *sph2* - 9R primer (5' – GCGGAGCTGCCATTTTCTG - 3') using HotStar Taq DNA polymerase from Qiagen. PCR products were analysed by 1.2% agarose gel electrophoresis, gel extracted and sequenced.

2.11.3. Preparation of *sph2* constructs and transformation into *L. biflexa*

2.11.3.1. PCR amplification of $sph2_{Pomona}$

A 2715 bp DNA fragment inclusive of the coding sequence *sph2* (1947 bp), up – stream and down – stream region of 638 and 130 bp respectively was amplified from the genomic DNA of *L. interrogans* serovar Pomona using gene specific primers (Table 7). PCR amplification reactions were performed in a 50 µL volume containing 10 µL of 5X Phusion HF buffer (having 1.5 mM $MgCl_2$), 200 µM dNTPs, 10 pmol each of forward and reverse primers, 2.0 Units of Phusion High Fidelity DNA polymerase (Finnzymes, Woburn, MA), 200 ng of genomic DNA and water. Amplifications were carried out in the thermal cycler (Eppendorf Master Cycler gradient. USA), using an initial denaturation for 1 min (98°C) followed by 29 cycles of amplification (10 s at 98°C, 20 s at 64.6°C & 1 min at 72°C) and a final extension for 2 min at 72°C. The amplicon referred to as $sph2_{Pomona\ -\ 2715}$ was analysed on 1.2% agarose gel electrophoresis and purified using Qiagen PCR purification kit. $sph2_{Copenhageni}$ construct having similar upstream and downstream region was gifted by Dr. James Matsunaga (University of California Los Angeles).

2.11.3.2. Cloning into shuttle vector

1 µg of pRAT575 shuttle vector (Matsunaga & Coutinho, 2012) and $sph2_{Pomona\ –\ 2715}$ were individually subjected to double digestion. Reactions were set in digestion tubes in a final volume of 20 µL containing 10 units each of KpnI and XhoI, 2 µL of

10X buffer 1 (10 mM Bis – Tris - Propane - HCl, pH 7.0 containing 10 mM $MgCl_2$, 1 mM Dithiothreitol) with BSA and milli - Q water. The tubes were incubated at 37°C for 5 h and preceded with Qiagen reaction clean - up kit as per manufacturer's instructions. Ligation of the purified digested products was done using Quick T4 DNA ligase (NEB) as per manufacturer's instructions and transformed into frozen competent *E. coli* NEB 5 - α cells (NEB). Transformants were screened and confirmed by plasmid isolation followed by restriction digestion. Positive recombinant plasmid (pRAT667) was further confirmed by DNA - sequencing (Laragen, Culver City, CA). A transcriptional T7 terminator (Table 7) was inserted at the KpnI site of pRAT667. The positive recombinant plasmid having T7 terminator (pRAT680) was verified by DNA sequencing.

Table 7. Primers and oligos used for preparation of *sph2* constructs

Gene	**Primer name**[#]	**Sequence (5'to 3')**
$sph2_{Pomona - 2715}$	Lic12631 (Kp) - 17F	GACCATGGTACCAGCGAGACGCTGAGTCTGA
	Lip0980 (Xh) - 4R	CAACTCGAGGGTATTTTATTGAATAAGATTGGGAAGGT
T7 terminator oligos	T7Te(Kp) - 2F	CTAATCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATAAGGAGGTAC
	T7Te(Kp) - 2R	CTCCTTATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGATTAGGTAC

F: Forward primer; R: Reverse primer;

2.11.3.3. Transformation into *L. biflexa*

Recombinant plasmids pRAT680 containing $sph2_{Pomona\ -\ 2715}$ and pRAT778 containing $sph2_{Copenhageni\ -\ 2592}$ (gifted by Dr. James Matsunaga, University of California Los Angeles) were transformed into *L. biflexa* as follows. *L. biflexa* serovar Patoc was grown in 1X Probumin™ Vaccine Grade Solution (Millipore, Billerica, MA) (Pinne & Haake, 2009) containing 5 - fluorouracil (100 µg / mL) at 30°C. After reaching a density of 0.3 to 0.4 (at 420 nm) cells were pelleted by centrifugation at 3,000xg at room temperature and washed with autoclaved milli - Q water. Cell pellet was re - suspended and diluted using autoclaved milli - Q water to a density of 3 x 10^{10} cells / mL. 3.5 µg of each of the recombinant plasmids was mixed with 100 µL of re - suspended cells separately and transferred to chilled electroporation cuvette.

The cuvette was placed in the electroporation unit (BioRad Gene Pulser II) and subjected to electroporation (1.8 kV, 25 µF, and 200 Ω) (Girons *et al.*, 2000). 1 mL of 1X Probumin^{TM} Vaccine Grade Solution was added to the electroporation cuvette, mixed gently, contents transferred to a 14 mL polypropylene tube and incubated overnight at 28°C with shaking. 200 µL of the grown culture was plated onto Probumin - Agar plates (containing 40 µg / mL spectinomycin, 100 µg / mL 5 – fluorouracil, 0.55% bacto agar) and incubated for 10 - 16 days at 30° C until leptospiral colonies appeared.

2.11.4. Expression analysis and immunoblotting

L. biflexa serovar Patoc transformants were inoculated and grown in 1X Probumin^{TM} Vaccine Grade Solution containing spectinomycin (40 µg / mL) and 5 - fluorouracil (100 µg / mL) to a density of 3 to 4 x 10^8 cells / mL. Cultures were harvested and processed as mentioned in Sections 2.3.4 and 2.3.5. Expression of $Sph2_{Pomona}$ and $Sph2_{Copenhageni}$ was assessed by immunoblotting.

CHAPTER 3

RESULTS

3. Results

3.1. Structural analysis of leptospiral sphingomyelinases

3.1.1. Multiple sphingomyelinases in *L. interrogans* serovar Lai and their corresponding homologs in other pathogenic *Leptospira*

Genome sequencing uncovered the multiple sphingomyelinases in several pathogenic *Leptospira*. Sphingomyelinase genes of *L. interrogans* serovar Lai encode Sph1 (68.19 kDa), Sph2 (71.03 kDa), Sph3 (65.33 kDa), Sph4 (27.92 kDa) and SphH (64.43 kDa). All the five orthologs are present in serovars Pomona, Copenhageni, and Manilae while only three orthologs (*sphA*, *sphB* and *sph4*) are present in *L. borgpetersenii* serovar Hardjobovis strains JB197 and L550. *L. borgpetersenii* sphingomyelinases LBJ0291 & LBL2785 (annotated as SphA) are 64% similar to Sph1 & Sph2 while LBJ0527 & LBL2552 (annotated as SphB) are 70% similar to Sph1, Sph2, SphA, SphH and Sph3. Orthologs of Sph1 and SphH are absent in *L. borgpetersenii*. The non - pathogen *L. biflexa* lacks all the five sphingomyelinase orthologs (Picardeau *et al.*, 2008).

3.1.2. Phylogenetic analysis of leptospiral sphingomyelinases

Leptospiral sphingomyelinases Sph1, Sph2, Sph3 and SphH are unique and form a separate cluster as compared to other bacterial sphingomyelinases (Fig. 8).

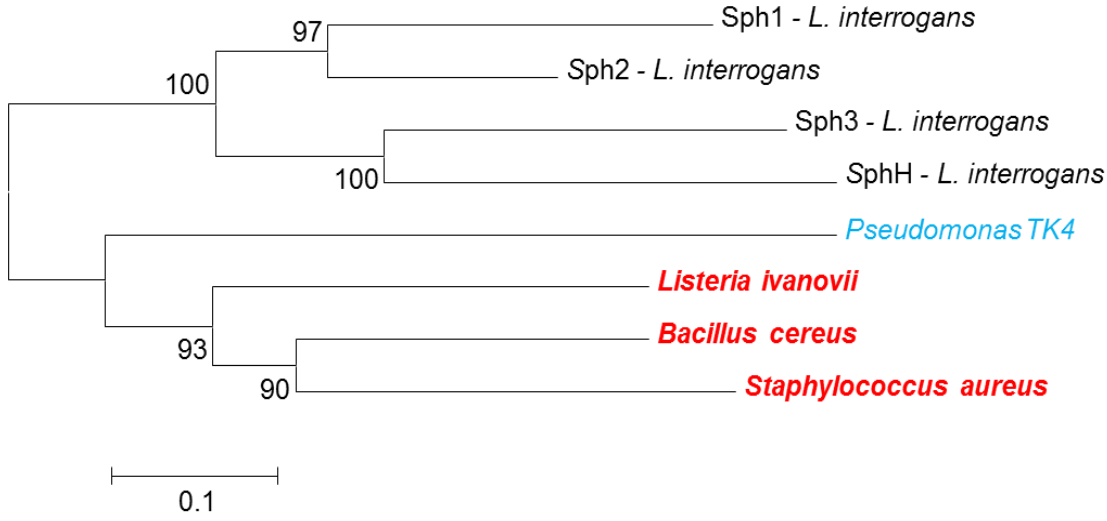


Fig. 8. Phylogenetic analysis of bacterial sphingomyelinases

The dendrogram represents the phylogenetic relationship of leptospiral sphingomyelinases with other bacterial sphingomyelinases.

The dendrogram of leptospiral sphingomyelinase proteins from four different strains of *L. interrogans* and two strains of *L. borgpetersenii* (Fig. 9) revealed six clusters with respective proteins from the two species *L. interrogans* and *L.*

borgpetersenii proteins forming separate clusters. The genes encoding Sph1 and Sph2 in *L. interrogans* appear to have arisen from a relatively recent duplication event consistent with *sph1* and *sph2* being located next to each other on the *L. interrogans* chromosome. In contrast, only one copy of *sphA* is present in the same genomic position in *L. borgpetersenii*.

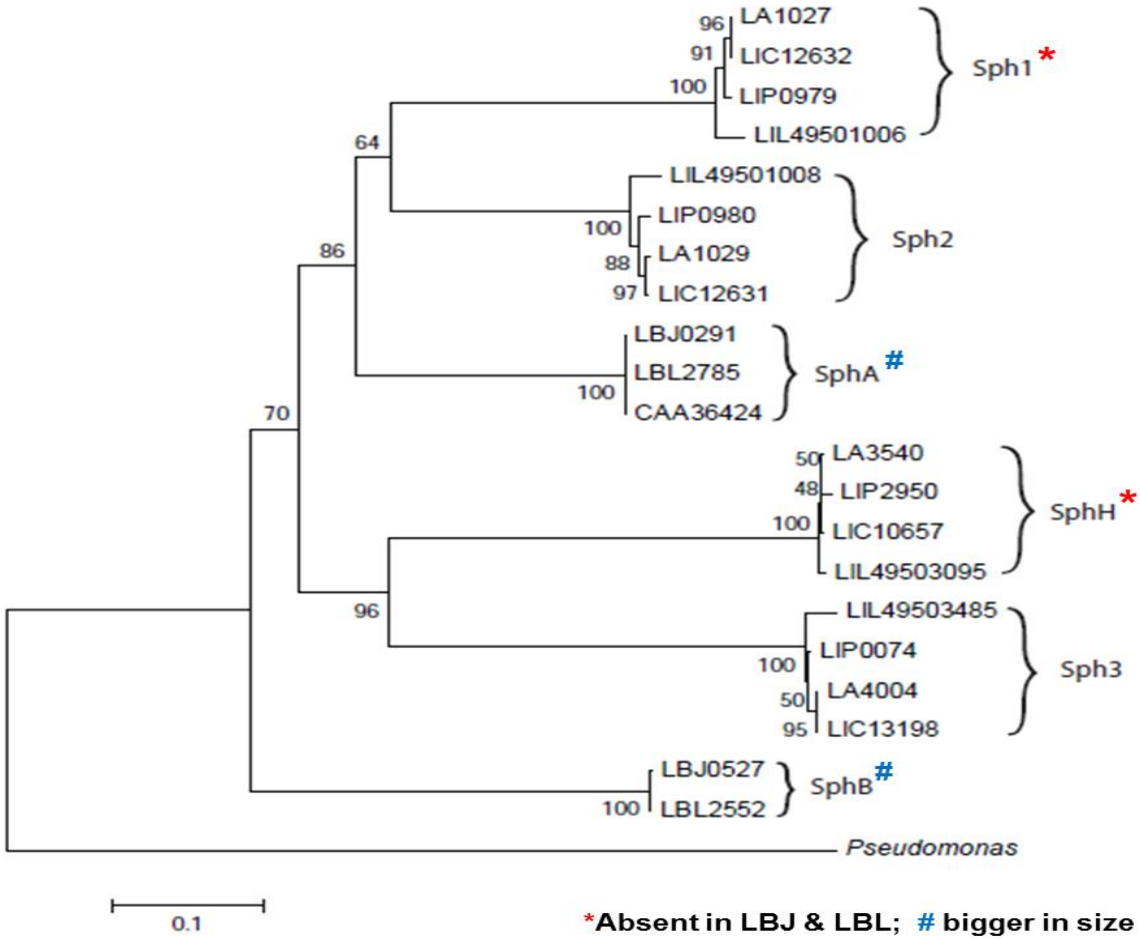


Fig. 9. Phylogenetic analysis of different leptospiral sphingomyelinases

The dendrogram represents the phylogenetic relationship among different leptospiral sphingomyelinases. The tree was rooted with the *Pseudomonas* species TK4 sphingomyelinase; LA - *L. interrogans* serovar Lai; LIC - *L. interrogans* serovar Copenhageni; LIP - *L. interrogans* serovar Pomona; LIL495 - *L. interrogans* serovar Manilae; LBJ - *L. borgpetersenii* serovar Hardjobovis strain JB197; LBL - *L. borgpetersenii* serovar Hardjobovis strain L550; CAA36424 – Partial sphingomyelinase sequence from *L. interrogans* serovar Hardjo (Segers *et al.,*1980).

3.1.3. Presence of exo – endo – phosphatase domain in leptospiral sphingomyelinases

All bacterial sphingomyelinases possess conserved exo - endo phosphatase domain (EEPD) as predicted by NCBI CDD (Fig. 8). In *L. interrogans* serovar Lai Sph1,

Sph2, Sph3 and SphH possess exo - endo phosphatase domain whereas Sph4 lacks it. Hence Sph4 was excluded from further *in silico* analysis.

Detailed analysis of leptospiral sphingomyelinases revealed the presence of putative signal sequences at the N – terminal end. In addition, they also possessed the N - terminal and C - terminal extensions flanking the central EEPD domain (Fig. 10; Table 8). Analysis of the sequences attached to the N - termini of the enzymatic domain using RADAR revealed the presence of two to seven short N - terminal imperfect repeats (NTRs) in Sph1, Sph2, and SphB (Fig. 10; Table 8). Comparison of the full - length amino acid sequences showed the presence of a C - terminal extension of 186 amino acids in the leptospiral sphingomyelinases (and in *Pseudomonas* sp. TK4) that was absent in other bacterial sphingomyelinases (Fig. 10).

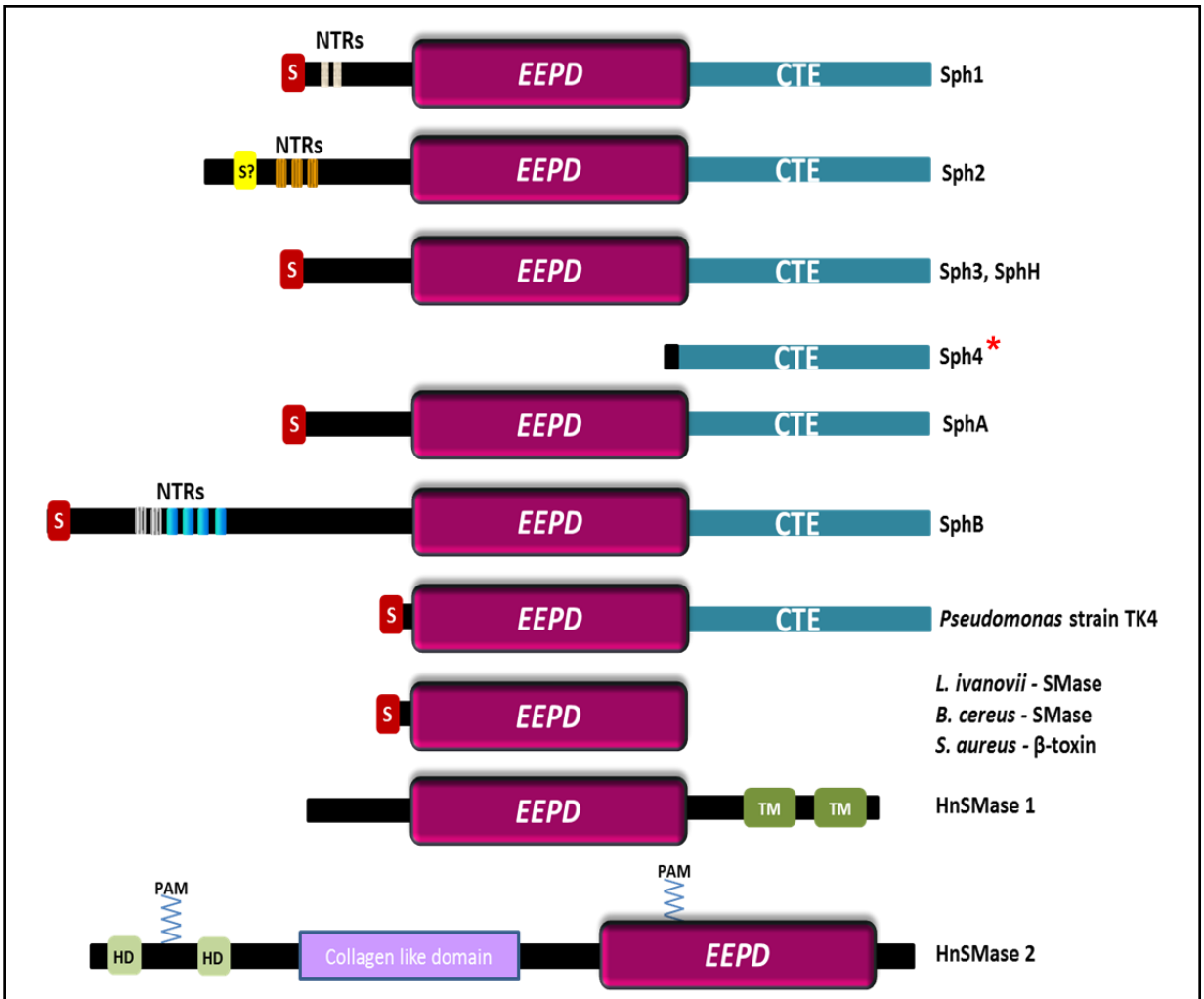


Fig. 10. Graphical representation of sphingomyelinases

NTRs, N - terminal repetitive sequences; S, signal peptide; EEPD, Exo - Endo Phosphatase Domain; CTE, C - terminal extension; TM, transmembrane domain; HD, hydrophobic domain; PAM, palmitate.

Table 8. N - terminal repeats of leptospiral sphingomyelinases

Protein	Locus tag	Signal peptide *	Number of repeats	Repetitive sequences #
Sph1	LA1027	Yes (39-40)	2	60-70 (NVNEKIEDSTN) 76-86 (NVNEEDENSIN)
	LIC12632	Yes (38-39)	2	59-69 (NVNEKIEDSTN) 75-85 (NVNEEDENSIN)
	LIP0979	Yes (38-39)	2	59-69 (NVNEKIEDSTN) 75-85 (NVNEEDENSTN)
	LiL49501006	No	2	59-69 (NVNEENENVTN) 75-85 (NVNEKDENATN)
Sph2	LA1029	No	3	49-67 (NQVNSVSINNDPANPNPVN) 74-92 (NQVNAVPENDDPANLNPVN) 99-117(NQVNAAPENGSPADPNPAN)
	LIC12631	No	3	49-67 (NQVNSVSINNDPANPNPVN) 74-92 (NQVNAVPENDNPANLNPVN) 99-117(NQVNAAPENGSSADPNPAN)
	LIP0980	No	4	55-77 (SINNDPANPNPVNPASANNNQVN) 80-102 (PENDNPANLNPVNPASANSNQVN) 105-127(PENDNPANLNPVNPASANSNQVN) 130-152(PENGSPTDPNPANLASANNNQVN)
	LiL49501008	No	3	27-48 (DPTNPNPVNPASATSNQVNAVP) 52-73 (DPANPNPVNPASANNNQVNAVP) 77-98 (NPADPNPANSASANNNQVNAVP)
Sph3	LA4004	Yes (38-39)	-	-
	LIC13198	Yes (38-39)	-	-
	LIP0774	No	-	-
	Li L49503485	No	-	-
SphH	LA3540	No	-	-
	LIC10657	No	-	-
	LIP2950	Yes (44-45)	-	-
	LiL49503095	No	-	-
SphB	LBJ 0527	Yes (38-39)	6	71-90 (GYDPISSGPASPTSpAGPGP) 92-110 (DLDPSNPDTANSSS-TNSGS) 112-130(NSSSTSSGSANSSS-TSSGS) 142-160(NSSSTSSGSANSSS-TSSGS) 162-180(NSSSTSSGSANSSS-TSSGS) 182-199(NSSSTSSGSANSSS--KAPP)
	LBL 2552	Yes (38-39)	7	79-109 (PASPTSPAgpgpGDLDPSNPDTANSSSTSSG) 110-139(SANPDTAN-sssTSSGSANPDTANSSSTSSG) 140-169(SANPDTAN-sssTSSGSANPDTANSSSTNSG) 170-184(SANPDTAN----------SSSTSSG) 185-214(-ANPDTAN-sssTNSGSANPDTANSSSTSSG) 215-244(SANPDTAN-sssTNSGSANPDTANSSSTSSG) 245-274(SANPDTAN-sssTNSGSANPDTANSSSTSSG)
SphA	LBJ 0291	Yes (26-27)	-	-
	LBL 2785	Yes (26-27)	-	-

*The number in parentheses represents the amino acids flanking the putative signal peptidase cleavage site. #The number represents the amino acid position in the protein sequence. Lower case characters are used for amino acid residues that are not aligned. Gaps are represented by ' –'.

3.1.4. Homology modeling of sphingomyelinases: three - dimensional folding by Insight II Modeler

The exo – endo phosphatase domain was selected for homology modeling of the leptospiral sphingomyelinases against the *L. ivanovii* and *B. cereus* sphingomyelinases. Sph1, Sph2, Sph3 and SphH showed 85% homology with the sphingomyelinases from *L. ivanovii* (SmcL; PDB: 1ZWX), *B. cereus* (BC SMase; PDB: 2DDR), *S. aureus* and *Pseudomonas* spp. strain TK4(BAB69072.1) (Fig. 11). Using Insight II Modeler the three - dimensional folding of Sph1, Sph2, SphH and Sph3 were predicted employing sphingomyelinases of *L. ivanovii* (1ZWX) and *B. cereus* (2DDR) respectively as templates (Fig. 12). Despite differences in the primary sequence (Fig. 11), all the four sphingomyelinases showed similarity in protein folding (Fig. 12). The predicted three - dimensional structure of leptospiral sphingomyelinases consisted of a central β - sandwich architecture flanked by α - helices and loops that were identical to the templates (Fig. 12). The main chain conformations of all the modeled structures were within the favoured regions (more than 92% of the residues) as seen in the Ramachandran plot (Fig. 13; Table 9). Defined regions in SmcL and BC SMase, namely the hydrophobic loop, solvent exposed loop and β - hairpin loop were identified in leptospiral sphingomyelinases upon superimposition of modeled sphingomyelinases with respective templates (Fig. 12). There were differences in these regions, notable being the absence of the β - hairpin loop specifically in leptospiral sphingomyelinases.

3.1.4.1. Amino acids at the active site: presence of conserved residues in Sph2

Based on the information from SmcL and BC Smase, an in - depth analysis for the presence of conserved amino acids at the active site of these molecules identified the central core that contained the deep solvent pouch for substrate binding as well as the negatively - charged metal - binding site. From a comparative analysis of the amino acid residues in the catalytic and metal - binding sites (Fig. 14), it was clear that only Sph2 closely resembled SmcL and BC SMase. The glutamate (Glu) reside at position 53 in the metal - binding site, essential for binding Mg^{2+} in SmcL and BC SMase was present only in Sph2 as also the two histidine (His151 and His286) residues in the catalytic site; one His residue was seen in Sph3, with none in Sph1 and SphH. Asp195, involved in maintaining the appropriate spatial arrangement of the catalytic histidine residues (Fig. 15a) is seen only in Sph1 and Sph2.

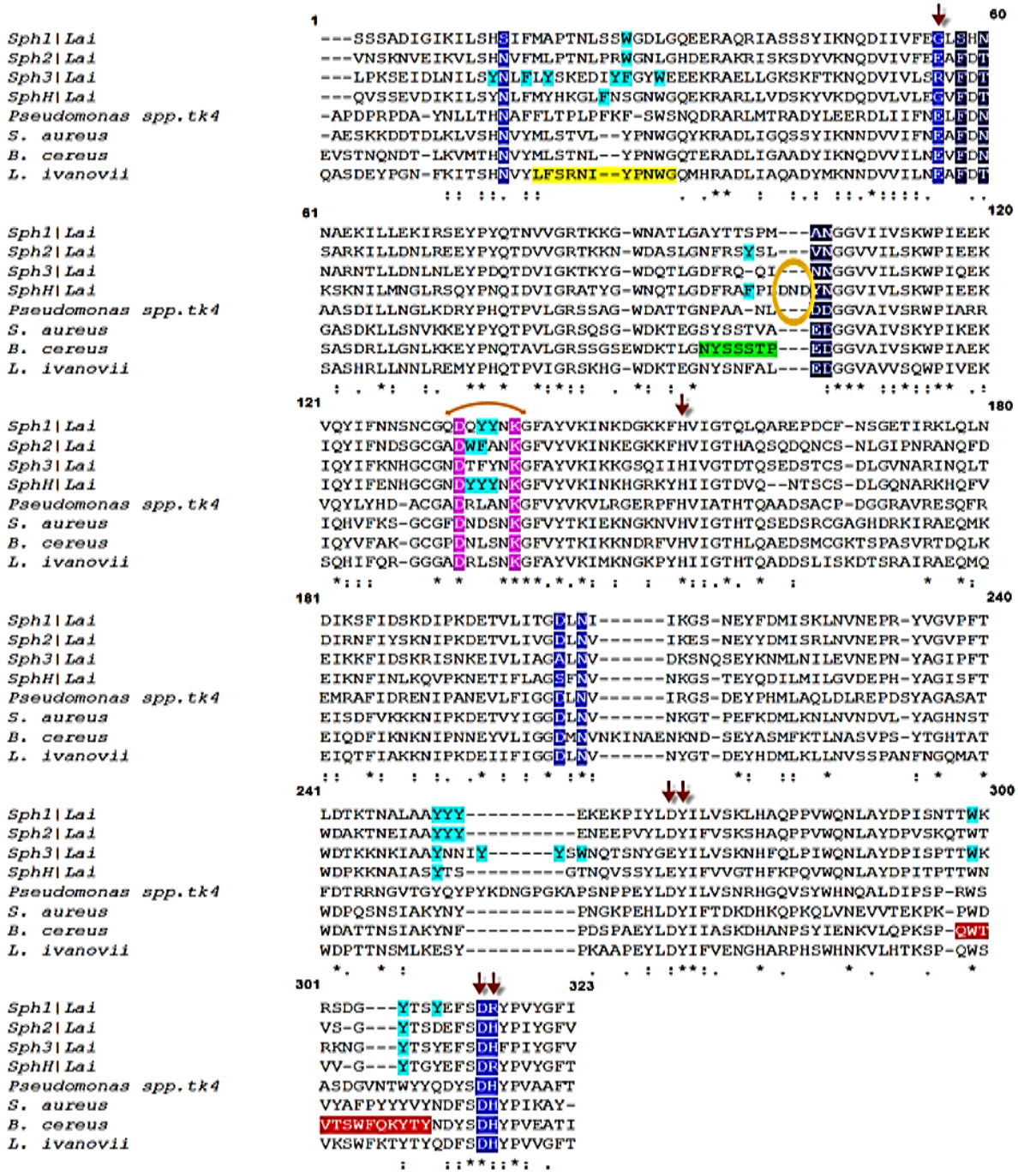


Fig. 11. Multiple sequence alignment of the leptospiral sphingomyelinases

Multiple sequence alignment was done using the exo – endo phosphatase domains of Sph1, Sph2, Sph3, SphH (*L. interrogans* serovar Lai), SmcL (*L. ivanovii*), BC SMase (*B. cereus*), SMase (*S. aureus*) and sphingomyelinase from *Pseudomonas* sp. strain TK4. Amino acids present in the central and edge metal - binding sites are highlighted in light & dark blue colours; others include the amino acids in the catalytic site (brown arrows), solvent exposed loop (green), hydrophobic loop (yellow), P - loop (pink, with double headed arrow) and β - hairpin region (red). Amino acids in turquoise are surface - exposed and are predicted to be involved in host membrane interaction. The DND motif, circled in orange is unique to SphH.

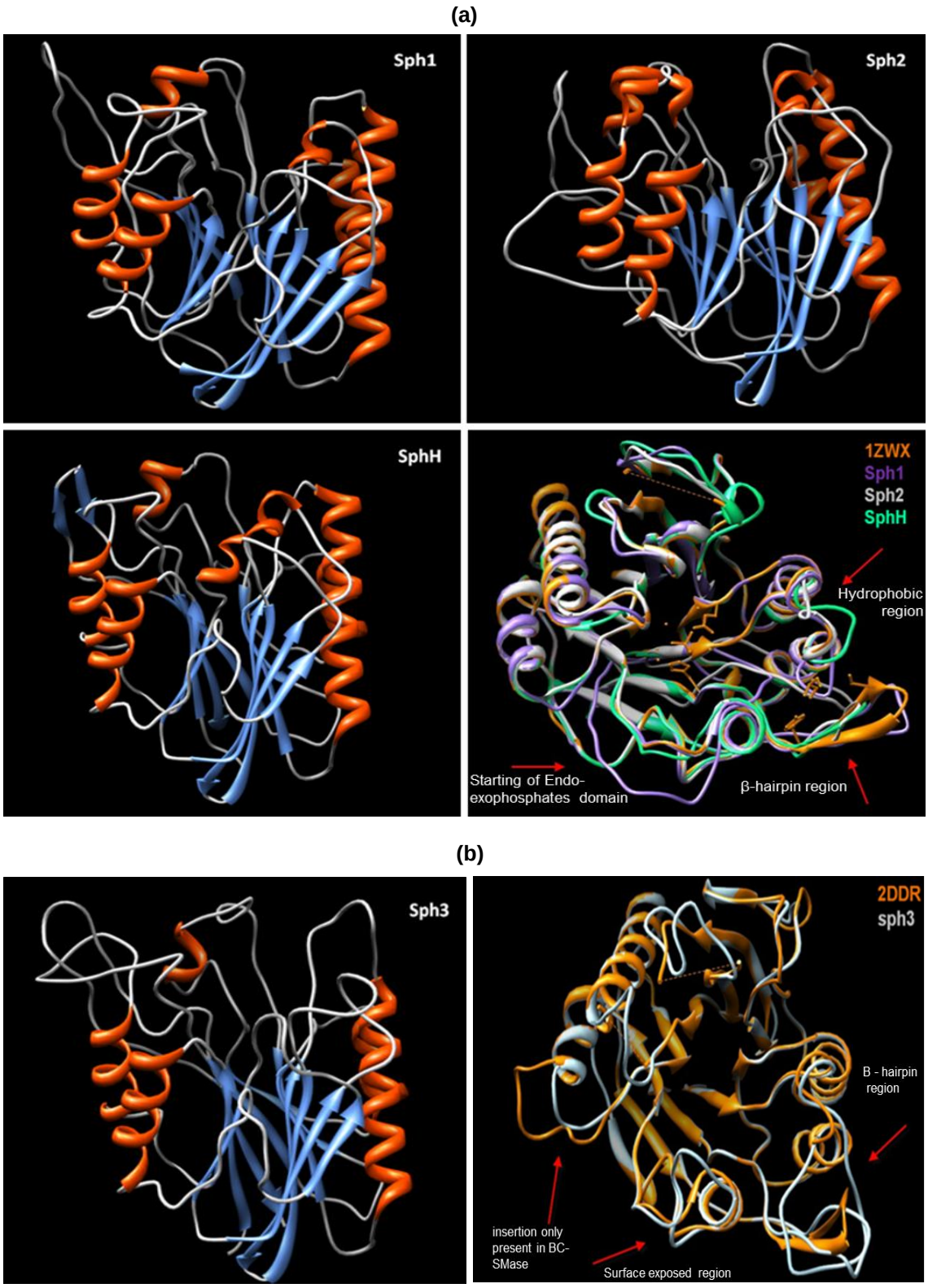


Fig. 12. Structure prediction of the leptospiral sphingomyelinases by homology modeling

Protein folding of Sph1, Sph2 and SphH built with SmcL (PDB: 1ZWX). Superimposing of all the three modeled structures on the template shows similarity in protein folding. (b) Sph3 modeled with BC SMase (PDB: 2DDR), and the superimposing of the structure on the template. The arrows indicate the differences seen in the hydrophobic loop, solvent exposed loop and the β - hairpin loop; the latter was absent in the leptospiral sphingomyelinases.

SphA from *L. borgpetersenii* possessed essential catalytic and metal binding residues like Glu53, His151 and His286 (Fig. 15b). In SphH, a unique triplet (DND), comprising an asparagine residue sandwiched between two negatively charged aspartic acid residues was located adjacent to the solvent exposed loop region, a feature not seen in any of the other sphingomyelinases. Protein folding revealed the surface exposure of this motif (Fig. 16).

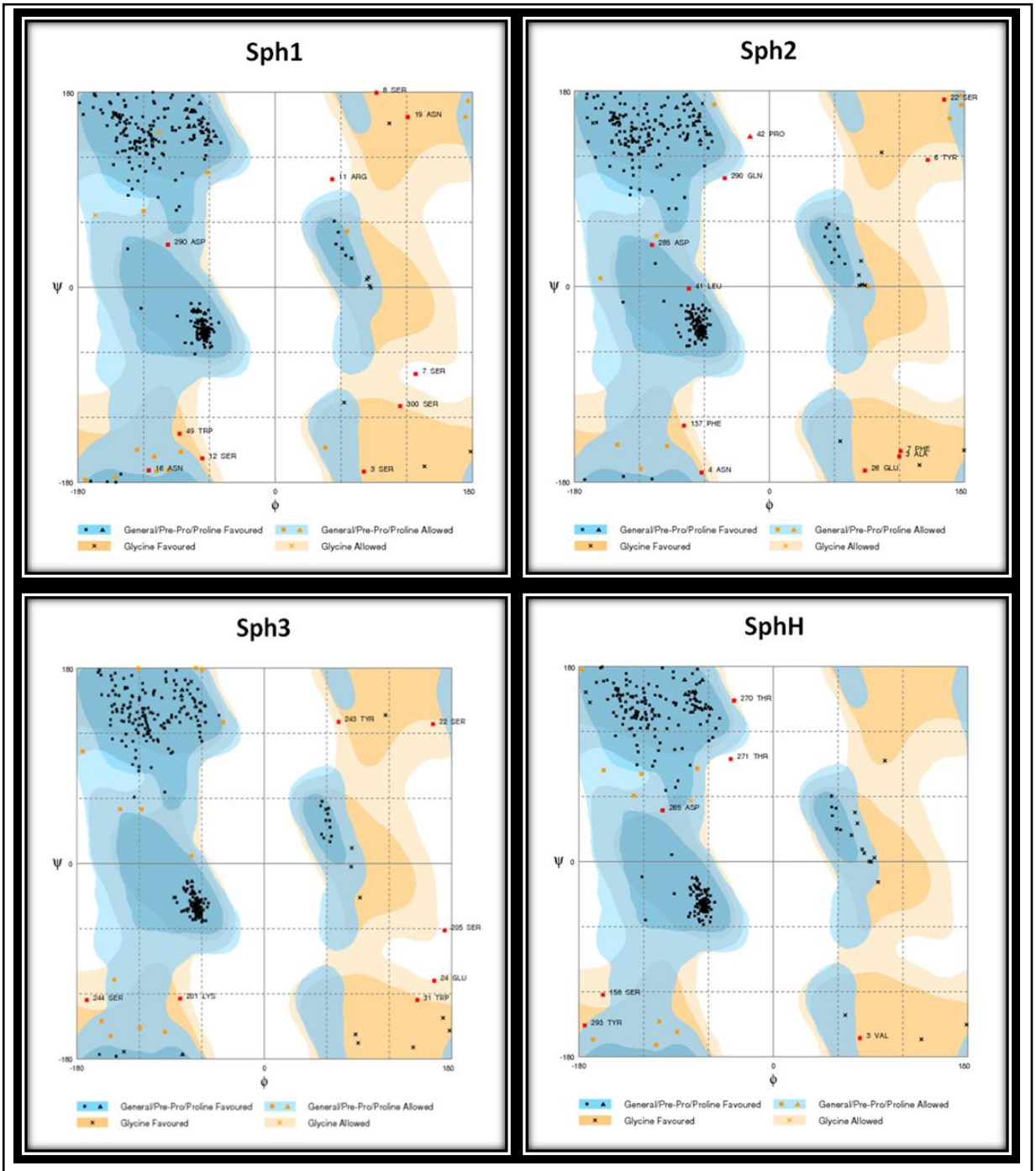
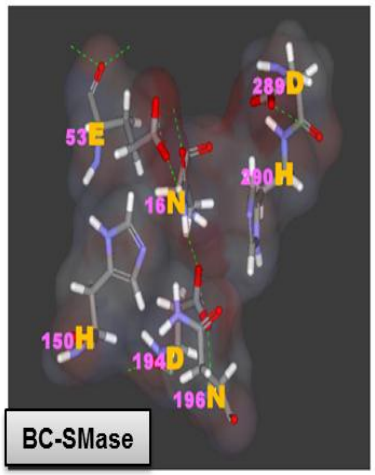


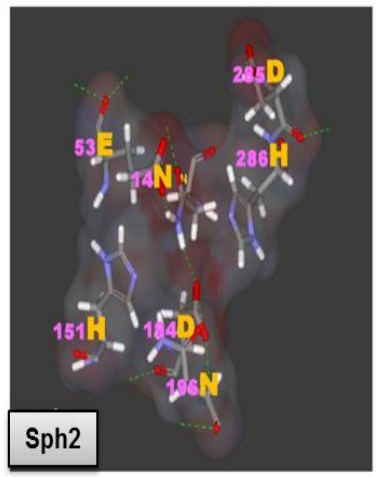
Fig. 13. Verification of the modeled structures by Ramachandran plot

The stability of the predicted structures was verified with the software Rampage. More than 92% of the residues were present in the favoured regions in the Ramachandran plot.

Table 9. The percentage of amino acids in the Ramachandran regions

	Number of residues in (%)		
	favored region	**allowed region**	**outlier region**
Sph1	92.1	4.7	3.2
Sph2	93.2	3.2	3.5
Sph3	93.4	4.3	2.3
SphH	94.5	3.4	2
Sph1	92.1	4.7	3.2

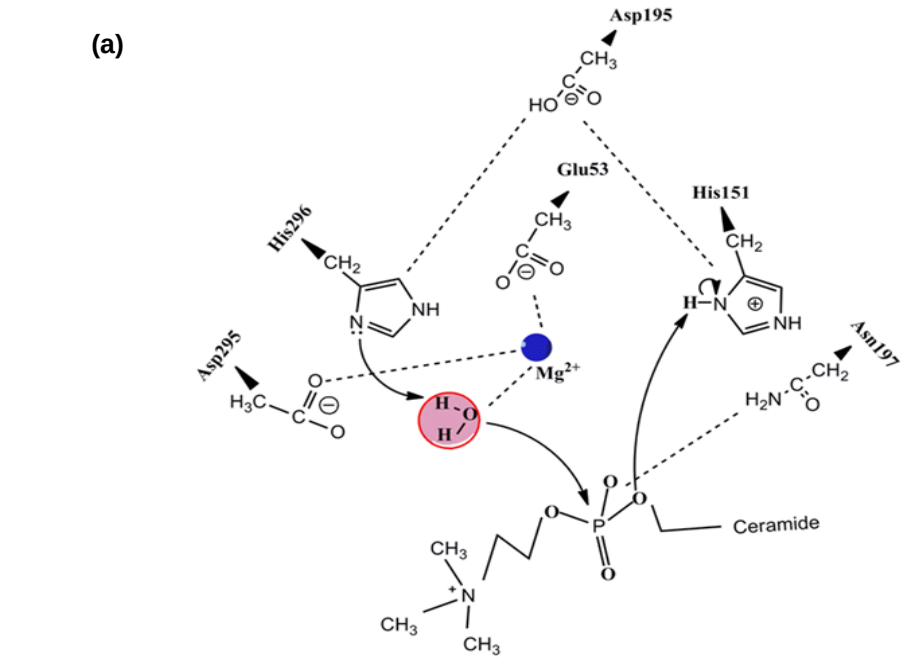




	Leptospira interrogans serovar Lai				*Pseudomonas tk4*	*S. aureus*	*B. cereus*	*L. ivanovii*
	Sph1	Sph2	Sph3	SphH				
Amino acid s in the Central Metal binding site	14S	14N	14N	14N	15N	16N	16N	16N
	53G	53E	53R	53G	53E	53E	53E	53E
	194D	194D	193A	195S	192D	194D	195D	194D
	196N	196N	195N	197N	194N	196N	197N	196N
	286D	285D	290D	287D	297D	288D	295D	289D
Amino acid s in Edge Metal binding site	57N	57T	57T	57A	60N	59N	59N	59T
	98A	98V	97N	101Y	97D	98E	99E	98E
	99N	99N	98N	102N	98D	99D	100D	99D
Amino acid s in Catalytic site	146H	146H	145H	149H	144H	145H	146H	145H
	246D	246D	250E	248E	255D	246D	253D	247D
	247Y	247Y	251Y	249Y	256Y	247Y	254Y	246Y
	151Q	151H	150D	154D	149H	150H	151H	150H
	287R	286H	291H	288R	298H	289H	296H	290H

Fig. 14. Catalytic and metal binding sites of the leptospiral sphingomyelinases

The highlighted residues (red) represent the amino acid residues associated with the catalytic site and the central & edge metal - binding sites. Glu53 is seen only in Sph2 and the differences in the His residues are also highlighted. The similarity in the catalytic site residues in Sph2 and SmcL (2DDR) is represented in the stereo view; these residues project outward to form the active site. The red colour indicates the negative charge on the specific residues.



(b)

		Glu-53	His-151	Asp-195	Asn-197	Asp-295	His-296
A.	*Listeria*	MKNNDVVILN**E**AFDT	IIGT**H**TPAD	FIGG**D**L**N**VN		DFS**DH**YPVV	
B.	*Bacillus*	IKNQDVVILN**E**VFDN	VIGT**H**LQAE	LFGG**D**M**N**VN		DYS**DH**YPVA	
C.	*LA1029-Sph2*	VKNQDVIVFE**E**AFDT	VIGT**H**AQSQ	LIVG**D**L**N**VI		EFS**DH**YPIY	
D.	*LA1027-Sph1*	IKNQDIIVFE**G**LSHN	VIGT**Q**LQAR	LITG**D**L**N**II		EFS**DR**YPVY	
E.	*LA4004-Sph3*	TKNQDVIVLS**R**VFDT	IVGT**D**TQSE	LIAG**A**L**N**VD		EFS**DH**FPIY	
F.	*LA3540-SphH*	VKDQDVLVLE**G**VFDT	IIGT**D**VQN-	FLAG**S**F**N**VN		EFS**DR**YPVY	
G.	*LBJ0291-SphA*	IQNQDVIVFD**E**AFDT	IIGT**H**VQAQ	LIAG**D**L**N**VI		EFS**DH**YPVY	
H.	*LBJ0527-SphB*	IKHQDIIVFE**G**LSDA	VIGT**Q**VQMV	LIAG**D**L**N**VT		EFS**DS**YPVY	
I.	*Staphylococcus*	IKNNDVVIFN**E**AFDN	VIGT**H**TQSE	YIGG**D**L**N**VN		DFS**DH**YPIK	
J.	*Pseudomonas*	LEERDLIIFN**E**LFDN	VIAT**H**TQAA	FIGG**D**L**N**VI		DYS**DH**YPVA	
K.	*HnSMase1*	QESFDLALLE**E**VWSE	AYVT**H**LHAE	LLCG**D**L**N**MH		PLS**DH**EALM	
L.	*HnSMase2*	LQNLDFLCLQ**E**VFDK	IACT**H**LHAP	VVCG**D**F**N**FD		GLT**DH**LPVA	

Fig. 15. Proposed function of amino acids at the active site

Panel (a) represents the diagram showing the proposed function of amino acids at the catalytic site of BC SMase (adapted from Obama *et al*., 2003a). Asn197 interacts with the phosphate group of sphingomyelin, and Glu53 and Asp295 coordinate with Mg^{2+}. His296 and His151 function as the acid - base catalytic residues; His296 and the metal ion activate the water molecule that attacks the phosphate of sphingomyelin, resulting in the hydrolysis to phosphocholine and ceramide. Asp195 maintains the appropriate spatial arrangement of the catalytic histidine residues. Panel (b) represents multiple sequence alignment showing six of the amino acids (highlighted in red) conserved in all members of the extended neutral sphingomyelinase family, including the two human neutral sphingomyelinases. It may be noted that Glu53, His151, Asp195 and/or His296 are not conserved (highlighted in grey) in Sph1, Sph3, SphH and Sph4.

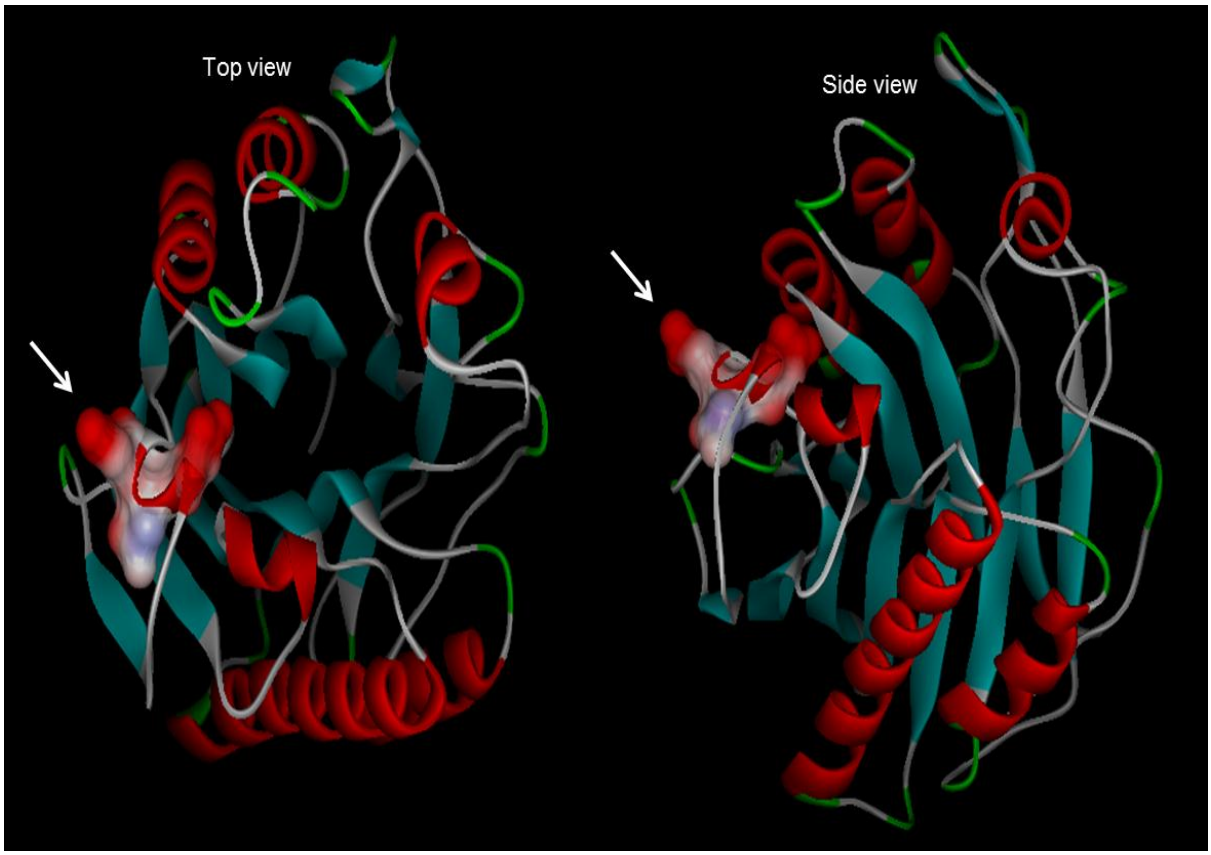


Fig. 16. Surface exposed DND residues in SphH

The top and side views of SphH show the novel surface - exposed DND motif (indicated by arrows).

3.1.4.2. Unique features of leptospiral sphingomyelinases

The β - hairpin loop was clearly missing in all the four leptospiral sphingomyelinases (Fig. 11 & 12). This aromatic amino acid - rich hydrophobic region present in SmcL and BC SMase was responsible for host - membrane interaction. Its absence in leptospiral sphingomyelinases clearly indicated alternative mechanism of membrane interaction. Three – dimensional analysis revealed that several of the aromatic amino acids projected outwards from the surface of the molecule (Fig. 17a) forming hydrophobic clusters. Sph3 showed the maximum residues (7Y + 3W + 2F = 12) with 9 in Sph1 (7Y + 2 W), 8 in Sph2 (5Y + 2W + 1F) and 7 in SphH (5Y + 2F) respectively (Table 10; Fig. 17b). These amino acids are clearly absent in SmcL and BC SMase (Table 10). We hypothesise that leptospiral sphingomyelinases interact with the host cell membrane via these pin – like hydrophobic clusters (Fig. 17b).

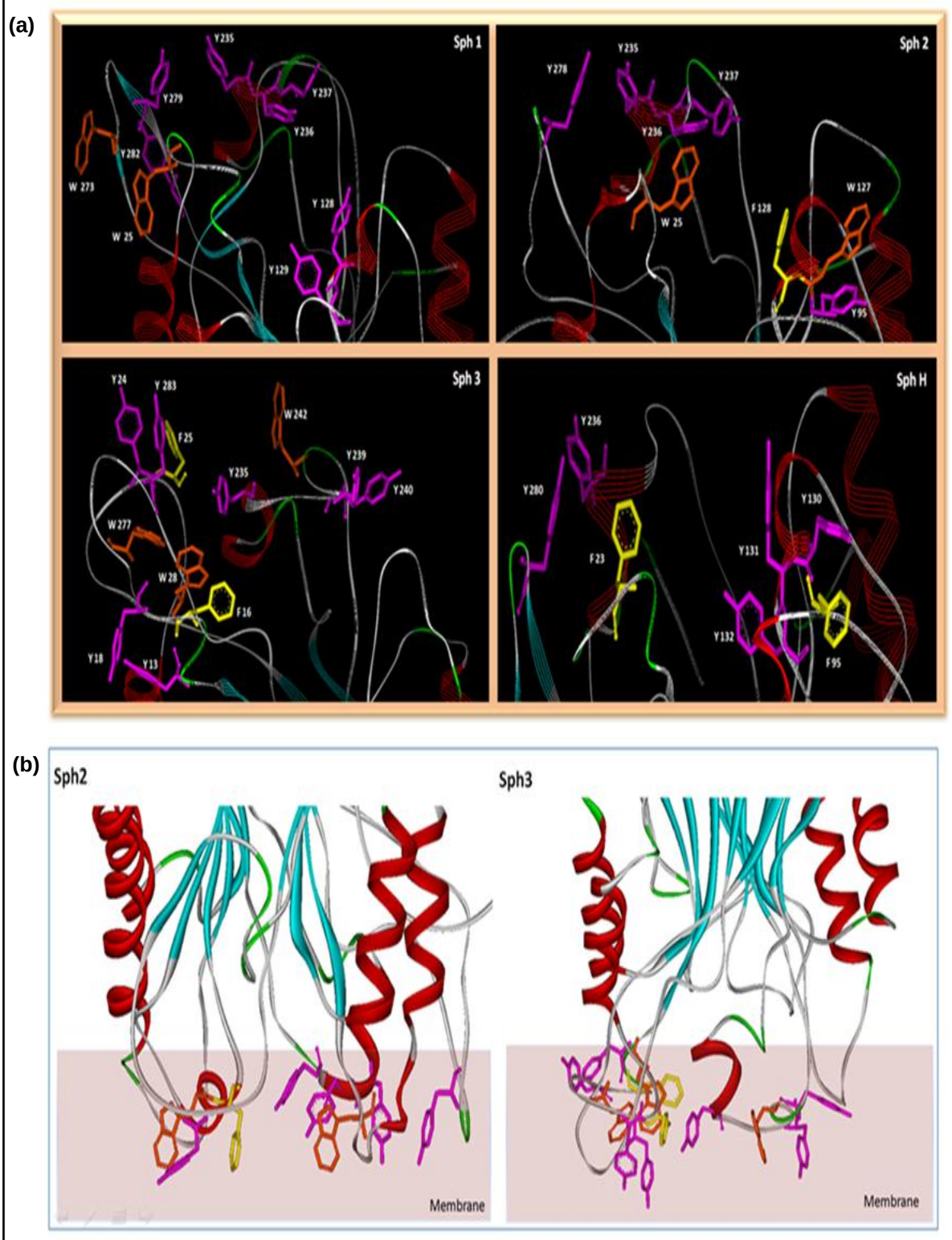


Fig. 17. Membrane interaction of the surface - exposed aromatic amino acids in leptospiral sphingomyelinases

In panel (a) aromatic amino acids in the hydrophobic loop region, solvent exposed loop, P - loop region represented in turquoise in Fig. 11 project outward from the surface of the four leptospiral sphingomyelinases. (b) The hydrophobic regions formed by these aromatic residues in Sph2 and Sph3 and their interactions with a schematic model of the host cell membrane.

Table 10. Surface exposed aromatic amino acids in sphingomyelinases

Surface exposed aromatic amino acids	Sph1	Sph2	Sph3	SphH	1ZWX	2DDR
	25W	25W	13Y	23F	273W	273W
	128Y	95Y	16F	95F	274F	274F
	129Y	127W	18Y	130Y		
	235Y	128F	24Y	131Y		
	236Y	235Y	25F	132Y		
	237Y	236Y	28W	236Y		
	273W	237Y	235Y	280Y		
	279Y	278Y	239Y			
	282Y		240Y			
			242W			
			277W			
			283Y			

3.2. Characterisation of Sph2 as a Mg^{2+} - dependent sphingomyelinase

3.2.1. Cloning and expression of recombinant Sph2

sph2 and *sph4* were cloned into pET28a as detailed in Materials and Methods to generate recombinant plasmid harbouring *sph2* (pMS510) and *sph4* (pMS511) respectively. Positive constructs were confirmed by double digestion (Fig. 18 & 19). Recombinant Sph2 and Sph4 were obtained as inclusion bodies upon induction with IPTG (Fig. 18d & 19d). Proteins were purified to apparent homogeneity using BugBuster Ni - NTA His - bind resin affinity chromatography under denaturing conditions, as evidenced by the single band of 74.5 kDa and 20 kDa respectively on silver stained SDS - PAGE gel (Fig. 18f and 19f) followed by immunoblot analysis using anti - His antibodies (Fig. 18e and Fig. 19e). Various strategies like induction with different concentrations of IPTG ranging from 0.3 mM to 1 mM, lysis of cell pellets using different buffers and co - expression with chaperone proteins were attempted but none of them resulted in the production of soluble Sph2 (Fig. 20). However immunoblot analysis showed that a small amount of soluble protein was present in the supernatant (Fig. 18e); hence whole cell sonicates of recombinant Sph2 or Sph4 clones were used for hemolytic and sphingomyelinase assays.

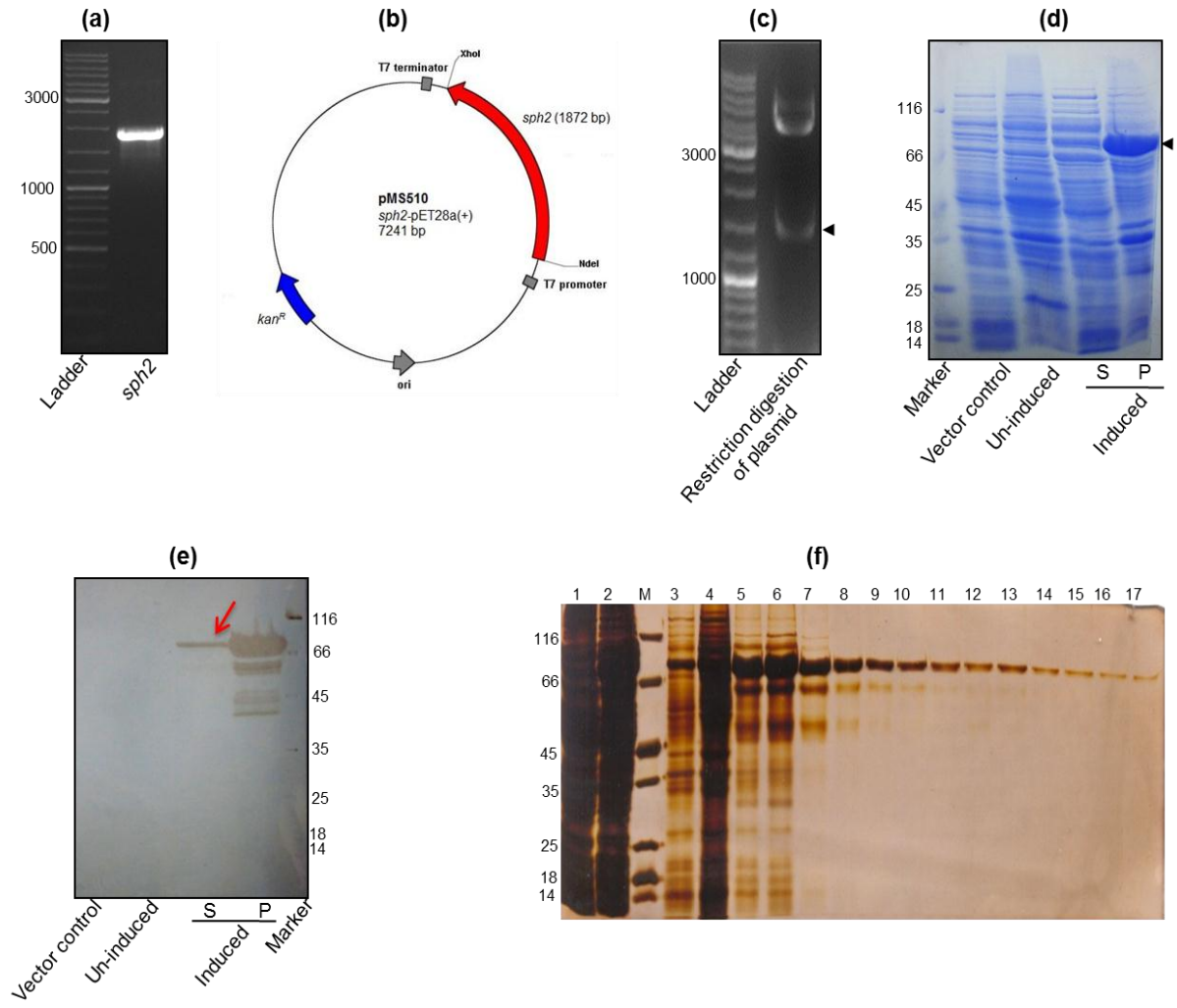


Fig. 18. Cloning, expression and purification of Sph2

The 1872 bp *sph2* amplicon (Panel a) was cloned into pET28a to generate pMS510 (Panel b). The recombinant was verified by restriction digestion (Panel c). The recombinant protein induced by IPTG was identified by SDS – PAGE (Panel d) and immunobloting with anti – Sph2 antibodies (Panel e). Sph2 was purified by Ni – NTA column chromatography (Panel f), different lanes represent whole cell sonicates of un – induced (lane 1), and induced (lane 2) *E. coli* BL21 (DE3) containing pMS510, molecular weight marker (lane M), flow through (lane 4), wash (lane 3) and elute fractions (lanes 5 to 17). 'S' and 'P' represents supernatant and pellet respectively of whole cell sonicate.

3.2.2. Biological activity of rSph2

3.2.2.1. Hemolytic activity

The hemolytic activity of recombinant Sph2 was comparable to the hemolysis of erythrocytes by the purified sphingomyelinases from *S. aureus* (commercial preparation from Sigma). Presuming 100% lysis of the erythrocytes with double - distilled water (positive control) and zero lysis with isotonic saline (negative control), 84% lysis was achieved with purified *S. aureus* sphingomyelinase and recombinant Sph2 showed 44.5% hemolytic activity (Fig. 20). No endogenous activity in the host

E. coli was evident as reflected by the negligible effect in the two additional controls (*E. coli* with empty pET vector and recombinant HupB, a mycobacterial gene, respectively). The hemolytic activity due to Sph2 was confirmed by neutralization with specific anti - Sph2 antibodies. It was observed that hemolytic activity was inhibited significantly by 86% (Two - tail unpaired t test with P=0.0466) upon pre - incubation with anti - Sph2 antibodies when compared to whole cell sonicate (Fig. 21).

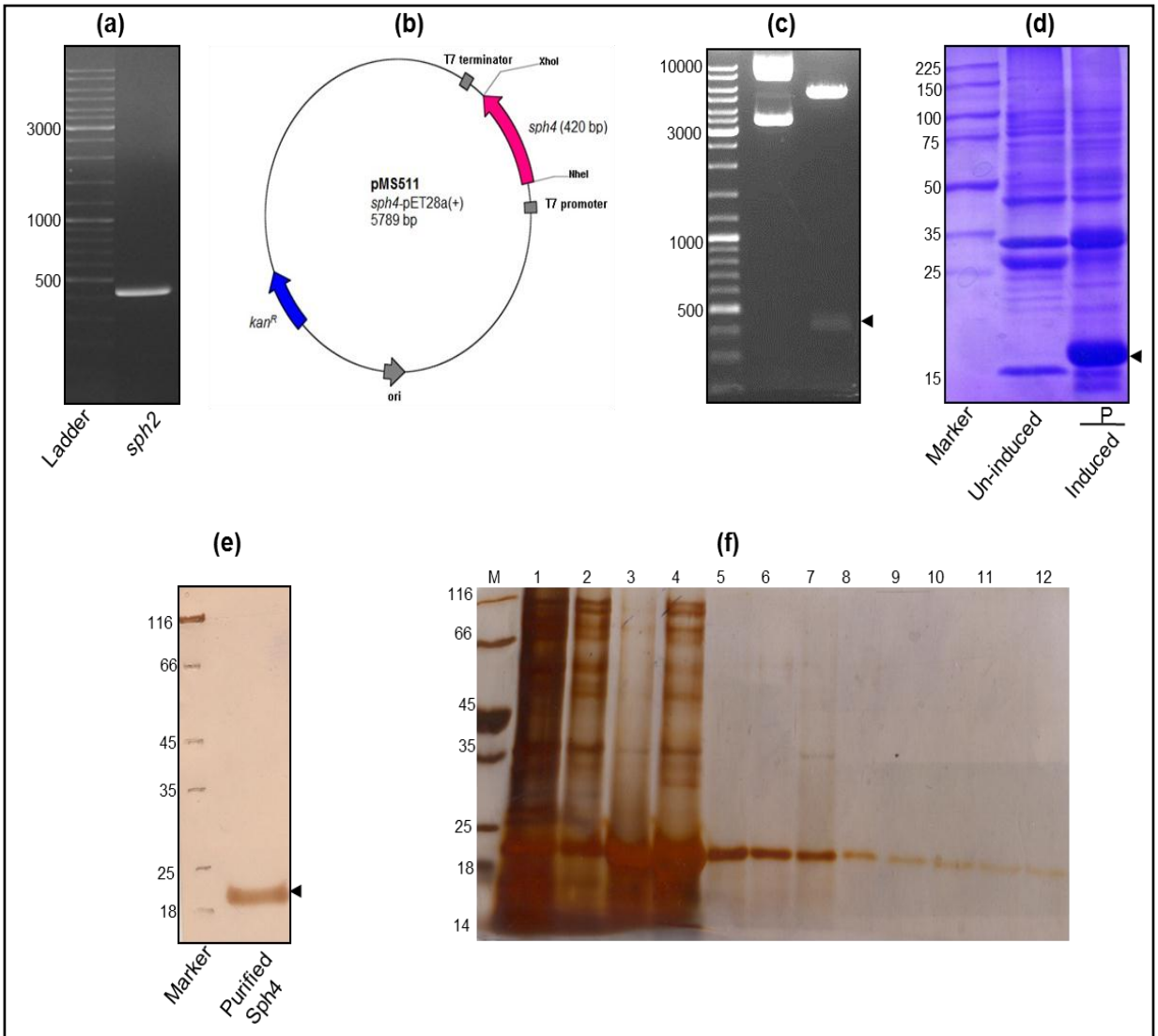


Fig. 19. Cloning, expression and purification of Sph4

The 420 bp *sph4* amplicon (Panel a) was cloned into pET28a to generate pMS511 (Panel b). The recombinant was verified by restriction digestion (Panel c). The recombinant protein induced by IPTG was identified by SDS – PAGE (Panel d) and immunobloting with anti – Sph4 antibodies (Panel e). Sph2 was purified by Ni – NTA column chromatography (Panel f), different lanes represent molecular weight marker (lane M), flow through (lane 1), wash (lane 2) and elute fractions (lanes 3 to 12). 'P' represents pellet of whole cell sonicate.

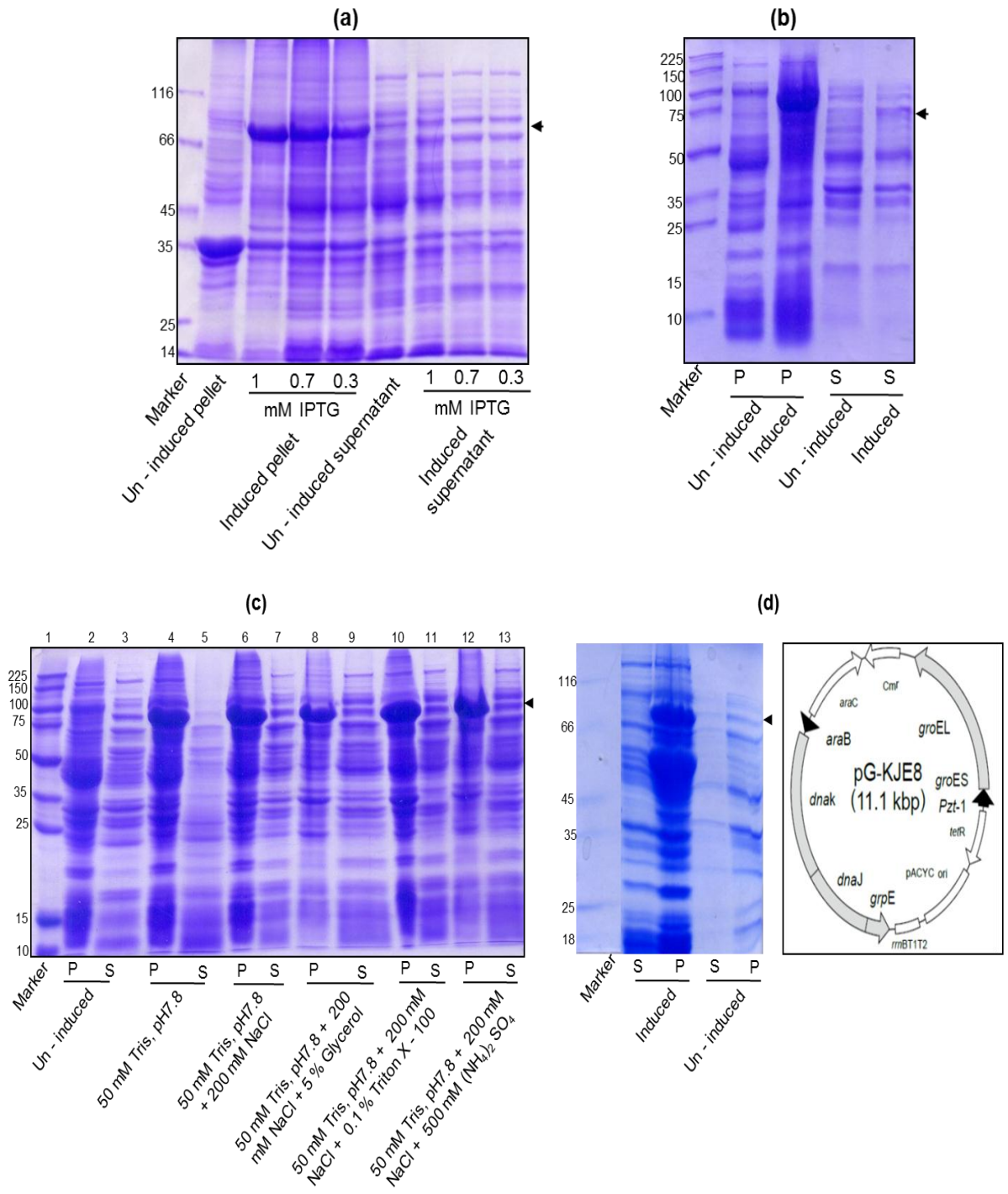


Fig. 20. Optimisation of the conditions for the soluble expression of Sph2

Panels (a), (b), (c) and (d) represents effect of different concentrations of IPTG, BugBuster protein extraction reagent, different lysis buffers and co – chaperones on the expression of Sph2. In Panels (b), (c), (d) 'S' and 'P' represents supernatant and pellet respectively of whole cell sonicate. Expression of Sph2 is indicated by arrow. In Panel (d) vector map of pG - KJE8 containing chaperone genes is shown. In Panel (c) cell pellets of *E. coli* BL21 (DE3) pLysE carrying pMS510 plasmid were subjected for lysis using different lysis buffers as indicated. Arrow represents the expressed Sph2 protein in lanes 4, 6, 8, 10 and 12.

3.2.2.1.1. Role of metal ions on the hemolytic activity: potentiating activity of Mg^{2+}

Upon addition of 10 mM $MgCl_2$, hemolytic activity of Sph2 increased to 62% (1.39 fold increase). As divalent metal ions, including Mg^{2+}, are likely to be present in the whole cell sonicate, possibly accounting for 44.5% hemolytic activity without the added magnesium, the assay was done after chelation of all divalent metal ions with EDTA. This drastically reduced the hemolytic activity to almost negligible levels. The addition of other divalent metal ions like 5 mM $MnCl_2$ and 10 mM $CaCl_2$ decreased the hemolytic activity (Fig. 20) and inclusion of magnesium ions to the reaction mixture did not restore the hemolytic activity.

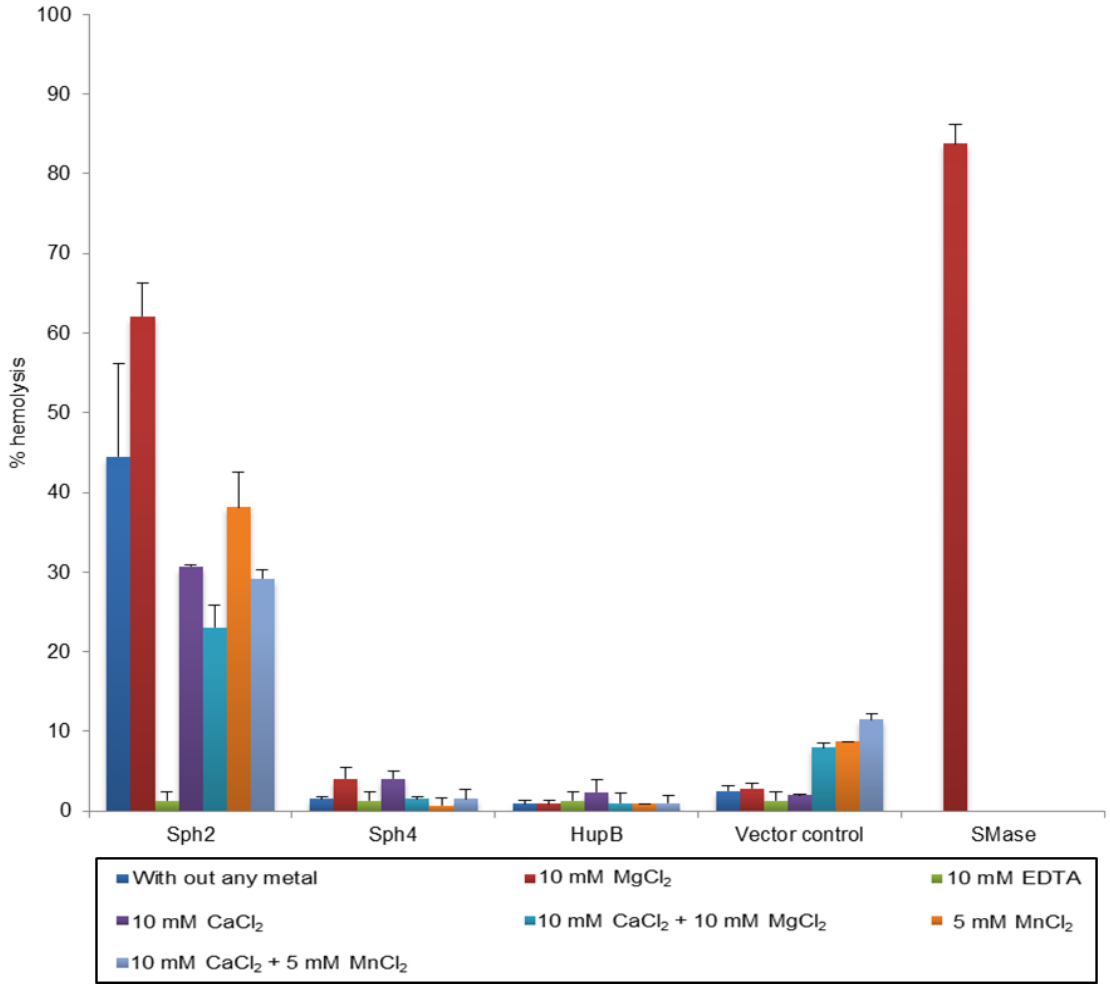


Fig. 21. Hemolytic assay with recombinant Sph2

Hemolytic assay was performed with the whole cell sonicates of *E. coli* expressing Sph2. Influence of various divalent metal ions on the hemolytic activity of recombinant Sph2 was analysed. The controls included the commercial sphingomyelinase from *S. aureus* (SMase), recombinant Sph4, *E. coli* with empty pET 28a vector and rHupB (unrelated mycobacterial protein). The % hemolysis was calculated as indicated in Material & Methods. Data are presented as mean ± SD of three independent experiments.

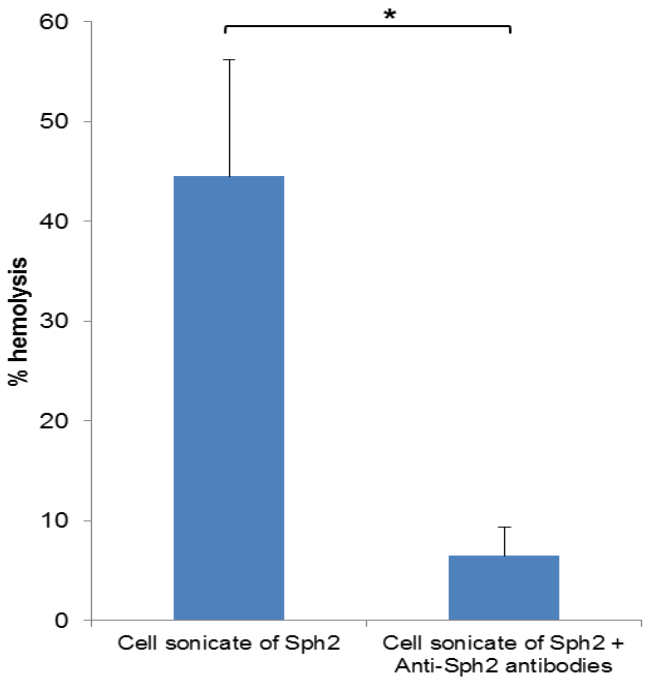


Fig. 22. Neutralisation of the hemolytic activity of Sph2 with anti - Sph2 antibodies

The graph represents the % hemolysis seen with rSph2 before and after pre - incubation with anti - Sph2 antibodies. Data are presented as mean ± SD of three independent experiments. *, P=0.0466 (Two - tail unpaired t test).

3.2.2.1.2. Dose - dependent effect of Mg^{2+} and Ca^{2+} on the hemolytic activity of Sph2

The dose - dependent effect of Mg^{2+} and Ca^{2+} further confirmed their effect on the ability of Sph2 to lyse the red blood cells (Fig. 22). Increasing the concentration of Mg^{2+} caused an increase in the hemolytic activity with maximal effect at 10 mM $MgCl_2$; a steep decline was seen upon further additions until at 100 mM final concentration of Mg^{2+}only 20% hemolysis was seen (Fig. 22a). The inhibitory effect of Ca^{2+} was evident even at low concentrations (Fig. 22b); only 10% hemolysis was seen at 100 mM final concentration.

3.2.2.2. Enzymatic activity of Sph2

The sphingomyelinase activity of rSph2 was evident from the ceramide detected upon chromatographic separation of the products after enzymatic digestion of sphingomyelin by rSph2 (Fig. 23). The mobility of this product (lane 4) was identical to the commercial ceramide (lane 3) and that generated by the sphingomyelinase from *S. aureus* (lane 2).

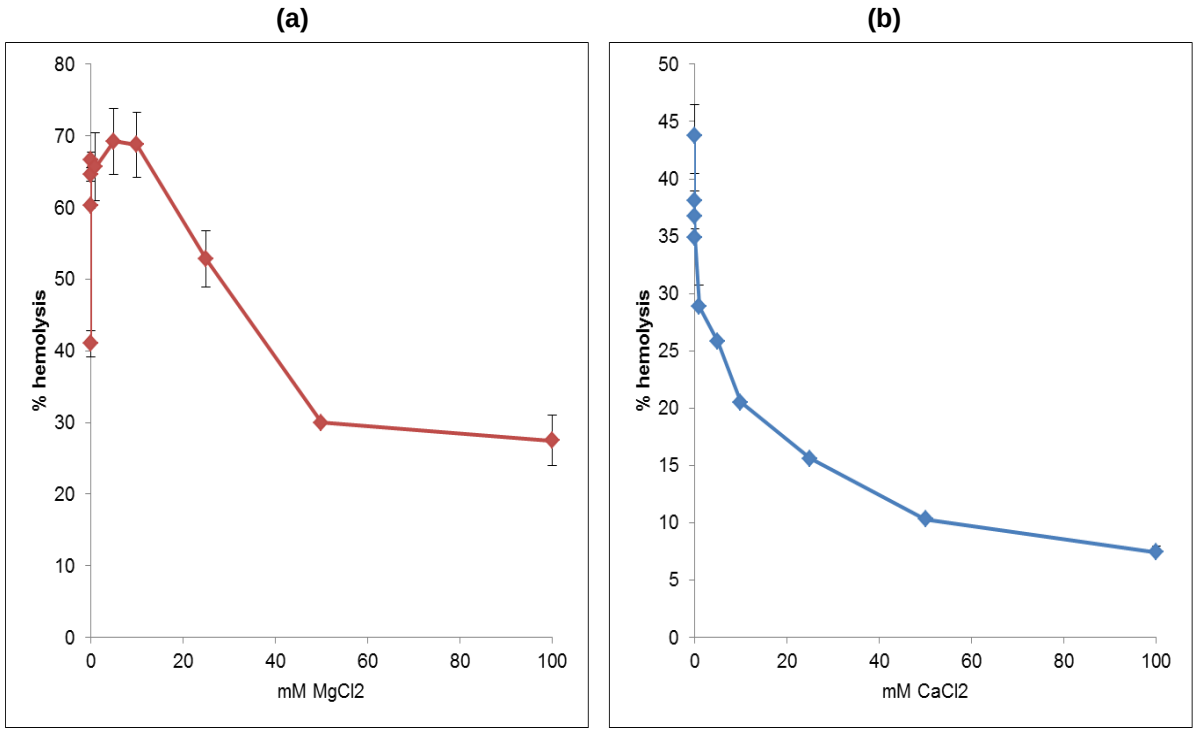


Fig. 23. Dose - dependent effect of Mg^{2+} and Ca^{2+} on the hemolytic activity of rSph2

Panels (a) and (b) showing the dose-dependent effect of Mg^{2+} and Ca^{2+} respectively on the hemolytic activity of Sph2. Data are presented as mean ± SD of three independent experiments.

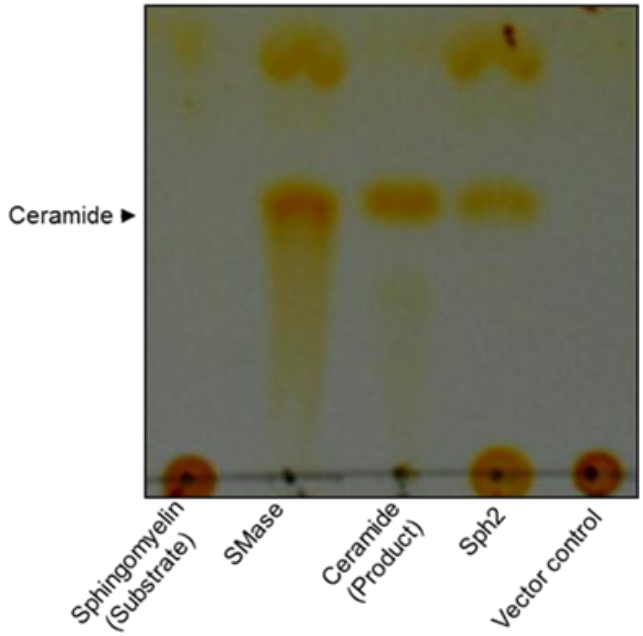


Fig. 24. Sphingomyelinase activity of recombinant Sph2

The figure represents the chromatogram of the products obtained upon incubation of sphingomyelin with the commercial sphingomyelinase from *S. aureus* (lane 2), whole cell sonicate of *E. coli* expressing Sph2 (lane 4) and *E. coli* with empty vector (lane 5). Lanes 1 and 3 represent purified sphingomyelin and ceramide (Sigma) respectively. The arrow arrow indicates ceramide.

3.3. Up - regulation of expression of leptospiral sphingomyelinases: influence of environmental conditions

The influence of environmental factors like iron limitation, addition of serum & salt and oxidative stress on the expression of the different sphingomyelinases was studied. This was done both at the transcript level by qRT - PCR and the protein by immunoblotting with specific antibodies. As different leptospiral species elaborate varying types of sphingomyelinases, several leptospiral serovars were used in this study. As it is unclear if sphingomyelinases undergo processing to form a potent toxin, both the cell sonicates and the spent growth medium were analysed for some of these proteins.

3.3.1. Effect of iron limitation on *L. interrogans* serovar Lai

The leptospires were subjected to iron limitation by the addition of 300 µM EDDA to EMJH – BSA medium prepared in iron - free water and the iron - replete organisms were grown in the presence of 10 µg Fe / mL at 30°C. qRT - PCR analysis of *sph1*, *sph2*, *sph3*, *sph4* and *sphH* done on these iron - regulated organisms showed a marked 11 – fold increase in the transcript level of *sphH* with about 1 – 2.5 fold rise in the other sphingomyelinases (Fig. 25; Table 11). When the temperature of growth was raised to physiological temperature of 37°C, the levels of all the sphingomyelinases dropped, with only a 5 – fold difference in the *sphH* transcript upon iron limitation.

Table 11. Effect of iron limitation on the expression of sphingomyelinases

Relative fold changes compared to EMJH control					
	***L. interrogans* serovar Lai**				
	sph1	***sph2***	***sph3***	***sph4***	***sphH***
Iron limitation at 30°C	1.43	1.93	2.43	0.94	11.06
Iron limitation at 37°C	0.83	0.94	1.78	0.67	5.31

The expression of SphH was analysed by immunoblotting with anti – $Sph2_{163}$ antibodies (recognises both Sph2 and SphH) in both whole cell lysate, spent growth medium and in outer membrane vesicles (OMVs). The latter was done as we had earlier reported a 42 kDa protein in the outer membrane vesicles as a sphingomyelinase; this band was recognised by anti - sphingomyelinase antibodies

raised against the common domain present in the different sphingomyelinases (Velineni *et al.*, 2009). In the whole cell lysates, SphH was observed as a 63 kDa protein in both high and low iron organisms. It could not be detected in the spent growth medium subjected to immunoprecipitation with anti – $Sph2_{163}$ antibodies. In the OMVs isolated from organisms grown at 30°C, no SphH could be detected but interestingly the OMVs of low iron organisms grown at 37°C (Fig. 25e) showed the 42 kDa band that was identified as SphH protein by immunoblotting. We could not detect any expression of Sph2.

3.3.2. Effect of salt, serum and a combination of salt and serum – studies with serovars Pomona and Manilae

Based on reports that addition of 120mM NaCl induced the expression of Sph2 (Matsunaga *et al.*, 2007b), the serovars Pomona and Manilae were subjected to addition of salt, serum and a combination of both salt and serum. In all the three conditions, notable changes at the transcript and the protein levels were observed in Sph2 and Sph1, with serovar Manilae showing an up - regulation of *sphH* transcript in the presence of added serum.

Upon incubation with 120 mM NaCl, the fold rise of *sph2* was 62 and 55 respectively in the serovars Pomona and Manilae (Fig. 26a & b) with a fold increase of 9 and 5 respectively of *sph1* (Table 12). While only a 2 - fold rise of *sph3* was noted, no major difference was seen in *sphH* in both the serovars. Addition of rat serum was optimised at 10% final concentration as no further changes in expression were observed at higher concentrations (Fig. 29a). When serum alone was added, there was a rise in *sph2* transcripts that was however considerably lower than that observed with salt. The fold rise was found to be 13 and 2.5 respectively in the serovars Pomona and Manilae, with an increase of approximately 2 and 1for *sph1* transcripts. Notable however was the 11 – fold increase in *sphH* in serovar Manilae that was not as dramatic in serovar Pomona that showed about a 2 – fold increase. But a combination of both 120 mM salt and 10% rat serum in the medium of growth caused a dramatic rise of *sph2* transcripts to values that were 184 – fold high in serovar Pomona with almost a similar fold rise (141) in serovar Manilae (Table 12). About 2 - fold increase in *sph3* was seen in both the serovars grown under these conditions, with some increase in *sphH* in serovar Manilae, as listed in Table 12.

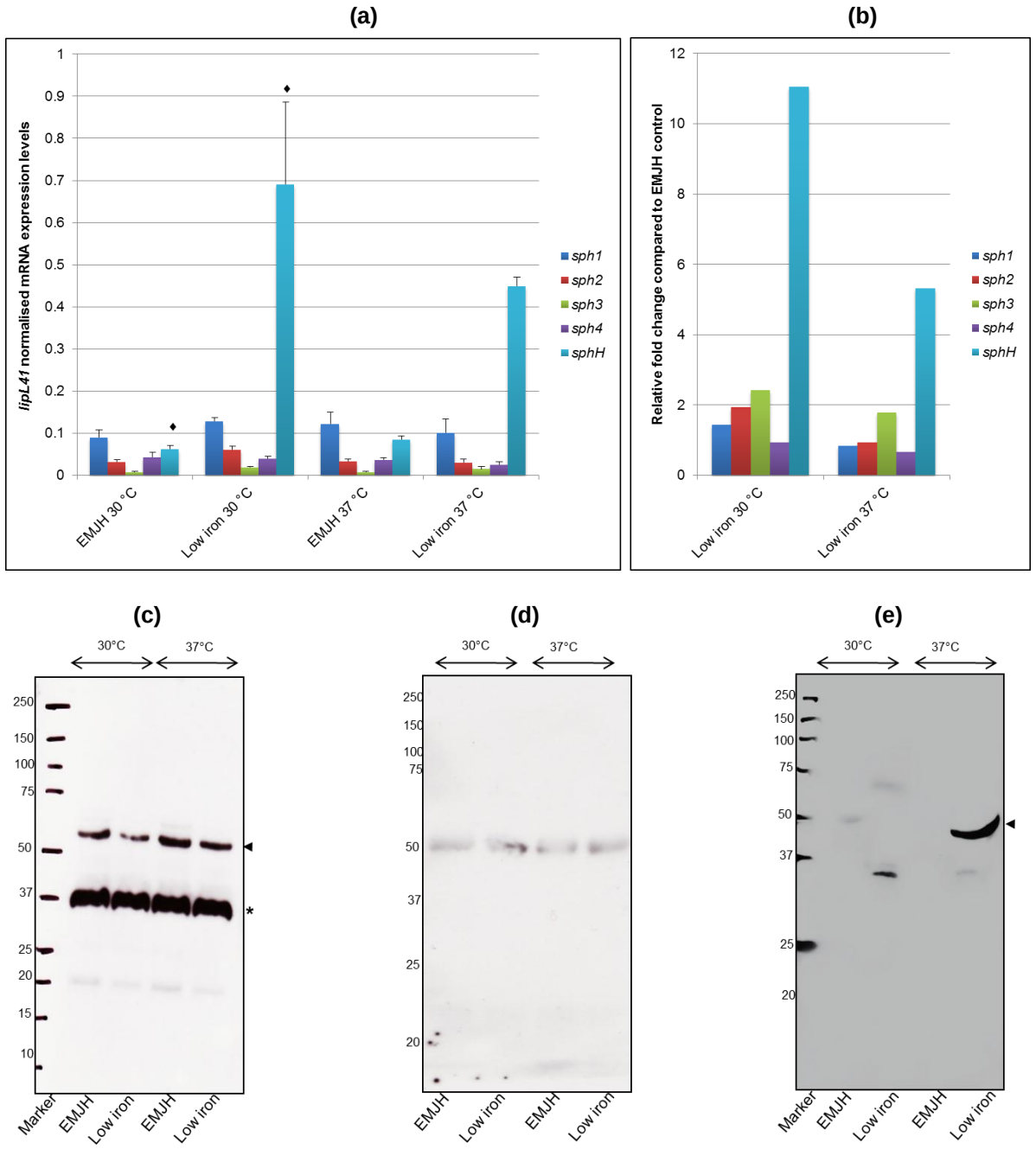


Fig. 25. Effect of iron limitation on the expression of sphingomyelinases in *L. interrogans* serovar Lai

Sphingomyelinase transcript levels were determined by qRT - PCR. *sph1, sph2, sph3, sph4 and sphH* signals were normalised to *lipL41* transcript levels. Representative results from three independent experiments are shown. Panel (a) represents mRNA expression levels, where in error bars denote the standard deviation. ♦, P=0.005 (Paired, two tailed, student t test). Panel (b) represents observed relative fold changes compared to EMJH control. Panels (c), (d) and (e) represents immunoblot analysis of whole cell lysates, immunoprecipitated samples from spent growth medium and outer membrane vesicle proteins respectively using anti - $Sph2_{163}$ antibodies. Bands corresponding to arrow arrow represent SphH specific signal, band corresponding to ***** represents LipL41 specific signal.

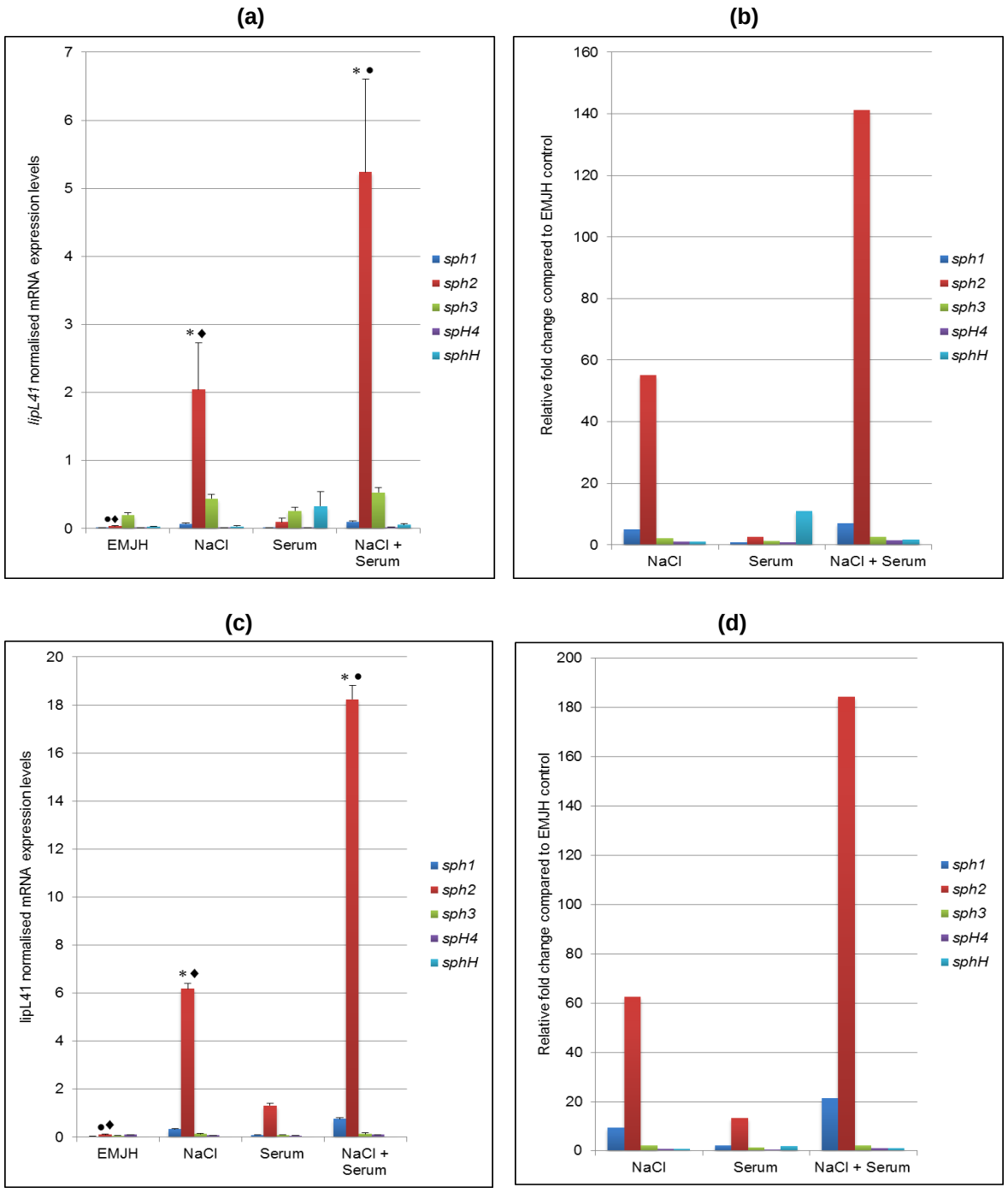


Fig. 26. Effect of salt, serum, salt and serum: quantification of sphingomyelinases by qRT - PCR

Panels (a) & (b) represents *L. interrogans* serovar Manilae; panels (c) & (d) represents *L. interrogans* serovar Pomona. Transcript levels were determined by qRT - PCR. Signals from *sph1*, *sph2*, *sph3*, *sph4* and *sphH* were normalised to *lipL41* transcript levels. Representative results from three independent experiments are shown. Panels (a) & (c) represents mRNA expression levels while Panels (b) & (d) represents observed relative fold changes compared to EMJH control. Error bars in panel (a) denote the standard deviation. *, P<0.001; ♦, P<0.001; ●, P<0.005 (one - way ANOVA, Student - Newman - Keuls multiple comparisons post test). Error bars in panel (c) denote the standard deviation. *, P<0.001; ♦, P<0.001; ●, P<0.001 (one - way ANOVA, Student – Newman - Keuls multiple comparisons post test).

Table 12. Effect of salt, serum and salt + serum on the expression of sphingomyelinases

	Relative fold changes compared to EMJH control									
	***L. interrogans* serovar Pomona**					***L. interrogans* serovar Manilae**				
	sph1	***sph2***	***sph3***	***sph4***	***sphH***	***sph1***	***sph2***	***sph3***	***sph4***	***sphH***
Salt	9.3	62.3	2.1	0.6	0.8	5.0	55.0	2.2	0.9	0.9
Serum	2.2	13.2	1.3	0.5	1.8	0.9	2.5	1.3	0.8	11.0
Salt + Serum	21.5	184.3	2.2	1.0	1.0	6.9	141.2	2.6	1.3	1.8

Sph2 expression in both whole cell lysates and spent growth medium were analysed with anti - $Sph2_{163}$ and anti - SphH antibodies. While the former recognised both Sph2 and SphH (Fig. 27), the latter, raised against a unique SphH peptide recognised only SphH (Fig. 28). Sph2 was identified as an 89 kDa protein in serovar Manilae with a slightly larger size of 95 kDa in the serovar Pomona. Other leptospiral serovars including Lai and Copenhageni expressed the 89 kDa protein, thus making the 95 kDa protein unique to serovar Pomona (Fig. 27a, b, c & d). Table 13 summarises the influence of salt, serum and both salt and serum in the medium of growth. Addition of heat inactivated rat serum has no effect on the expression of Sph2 (Fig. 29b).

Anti - $Sph2_{163}$ antibodies also recognised SphH as a 63 kDa protein in these samples (Fig. 27). Immunoblot analysis with anti - SphH antibodies clearly indicates that SphH was unaffected by the inclusion of salt and serum in the medium of growth (Fig. 28). In the serovar Pomona, additional low molecular weight proteins of approximate molecular mass of 27, 34, 43 and 55 kDa were seen (Fig. 27d).

From the Sph2 - specific immunoblot profile of the spent growth medium of serovar Pomona, both the 75 and 85 kDa proteins were up - regulated in the presence of salt, with traces of the former seen in the presence of serum alone. However, in the presence of both salt and serum, the full length 95 kDa band was clearly up - regulated with the detection of the 75 kDa band and traces of the 85 kDa protein (Fig. 30). No Sph2 was seen in serovar Manilae as well as Copenhageni and Lai. In all the immunoblot analysis, LipL41 was used as control.

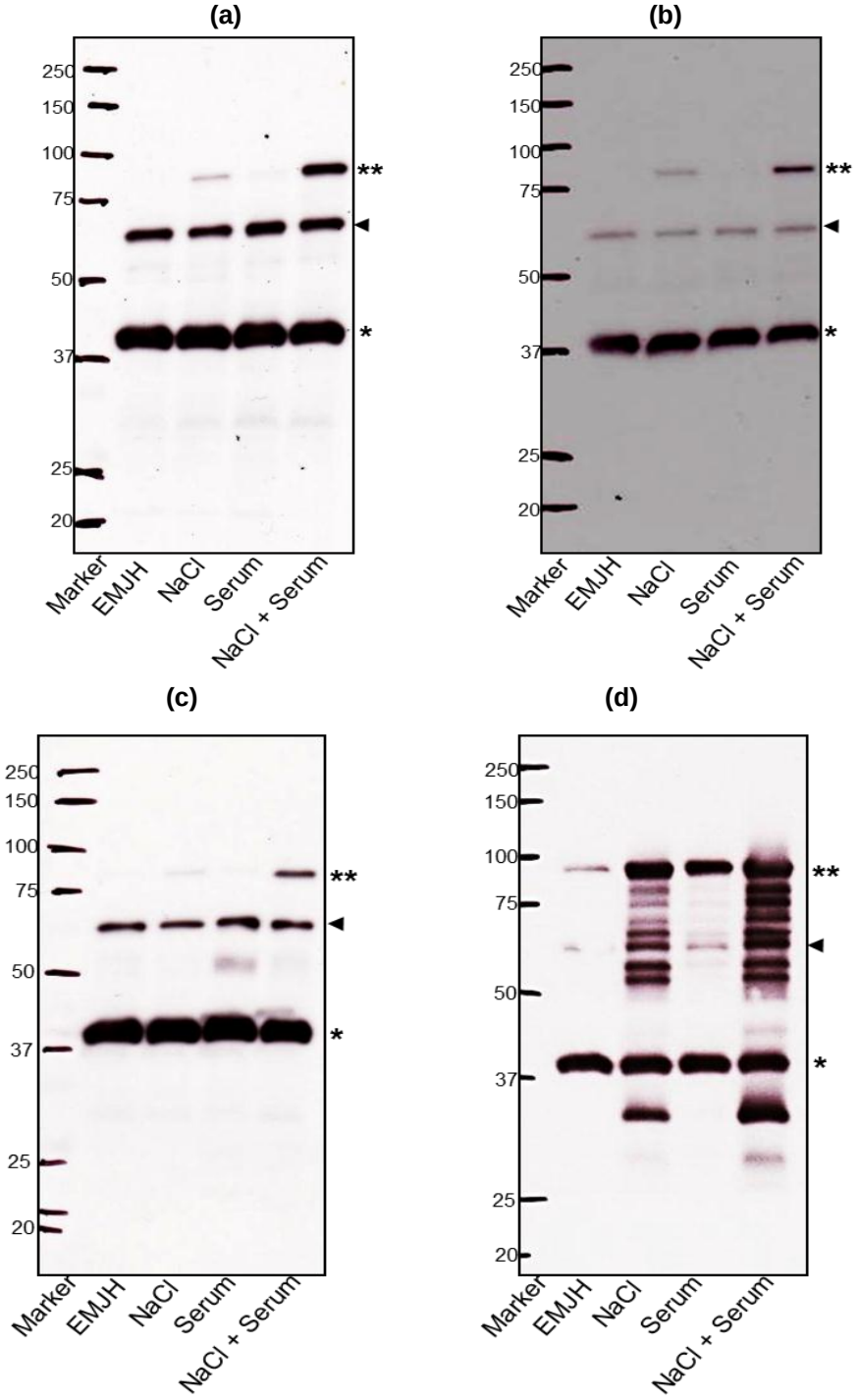


Fig. 27. Effect of salt and serum on Sph2 and SphH protein levels

L. interrogans serovar Manilae (panel a), Lai (panel b), Copenhageni (panel c) and Pomona (panel d) were grown in EMJH, EMJH supplemented with 120 mM NaCl or 10% rat serum or 120 mM NaCl along with 10% rat serum for 4 h at 30°C. Whole cell lysates were analysed by immunobloting with anti - $Sph2_{163}$ and anti - LipL41 antibodies. Bands corresponding to arrows represent SphH specific signal, bands corresponding to * represents LipL41 specific signal and bands corresponding to ** represents Sph2 specific signal.

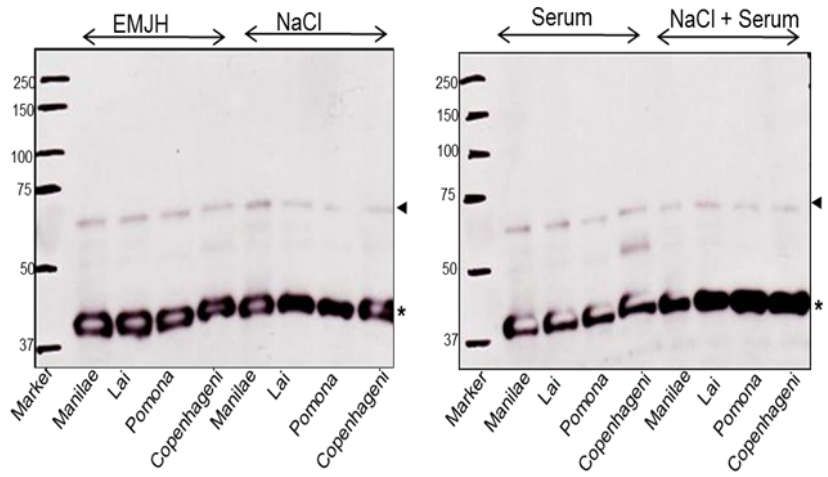


Fig. 28. Detection of SphH protein using anti - SphH peptide antibodies

Different *L. interrogans* serovars were grown in EMJH, EMJH supplemented with 120 mM NaCl, EMJH supplemented with 10% rat serum, and EMJH supplemented with 120 mM NaCl + 10% rat serum for 4 h at 30°C. Whole cell lysates were analysed by immunobloting with anti - SphH peptide antibodies and anti - LipL41 antibodies. Bands corresponding to arrows represent SphH specific signal, bands corresponding to ***** represents LipL41 specific signal.

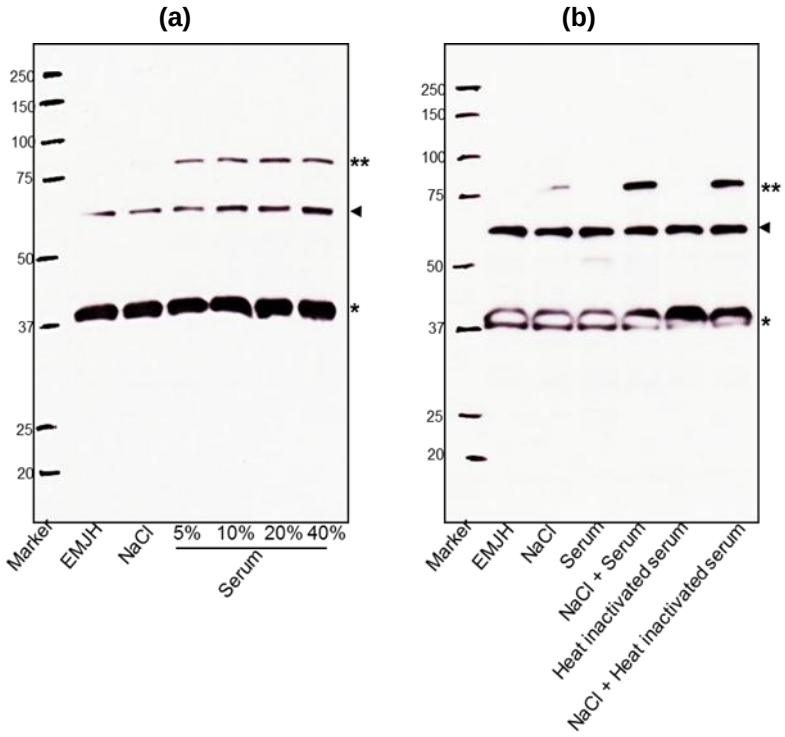


Fig. 29. Effect of serum on the expression of Sph2

Panels (a) and (b) represents the immunoblot analysis of whole cell lysates using anti - $Sph2_{163}$ and anti - LipL41 antibodies. Panel (a) represents the dose - dependent effect of serum whereas Panel (b) represents the effect of heat inactivated rat serum. Bands corresponding to arrows represent SphH specific signal, bands corresponding to ***** represents LipL41 specific signal and bands corresponding to ** represents Sph2 specific signal.

Table 13. Effect of salt and serum on the expression of Sph2

	Pomona*	**Manilae**	**Copenhageni**	**Lai**
Salt	++++	+	+ / -	+
Serum	++++	-	-	-
Salt + serum	+++++	++++	++	++

*There were several bands of similar intensity over a range between 95 kDa to 27 kDa in this serovar. + / - represents traces of protein detected, + to +++++ represents increasing intensity of the expressed protein.

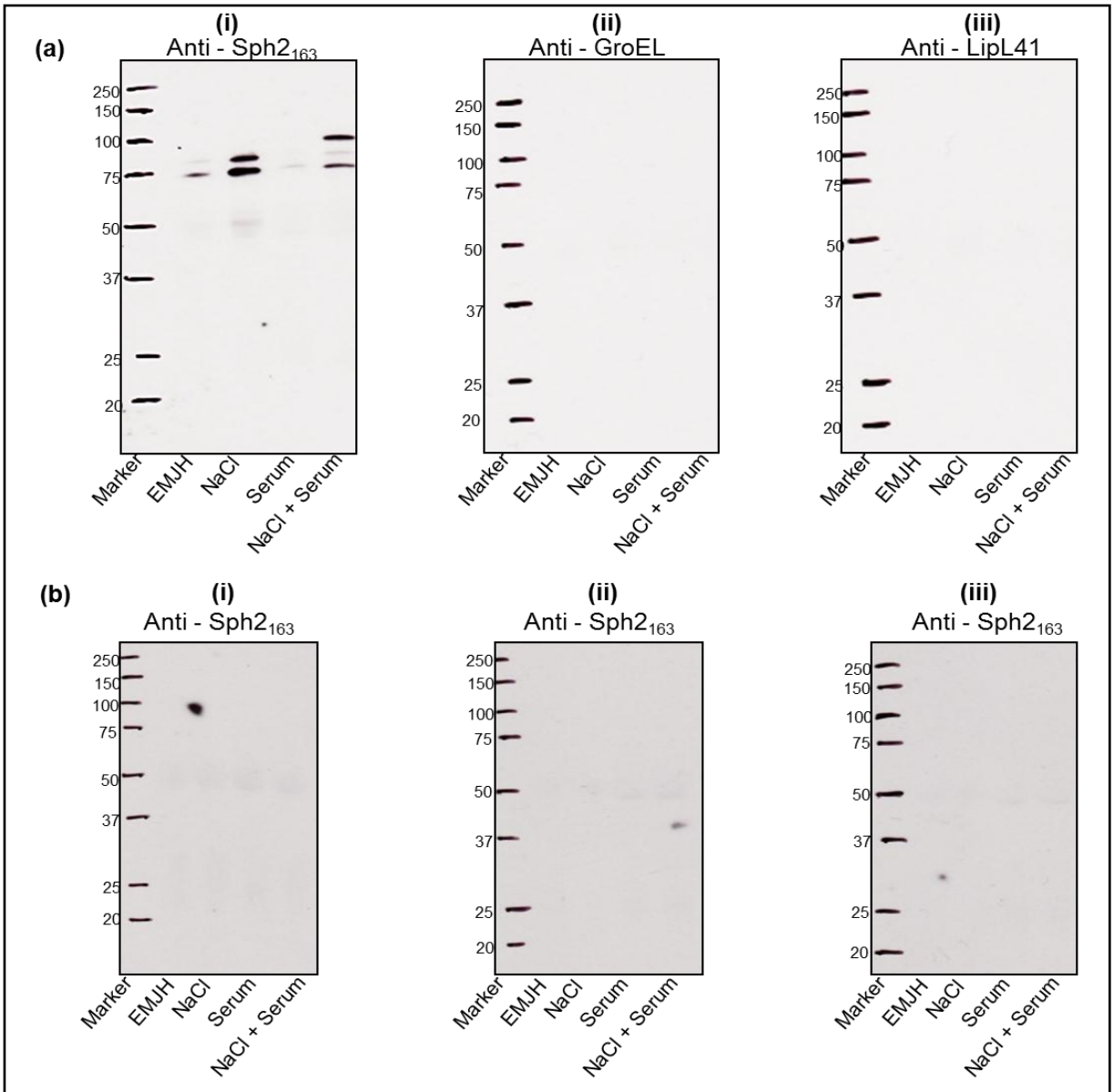


Fig. 30. Immunoprecipitation of Sph2 protein from spent growth medium

Panel (a) represents immunoprecipitation of Sph2, GroEL and LipL41 antigens from spent growth medium of *L. interrogans* serovars Pomona grown under different conditions as mentioned. Panel (b) represents immunoprecipitation of Sph2 antigen from spent growth medium of *L. interrogans* serovars Copenhageni (i), Lai (ii) and Manilae (iii).

Both hemolytic and sphingomyelinase activities were tested in the spent growth medium of the serovar Pomona and Manilae grown in the presence of salt (serum and combination of serum and salt was not done). Serovar Pomona showed hemolytic activity even in the absence of added salt that however increased 3 – fold upon addition of 120 mM NaCl (Fig. 31). This was also reflected in the sphingomyelinase assay (Fig. 32). While negligible hemolytic activity was exhibited by serovar Manilae, it shows a marked increase upon salt addition, though the overall hemolytic activity was lower than serovar Pomona (Fig. 31).

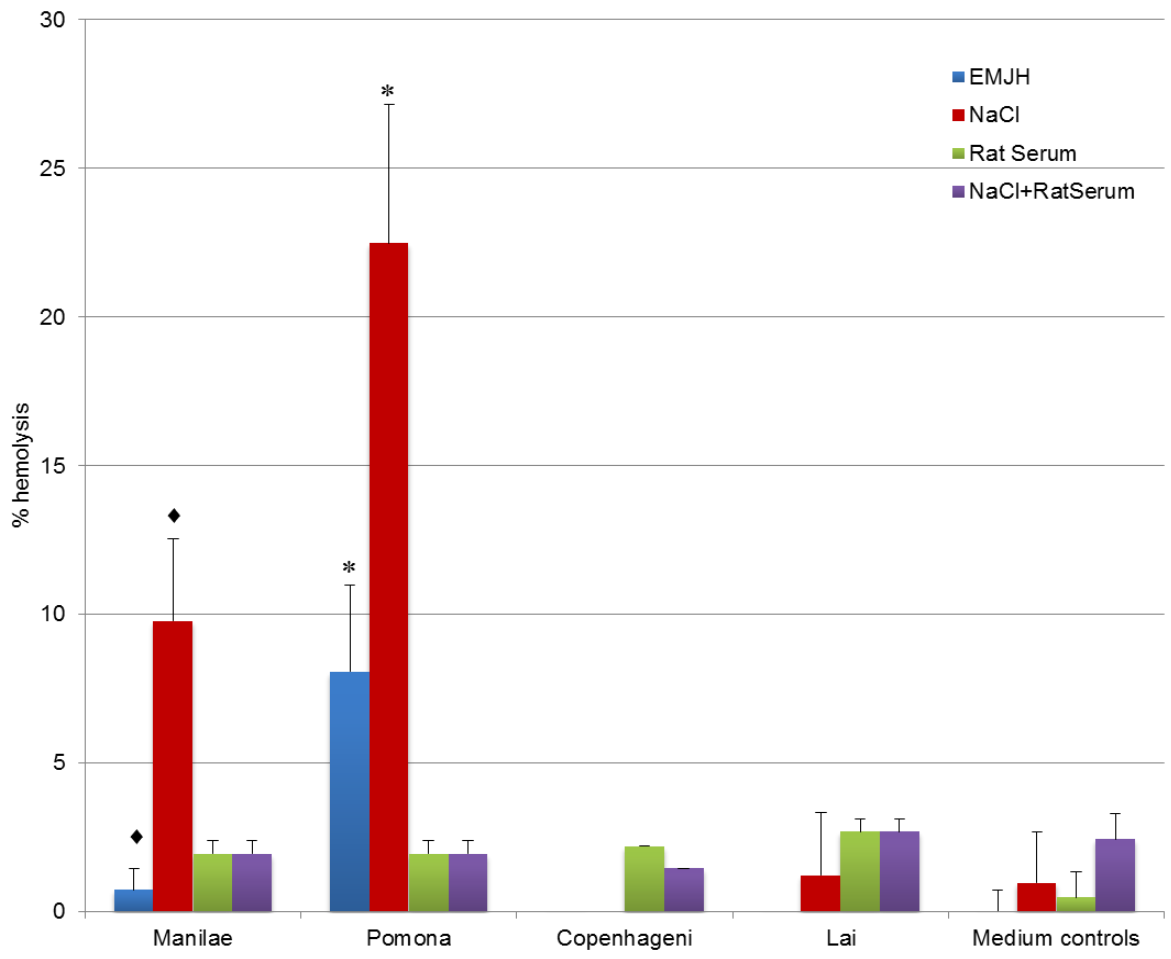


Fig. 31. Hemolytic activities of the spent growth medium

Hemolytic assay was performed with the spent growth medium of *L. interrogans* serovars Manilae, Pomona, Copenhageni and Lai grown in EMJH, EMJH supplemented with 120 mM NaCl or 10% rat serum or 120 mM NaCl along with 10% rat serum for 4 h at 30°C. The % hemolysis was calculated as indicated in Materials & Methods and represents the hemolytic activity of the respective samples as compared to the complete hemolysis of erythrocytes with water. Data are presented as mean ± SD of triplicates. Error bars denote the standard deviation. *, P<0.001; ♦, P<0.001; (one - way ANOVA, Tukey multiple comparisons post test).

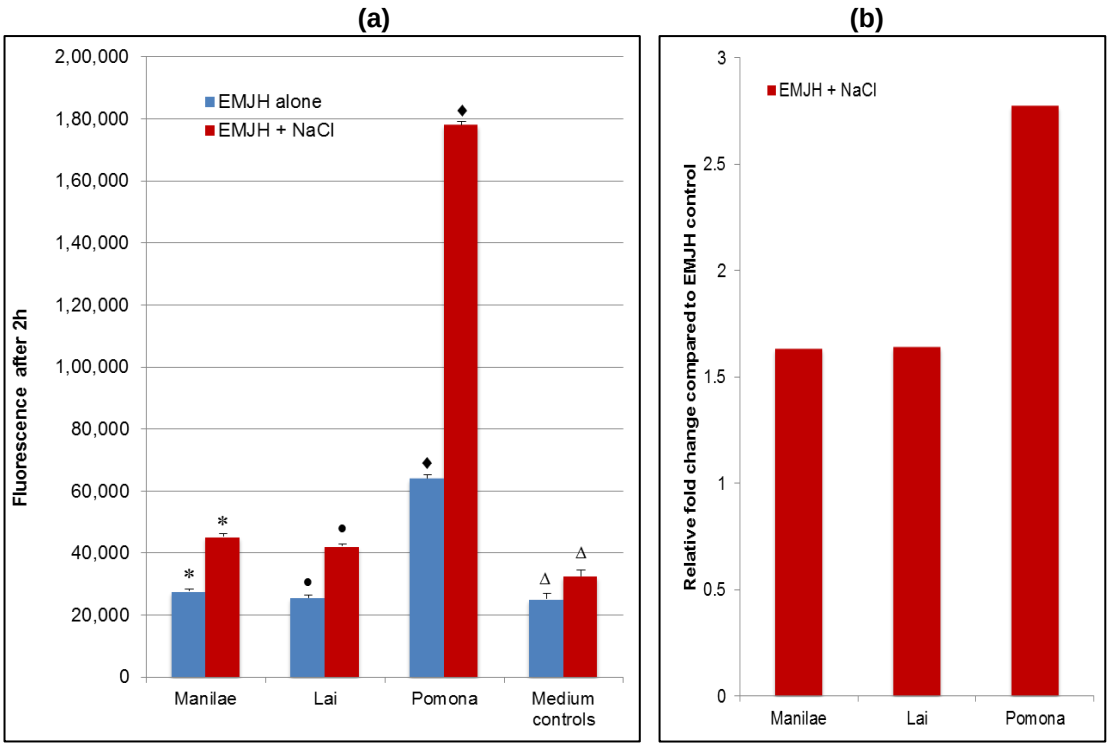


Fig. 32. Sphingomyelinase assay with spent growth medium

Sphingomyelinase assay was performed with the spent growth medium of *L. interrogans* serovar Manilae, Lai, Pomona and Lai grown in EMJH, EMJH supplemented with 120 mM NaCl for 4 h at 30°C. Panel (a) represents the observed fluorescence and Panel (b) represents relative fold change compared to EMJH control. Data are presented as mean ± SD of triplicates. Error bars denote the standard deviation. *, P=0.0009; ●, P=0.009; ♦, P<0.000; Δ, P = 0.1267 (Not significant) (Paired, two tailed student t test).

3.3.3. Effect of hydrogen peroxide

Oxidative stress, induced by adding increasing amounts of H_2O_2 caused the marked increase in the levels of *sphH* transcripts in both serovar Pomona and Manilae with approximately a 3 - fold higher increase in the latter (Fig. 33, Table 14). This however was not reflected in the protein profile in whole cell lysates of these two serovars (Fig. 34). The effect on the other sphingomyelinases was only marginal.

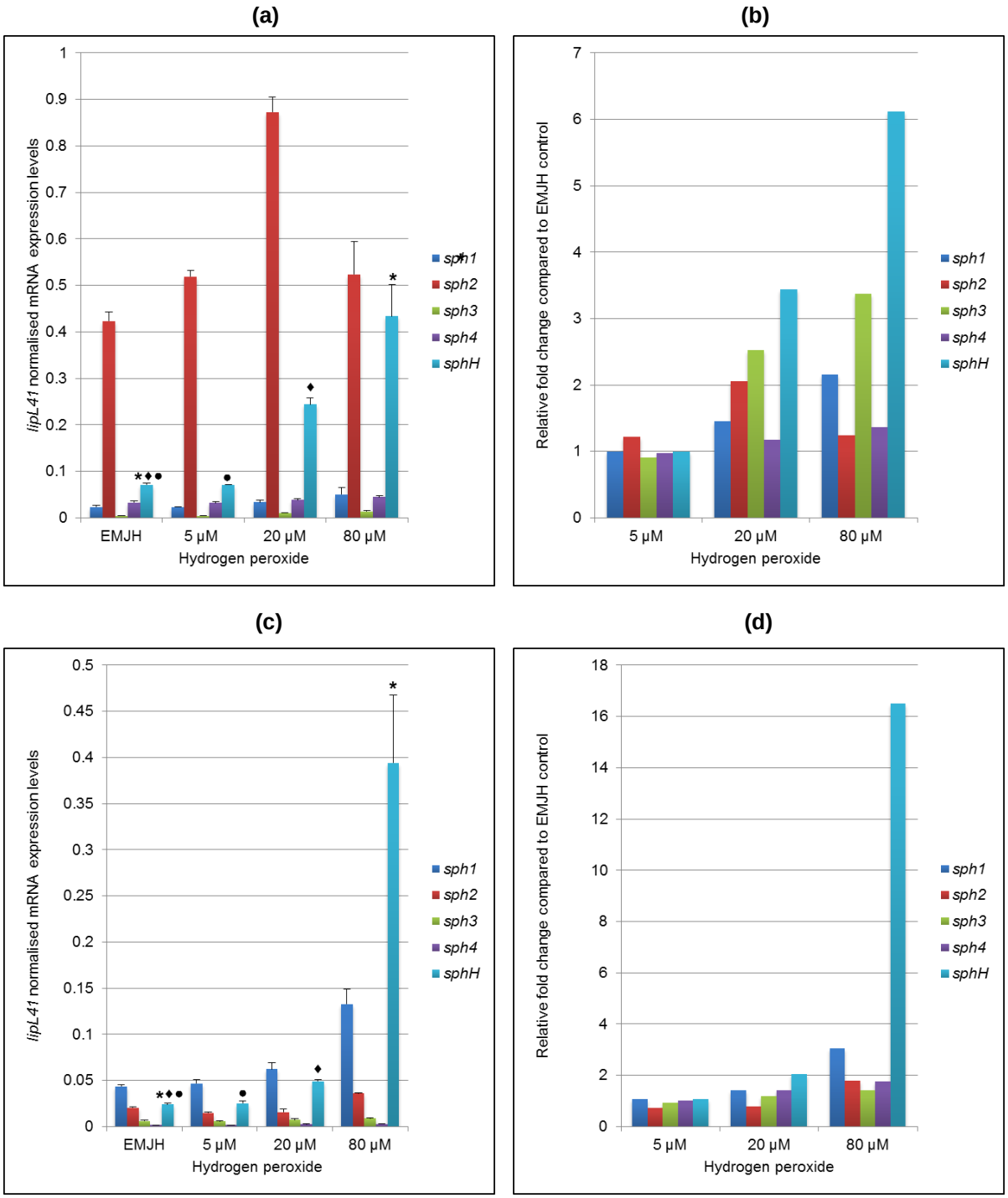


Fig. 33. Effect of oxidative stress on the expression of sphingomyelinases

Panels (a) & (b) represent *L. interrogans* serovar Pomona and Panels (c) & (d) represent *L. interrogans* serovar Manilae. Transcript levels were determined by qRT - PCR. Signals from *sph1*, *sph2*, *sph3*, *sph4* and *sphH* were normalised to *lipL41* transcript levels. Representative results from three independent experiments are shown. Panels (a) & (c) represents mRNA expression levels, Panels (b) & (d) represents observed relative fold change compared to EMJH control. Error bars in panel (a) denote the standard deviation. *, P<0.001; ♦, P<0.01; ●, P>0.05 (not significant) (one- way ANOVA, Tukey - Kramer multiple comparisons post test). Error bars in panel (c) denote the standard deviation. *, P<0.001; ♦, P>0.05; ●, P>0.05 (one- way ANOVA, Tukey - Kramer multiple comparisons post test).

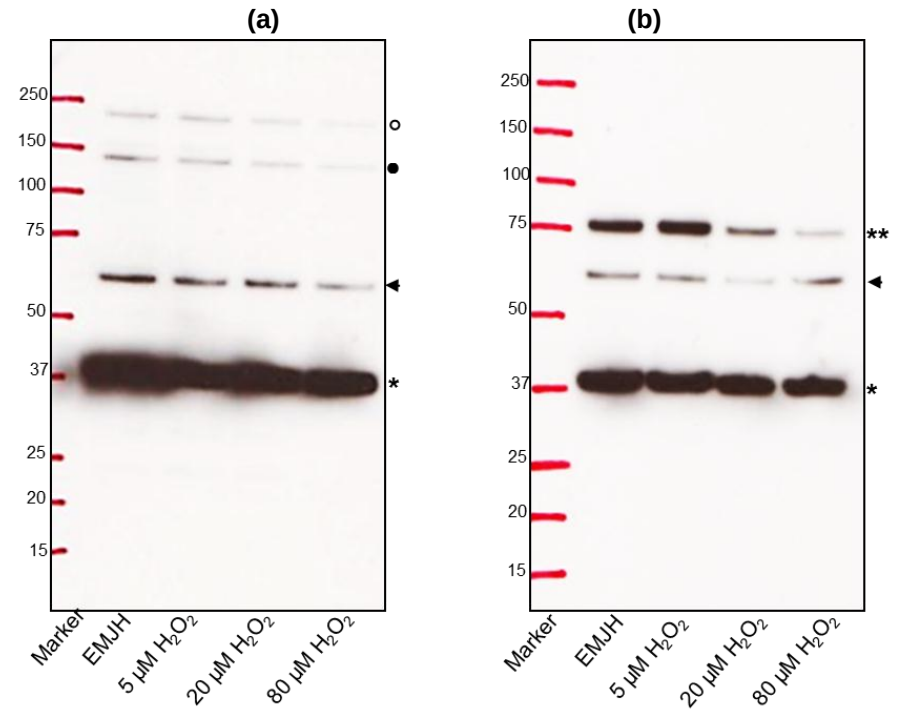


Fig. 34. Effect of H_2O_2 on the expression of leptospiral sphingomyelinases

Panels (a) and (b) represent *L. interrogans* serovars Manilae and Pomona respectively. Leptospires were grown in EMJH, EMJH supplemented with different concentrations of H_2O_2 as indicated. Immunoblot analysis of whole cell lysates using anti - $Sph2_{163}$ and anti - LipL41 antibodies revealed unaltered expression of SphH under different concentrations of H_2O_2 tested. Bands corresponding to arrows represent SphH specific signal, bands corresponding to * represents LipL41 specific signal and bands corresponding to ** represents Sph2 specific signal. Bands corresponding to ° and ● represent LigB and LigA specific signals respectively.

Table 14. Effect of hydrogen peroxide on the expression of sphingomyelinases

	Relative fold changes compared to EMJH control									
	***L. interrogans* serovar Pomona**					***L. interrogans* serovar Manilae**				
	sph1	***sph2***	***sph3***	***sph4***	***sphH***	***sph1***	***sph2***	***sph3***	***sph4***	***sphH***
5 µM H_2O_2	0.99	1.22	0.90	0.97	1.00	1.06	0.72	0.92	1.02	1.05
20 µM H_2O_2	1.45	2.06	2.53	1.18	3.43	1.42	0.77	1.17	1.42	2.06
80 µM H_2O_2	2.15	1.23	3.36	1.36	6.11	3.03	1.77	1.42	1.76	16.51

3.4. Molecular basis for elevated expression of Sph2 in *L. interrogans* serovar Pomona

From the above results, it was observed that serovar Pomona has higher levels of Sph2 expression when compared to other serovars. We wished to understand the molecular basis underlying the elevated expression of $Sph2_{Pomona}$. As it is likely that the upstream region of $sph2_{Pomona}$ may contain regulatory elements, the upstream region of *sph2* from serovar Pomona was compared with serovar Copenhageni.

3.4.1. Transcriptional start sites of $sph2_{Pomona}$ and $sph2_{Copenhageni}$ were identical

Since the genome sequence of *L. interrogans* serovar Pomona is not available, RNA was isolated and converted to cDNA using $sph2_{Pomona}$ gene specific primers. To identify the transcriptional start site (TSS) of $sph2_{Pomona}$ 5′ RACE was performed. Sequencing results (Fig. 30a, b & c) indicate that the TSS of $sph2_{Pomona}$ was located 27 nucleotides upstream of the ATG start codon and was identical to TSS of $sph2_{Copenhageni}$ (Fig. 34).

3.4.2. Identification of 330 nucleotide insertion sequence like - element in the upstream region of $sph2_{Pomona}$

Analysis of upstream regions of *sph2* from *L. interrogans* serovars Copenhageni, Pomona, Lai and Manilae revealed the presence of 330 nucleotide insertion sequence - like element in serovar Pomona and was absent in the others (Fig. 35c).

3.4.3. Expression of $Sph2_{Pomona}$ and $Sph2_{Copenhagnei}$ in *L. biflexa*

The full – length *sph2* of Pomona along with a 638 bp upstream region and a 130 bp downstream region, totally to a DNA fragment of 2715 bp was PCR amplified and expressed in the non – pathogenic *L. biflexa* as mentioned in materials and methods. The corresponding segment from the serovar Copenhageni was similarly expressed in *L. biflexa.* Transformants carrying $sph2_{Pomona}$ or $sph2_{Copenhageni}$ were designated pRAT680 and pRAT678 respectively (Fig. 36). Expression of rSph2 protein by *L. biflexa* transformants, detected with anti - $Sph2_{163}$ antibodies (Fig. 37) identified $Sph2_{Pomona}$ and $Sph2_{Copenhagnei}$ as 95 kDa and 89 kDa bands respectively. Over expression of $Sph2_{Pomona}$ protein (Fig. 37a; lane 2) was observed when compared to $Sph2_{Copenhagnei.}$ (Fig. 37a; lane 3). Expression of Sph2 was not seen with *L. biflexa* containing vector control while whole cell sonicates of *L. interrogans* serovars Pomona and Copenhageni grown in EMJH and EMJH supplemented with salt were used as controls.

The 330 nucleotide insertion sequence like – element in the serovar Pomona was deleted and the recombinant, designated pRAT696 was transformed into *L. biflexa*. There was no difference in expression of the rSph2 protein by *L. biflexa* (Fig. 37c) indicating that the *IS* – like element has no role in the observed overexpression.

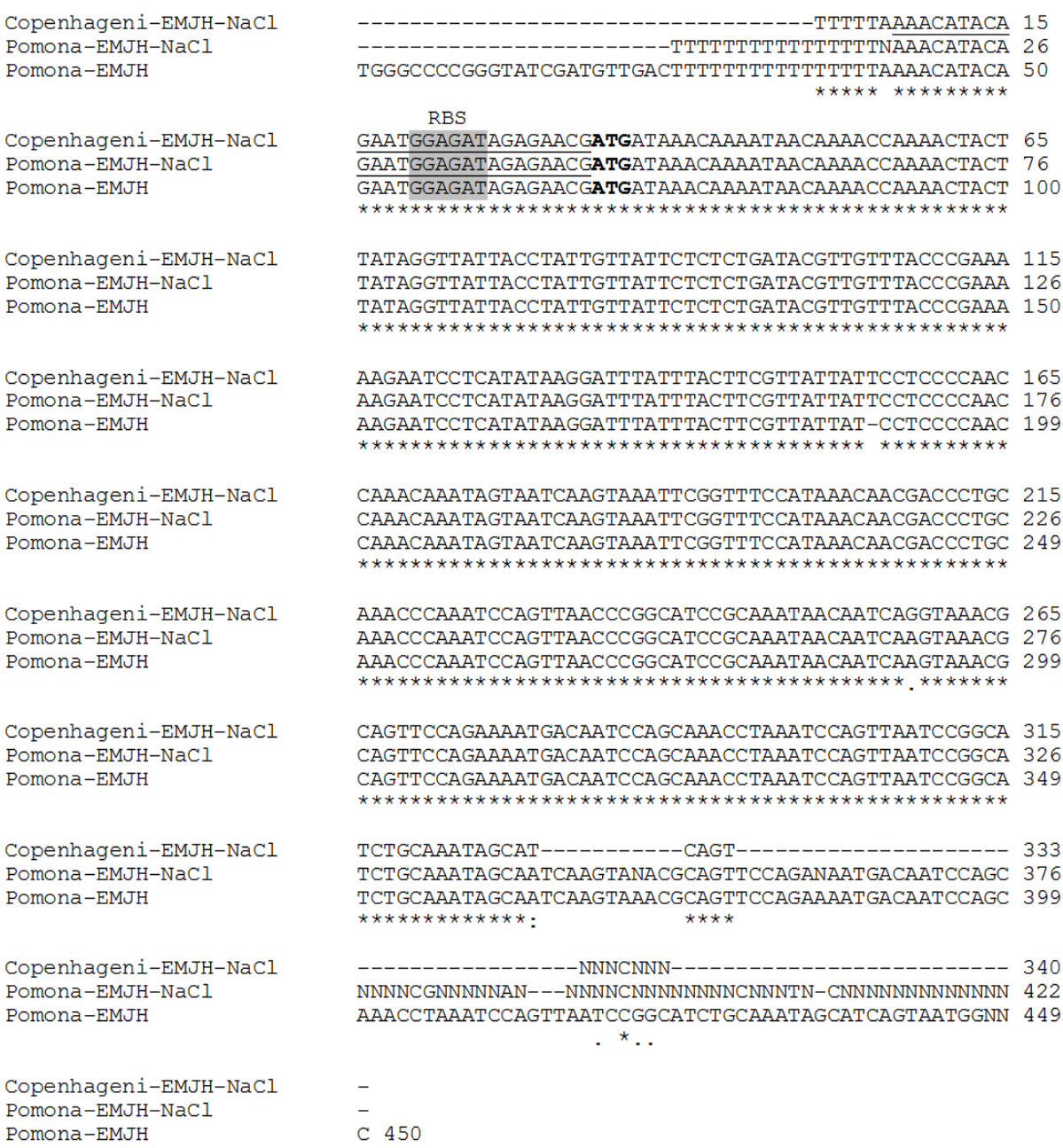
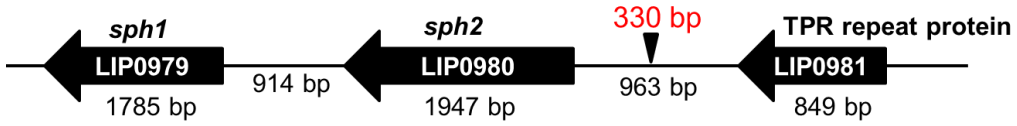


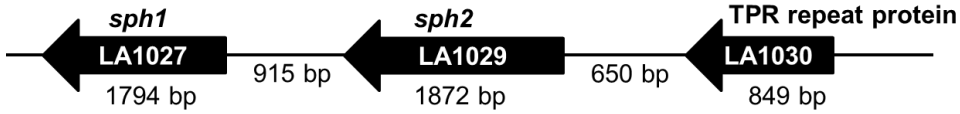
Fig. 35. Prediction of transcriptional start site and multiple sequence alignment of upstream regions of sph2 of Pomona and Copenhageni

For predicting the transcriptional start site leptospires were grown as mentioned in Materials and Methods. Sequences shown were obtained by 5'RACE. The transcriptional start site of *sph2*$_{Pomona}$ was identical to that of *sph2*$_{Copenhagenii}$ (underlined). Stat codon "ATG" is represented in bold and the ribosomal binding site (RBS) in grey.

(a)



(b)



(c)

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LIP0980         GCGACCATAGAAGCGAGACGCTGAGTCTGAAAGAGCGTTTTGTTAAATCTCCCCTCACAT
LA1029          GCGACCATAGAAGCGAGACGTTGAGTCTGAAAGAGCGTTTTGTTAAATTTCCCCTCACAT
LIL49501008     GCGACCATAGAAGCGAGACACTGGTTTCGAAAGAACGTTTTGTTAAACTTCCCTTCACAT

LIC12631        CGCCCCCTTCGCGCTATACTAAATTT----------------------------------
LIP0980         CGCCCCCTTCGCGTTATACTAAATTTCATCACCTTACCCACAAGTTGAATAGGATTTTAG
LA1029          CGCCCCCTTCGCGTTATACTAAATTT----------------------------------
LIL49501008     CGCCCCTTTCTCTTTATACCAAATTT----------------------------------

LIC12631        ------------------------------------------------------------
LIP0980         AATCAGAATGAACTCAACAGAACGGGCTTTCTATGGATCGCCGTTCTGGGCTCAAAAATG
LA1029          ------------------------------------------------------------
LIL49501008     ------------------------------------------------------------

LIC12631        ------------------------------------------------------------
LIP0980         CACATTTTTCAAGTGTTCCGACAAGAATGAGTCTTTTTACTTGCAAAAAGTATGTTTTTC
LA1029          ------------------------------------------------------------
LIL49501008     ------------------------------------------------------------

LIC12631        ------------------------------------------------------------
LIP0980         TGATAAAGAAAACCTTTCCACCTCCTCCCCCACCCAAAAATAGGGAAAACTCAGCACAAC
LA1029          ------------------------------------------------------------
LIL49501008     ------------------------------------------------------------

LIC12631        ------------------------------------------------------------
LIP0980         GCTCTCTATGAATCGCGTTGTGGGGCGGGAACTAAGTTTTACAGAAGATTTTGTCGTAAT
LA1029          ------------------------------------------------------------
LIL49501008     ------------------------------------------------------------

LIC12631        ------------------------------------------------------------ATGC
LIP0980         TCCGACAGATTTATATTGAGATCCAATTATTTGTGGGCAAGGTTATGACTAAATTTATGC
LA1029          ------------------------------------------------------------ATAC
LIL49501008     ------------------------------------------------------------ATGC

LIC12631        TATCATCCGCTTTCTCTAAAATTATTTAAAACAATATTCAATTTAAAGAATATTATAATA
LIP0980         TATCATCCGCTTTCTCTAAAATTA----------------TTTAAATAATATTATAATA
LA1029          TACCATCCACTTGTCCTCAAATTATTTAAAATAACATTCAATTTAAAGAATATTATAATA
LIL49501008     TACCATACACTTTCTCCAAATTCAGTTAAAATAACATTTTATTTAAATAATATTATAATA

LIC12631        AAATAA------------AATTTTATAAAAAATACAAATTTTAAATATCCCTAAAATAAC
LIP0980         AAATAA------------AATTTTATAAAAAATACAAATTTCAAATATCCCTAAAATAAC
LA1029          AAATAA------------AATTTTATAAAAAATACAAATTTCAAATATCCCTAAAATAAC
LIL49501008     AAATAATTTTTTAAATAAAATTTCATAAAAAATACAAATTTCAAATATCCCTAAAATAAC
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(Continued in next page)

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LIC12631        GCTCCTTAAGTCTTCAGTTCGATTACCTAAGTATTTTTAGATATTTTATGTTTATTAGTA
LIP0980         ACTCCTTAAGTCTTCAGTCAGATTACCTAAGTATTTTTATATATTTTATGTTTATTAGTA
LA1029          GCTCCTTAAGTCTTCAGTTCGATTACCTAAGTATTTTTAGATATTTTATGTTTATTAGTA
LIL49501008     GCTCCTTAAGTCTTCAGTCCGATTACCTAAGTATTTTTATATATTTTATCTTTATTAGTA


LIC12631        TTTTTTTATAAAATACTATTCATTTTTATGTTATATGATAACATAAAATACAAACATACA
LIP0980         TTTTTTTATAAAATACTATTCATTTTTATGTTATATGATAACATAAAATACAAACATACA
LA1029          TTTTTTTATAAAATACTATTCATTTTTATGTTATATGATAACATAAAATACAAACATACA
LIL49501008     TTTTTTTATAAATTACTATTCATTTTTATGTTATATGATAACATAAAATACAAACATATA


 LIC12631        GAATGGAGATAGAGAACG
 LIP0980         GAATGGAGATAGAGAACG
 LA1029          GAATGGAGATAGAGAACG
 LIL49501008     GAATGGAGACAGTGAACG
```

Fig. 36. Genome organisation of *sph2* locus and multiple sequence alignment of upstream regions of *sph2*

Panels (a) and (b) represents the genome organisation of *sph2* and the neighbouring genes in the serovars Pomona and Lai respectively. The 330 bp insertion sequence (IS) element upstream of *sph2* is seen in serovar Pomona (Panel a) that is absent in serovar Lai (Panel b). The complete IS element with its inverted repeats (shown in green) and the direct repeats (shown in yellow) can be seen in Panel (b) that shows the multiple sequence alignment of upstream regions of *sph2* from serovars Copenhageni (LIC12631), Pomona (LIP0980), Lai (LA1029), and Manilae (LIL49501008) using Kalign.

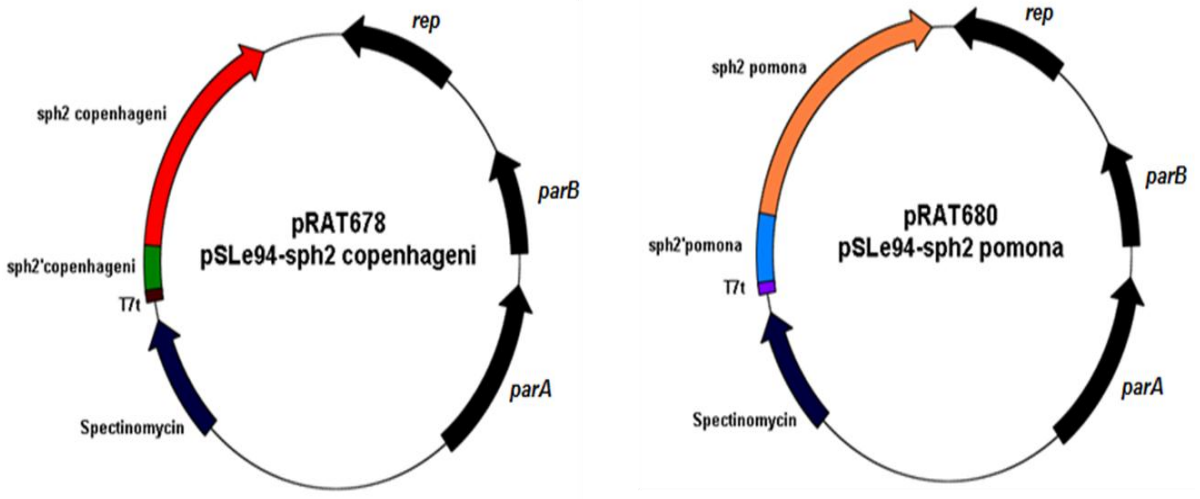

Fig. 37. Generation of *sph2*$_{\text{Copenhageni - 2592}}$ and *sph2*$_{\text{Pomona - 2715}}$ constructs for expression in *L. biflexa*

Schematic diagram of the plasmid constructs used to express *sph2*$_{\text{Copenhageni - 2592}}$ (a) and *sph2*$_{\text{Pomona – 2715}}$ (b). The determinants for replication in *L. biflexa* (*parA*, *parB* and *rep*), as well as spectinomycin resistance cassette is indicated. In Panel (a) the red arrow represents the *sph2* coding region in Copenhageni while the up - steam region is represented in green. In Panel (b) the orange arrow represents the *sph2* coding region in Pomona while the up - steam region is represented in blue.Both the genes were cloned downstream of spectinomycin resistance cassette. A T7 terminator was inserted at the 5' end, upstream of *sph2.* Vector maps were drawn using 'Plasm' software (http://biofreesoftware.com/plasm).

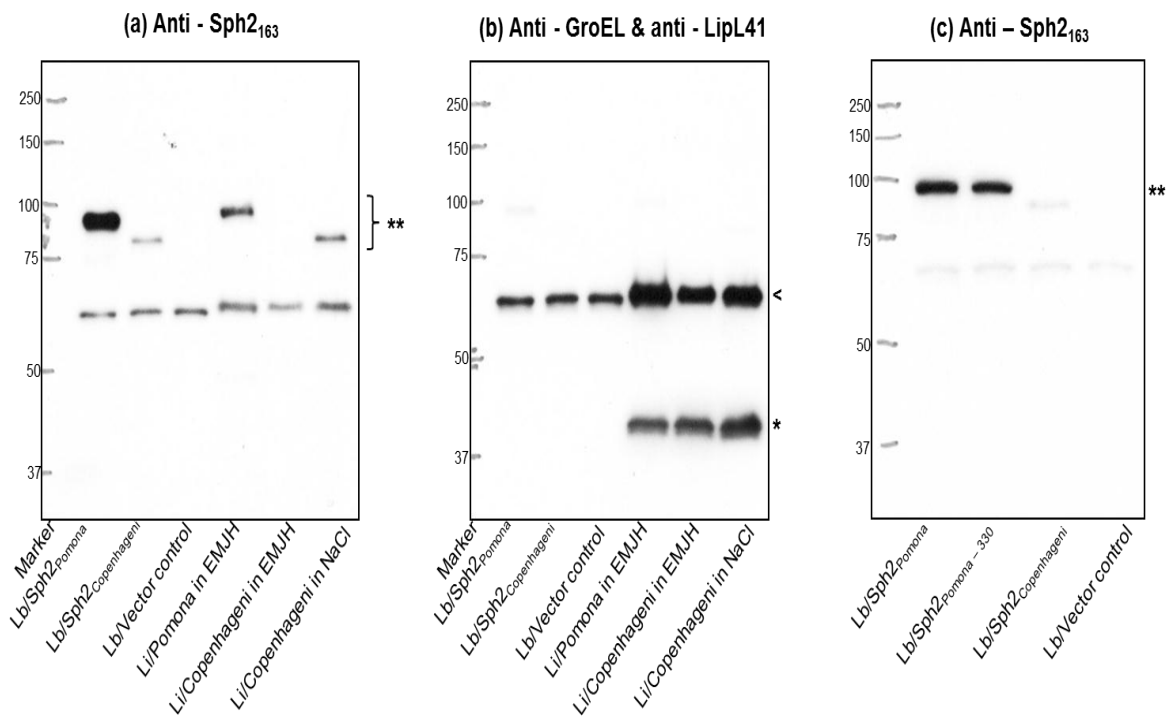

Fig. 38. Expression of Sph2 proteins by *L. biflexa* transformants

The constructs depicted in Fig. 37 were transformed into *L. biflexa*. Panel (a) shows Sph2 expression, detected with anti - $Sph2_{163}$ antibodies shows the high levels of expression in *L. biflexa* bearing the Pomona construct ($Lb/Sph2_{Pomona}$) and low levels of the protein in Lb/Sph2*Copenhageni*; no band was detected in the vector control. The other lanes include the serovars Pomona and Copenhageni (LI/Pomona and LI/Copenhageni) as controls. Panel (b) shows the immunoblot with anti – GroEL and anti – LipL41 that shows equal loading of protein samples in all the lanes. Deletion of the 330 bp *IS* element in serovar Pomona showed no effect on the expression of Sph2 (Panel c), where the first two lanes clearly show identical expression of Sph2 in $Lb/Sph2_{Pomona}$ & $Lb/Sph2_{Pomona\ -\ 330}$; $Lb/Sph2_{Copenhageni}$ and Lb/empty vector are shown as controls. * represents LipL41, ** represents Sph2 and the arrow represents GroEL.

CHAPTER 4

DISCUSSION

4. Discussion

Pathogenic *Leptospira* have adapted to the hostile environment of the mammalian host. Upon entry, they produce disease when they overcome the defense mechanisms of the host that attempts to get rid of the infecting pathogen. While the majority of patients recover from leptospirosis, the disease can manifest itself into the severe Weil's disease, often characterized by hemorrhage and tissue damage in several organs, including the liver, lungs and kidneys that more often results in death. The pathogenesis mechanisms are poorly understood. However, with advances in genome sequencing, comparative genomics is making possible the identification of potential virulence factors elaborated by these pathogens (Adler *et al.*, 2011).

In this study, we have focused on the structural analysis of the virulence factors sphingomyelinases, and the environmental conditions that regulate their expression. There is increasing evidence to implicate these proteins in some of the damage caused by this pathogen. In 1976, an experimental study in guinea pigs showed clear evidence of hemorrhage in almost all organs; vascular lesions were seen in which the majority of the vessels showed detached endothelial cells in the lumen (Higgins & Cousineau, 1977). After several studies subsequently (detailed in Review of Literature, Chapter 1), sphingomyelinases are implicated in the hemorrhagic manifestations of this disease. These proteins, expressed only by pathogenic leptospires are the subject of research in several laboratories (Carvalho *et al.*, 2010; Matsunaga *et al.*, 2007b; Velineni *et al.*, 2009; Zhang *et al.*, 2008). While our primary interest was to study the role of iron in the expression of these virulence factors, we have however made notable observations in this study, particularly on their three -dimensional structure, identification and characterisation of Sph2 as a true sphingomyelinase and the influence of different environmental conditions including iron limitation on the expression of these molecules. We observed variation in the regulation of these proteins among different serovars and an attempt has been made to understand the reason for the increased expression of Sph2 in the serovar Pomona.

Sphingomyelinases, classified as hydrolases belong to the DNase I superfamily by virtue of the presence of the common exo – endo phosphatase domain. This family comprises of members from several species including bacteria, yeast and eukaryotes. While they exhibit similar enzymatic activity, they show

variation in structure and substrate specificity (Matsuo *et al.*, 1996). Sphingomyelinases act on sphingomyelin to give ceramide and phosphocholine. In eukaryotes, the cleavage product ceramide and its derivatives act as potent regulators of cell proliferation, differentiation and apoptosis (Hannun & Obeid, 2008). Bacterial sphingomyelinases have not been well explored and till date, only three bacterial sphingomyelinases have been crystallized; they include SmcL from *L. ivanovii* (Gonzalez-Zorn *et al.*, 1999; Openshaw *et al.*, 2005), BC SMase from *B. cereus* (Ago *et al.*, 2006) and the sphingomyelinase from *S. aureus* (Huseby *et al.*, 2007) respectively. They are considered as virulence factors, as deletion mutants of *L. ivanovii* and *B. cereus*, failing to express the sphingomyelinases showed low infectivity in experimental animals (Bramley *et al.*, 1989; Gonzalez-Zorn *et al.*, 1999).

Pathogenic leptospires and not the saprophytic species elaborate sphingomyelinases. They are implicated in the hemorrhagic manifestations seen in the severe form of the disease. The genome of *L. interrogans* serovar Lai, the first leptospiral pathogen to be sequenced (Ren *et al.*, 2003) revealed the presence of five sphingomyelinase genes, namely *sph1*, *sph2*, *sph3*, *sph4* and *sphH* (*sph4* has been omitted in this study as it lacks the enzymatic domain). The multiplicity of these molecules is intriguing and their role in the survival of the pathogen *in vivo* remains to be understood. Species - specific differences in the expression of these molecules came to light with the sequence analysis of *L. borgpetersenii* that contained only three sphingomyelinase genes (*sphA*, *sphB* and *sph4*) in its genome and lacked the corresponding orthologs of *sph1* and *sphH*. It remains to be proven if the presence of the latter two genes in serovar Lai offers any advantage to this pathogen that can survive in the environment outside the mammalian host as against the host - host transmission of *L. borgpetersenii* (Bulach *et al.*, 2006b). However, it is clear that the leptospiral sphingomyelinases form a unique class of molecules as inferred from phylogenetic tree analysis that grouped them into a single cluster.

All the leptospiral sphingomyelinases, with the exception of Sph4 possess the exo – endo phosphatase domain that shows similar protein folding as other bacterial sphingomyelinases. The unique features of these molecules include the presence of the 186 amino acid C - terminal segment, presence of the N – terminal repeats and the absence of the β - hairpin loop, characteristically seen in other bacterial sphingomyelinases. The C - terminal extension, also seen in the sphingomyelinase from *Pseudomonas* strain TK4 possibly plays a role in its biological activity as

deletion of the gene in the latter completely abolished the hemolytic activity without affecting the sphingomyelinase activity (Sueyoshi *et al.*, 2002). The authors suggested that this stretch of amino acids facilitated interaction with the host membrane in such a manner as to position the enzymatic domain near the sphingomyelin substrate. Multiple short N – terminal repeats, enriched in disorder - promoting amino acids (a stretch of amino acids that does not exhibit secondary or tertiary structure) (Tompa, 2005) are seen in Sph1, Sph2 and SphB. It was envisaged, based on the known functions of intrinsically disordered sequences that these NTRs may harbour proteolytic sites and function as a flexible linker between the signal peptide and the enzymatic domain, or bind macromolecules or small ligands (Tompa, 2005).

Bacterial sphingomyelinases interact with the host cell membrane via the β – hairpin loop. This hydrophobic region, rich in aromatic amino acids is essential for interaction with the eukaryotic cell membrane (Ago *et al.*, 2006). So the question arose as to how the leptospiral sphingomyelinases that lack this region interacts with the host membrane. The clusters of hydrophobic residues exposed on the surface of Sph2 led us to propose a novel mechanism of interaction whereby these clusters interact via hydrophobic interactions with similar residues on the host membrane, making several points of interaction instead of a single region via the β – hairpin structure seen in other bacterial sphingomyelinases. Experimental validation by mutagenesis studies is however required to prove our hypothesis.

Among the five sphingomyelinases in serovar Lai, we demonstrated Sph2 to be a true sphingomyelinase by virtue of the presence of conserved amino acids at the metal - binding and catalytic sites and the dependence of the protein for Mg^{2+} for optimal activity. Sequence comparisons at the primary level and the identification of conserved amino acids in the folded tertiary conformation with other bacterial sphingomyelinases revealed that only Sph2 possessed Glu53 at the metal - binding site and the two His residues at positions 151 & 296 and Asp195 at the catalytic site. None of the other sphingomyelinase in serovar Lai showed these amino acids. SphA in *L. borgpetersenii* is most likely to function as a true sphingomyelinase by virtue of the presence of these conserved amino acid residues. Crystal structures of *B. cereus* sphingomyelinase showed that the active site of the protein contained the divalent metal cation Mg^{2+} necessary for catalytic activity (Ago *et al.*, 2006). Further, mutagenesis studies showed that these amino acids were essential for the biological

activity (Huseby *et al.*, 2007; Obama *et al.*, 2003a; Obama *et al.*, 2003b). It is highly likely that they are essential for the activity of Sph2. The dependence of Sph2 for Mg^{2+} was demonstrated experimentally with the recombinant Sph2 (to be discussed below in detail).

Bacterial sphingomyelinases act on eukaryotic cell membranes and lyse them by the enzymatic activity on sphingomyelin present in abundance in these membranes. If Sph2 is considered a true sphingomyelinase, it remains to be ascertained as to why these pathogens elaborate several sphingomyelinases. It is likely that the other sphingomyelinases play different roles. While SphH was demonstrated to lyse eukaryotic cells via its pore - forming property (Lee *et al.*, 2002), the mechanism of action and the roles of the other sphingomyelinases require further analysis. In other bacterial systems, sphingomyelinases have been demonstrated to exhibit additional roles. In *Helicobacter pylori*, the toxin VacA uses sphingomyelin as a receptor to enter the target cell (Gupta *et al.*, 2008). A novel role for sphingomyelinase has been described for the *S. aureus* β - toxin. It was implicated in biofilm formation as it covalently interacted with extracellular DNA, forming insoluble nucleoprotein complexes. Biofilm assembly occurred even when the two histidine residues responsible for catalytic activity were altered by mutation indicating that the residues involved in biofilm formation are distinct from the ones involved in catalysis (Huseby *et al.*, 2010).

Sph2 exhibits both hemolytic and sphingomyelinase activities. We demonstrated this with recombinant Sph2. The tendency of the latter to precipitate as insoluble inclusion bodies and the failure to re - solubilise the biologically active molecule has been a challenge for several researchers (Artiushin *et al.*, 2004; Carvalho *et al.*, 2010; Matsunaga *et al.*, 2007b). As we faced similar challenges despite alteration of the different conditions of growth and solubilisation, we chose to use whole cell extracts on the assumption that even small amounts of the soluble toxin would exhibit measurable biological activity as toxins are known to be potent at very low concentrations. In fact, the presence of low levels of rSph2 in the supernatant after sonication was proved by immunoblotting with Sph2 - specific antibodies. The hemolytic activity of Sph2 was clearly enhanced upon addition of Mg^{2+}. Considerable hemolytic (45%) activity in the absence of added Mg^{2+} was an anticipated observation as whole cell sonicates containing the released intracellular

Mg^{2+} promoted the hemolytic activity. The complete loss of hemolytic activity in the presence of the divalent metal ion chelator EDDA lends enough proof on the role of divalent metal ions on the biological activity of Sph2, the specificity of which was evident by the loss of the activity upon pre - incubation with anti - Sph2 antibodies. Further, we showed that Ca^{2+} inhibited the hemolytic activity. These observations differ from that of Zhang and his group (Zhang *et al.*, 2008) who reported that Mn^{2+} and Ca^{2+} promoted hemolytic activity. It may be argued that the hemolysis seen with the whole cell sonicates of *E. coli* could be due to the activity of the cryptic *E. coli* hemolysin SheA (Fernandez *et al.*, 1998). Inclusion of suitable controls however negates this argument; both the controls including *E. coli* clones carrying the empty vector and an unrelated mycobacterial gene showed negligible hemolysis. The negative effect of Ca^{2+} on the hemolytic activity further strengthens the effect due to rSph2 and not by SheA hemolysin, as the latter is unaffected by calcium chloride (Fernandez *et al.*, 1998). Finally, it may be mentioned that the source of the erythrocytes for the hemolytic assay is important. In this study, we used sheep erythrocytes as earlier studies (Kasarov, 1970; Russell, 1956) had shown that leptospiral sphingomyelinases were effective on sheep erythrocytes and were less effective on the human and rabbit erythrocytes. This can be attributed to the low levels of sphingomyelin (29%) in rabbit erythrocytes as against the 64% sphingomyelin in sheep RBC. Weak hemolytic activity was observed with Lk73.5 (Sph2) from a Pomona strain of *L. interrogans* using rabbit and horse RBC (Artiushin *et al.*, 2004) thereby clearly indicating that sheep RBC must be used for assaying leptospiral sphingomyelinases. No hemolysis was evident with rSph4, an anticipated finding due to the absence of the enzymatic domain in the molecule.

In addition to the hemolytic activity, Sph2 has been demonstrated to cleave sphingomyelin to yield ceramide and phosphocholine. The products were separated by thin layer chromatography. This was achieved with 300 µg / mL of the whole cell sonicate of *E. coli* expressing rSph2 that was markedly less than the 100 mg / mL concentration used in the study by Zhang *et al* (Zhang *et al.*, 2005). As the authors did not demonstrate the products and instead reported the decrease in the substrate sphingomyelin by HPLC, the high concentrations may have affected non - specific cleavage of sphingomyelin by the *E. coli* lipases.

There is increasing evidence that Sph2 is expressed *in vivo.* Anti - Sph2 antibodies were detected in serum from horses infected with *L. interrogans* serovar

Pomona (Artiushin *et al.*, 2004). Recently, it was reported that the serum from patients with leptospirosis showed anti - Sph2 antibodies and did not react with Sph1, Sph4, or SphH (Carvalho *et al.*, 2010). Moreover, *Leptospira* colonizing in the renal tubules of hamsters expressed SphH and Sph2 antigens owing to their possible role in mediating infection via colonization. Anti - Sph2 and anti - SphH antisera reacted with renal tubular epithelium of laboratory hamsters infected with *L. interrogans* (Carvalho *et al.*, 2010). The expression of these proteins *in vivo* and only by pathogenic leptospires clearly point to sphingomyelinases as virulence factors.

We thus wished to study the factors likely to be encountered by the pathogen within the mammalian host. The ability of leptospires to cause disease is due to the adaptation of the pathogen to various environmental cues outside and its ability to survive in the bloodstream and tissue fluids within the mammalian host. It is well known that pathogenic bacteria respond to changes in their immediate environment and there is a wealth of information on the effect of external factors on the expression of bacterial proteins including toxins (Boyle & Finlay, 2003; Konkel & Tilly, 2000; Litwin & Calderwood, 1993; Mekalanos, 1992; Schoolnik, 2002). Limitation of iron, for example up - regulates the expression of several bacterial virulence determinants like diphtheria toxin, aerobactin, hemolysins and others (Sritharan, 2000; Sritharan, 2006). When a pathogen enters a mammalian host, the latter limits iron to the invading pathogen by a process called ‘nutritional immunity’ (Kochan, 1977). This involves lowering the circulating free iron levels by decreasing the absorption from the intestine and by transferring the serum iron to proteins like transferrin, lactoferrin and the storage protein ferritin. It is therefore likely that these pathogens have adapted successfully to low iron conditions by elaborating novel iron acquisition machinery. As mentioned earlier (Review, Chapter 1), pathogenic leptospires have adapted by the elaboration of the direct acquisition machinery by expressing the hemin - binding protein HbpA. Intracellular iron levels control not only the expression of the components of the iron acquisition machinery but also the virulence determinants as seen in several bacterial systems; examples include diphtheria toxin by *Corynebacterium diphtheriae* , hemolysin by *E. coli* , Shiga toxin by *Shigella dysenteriae* , vero - cytotoxin by *E. coli* and exotoxin A by *Pseudomonas aeruginosa* (Litwin & Calderwood, 1993; Sritharan, 2000). We wished to see if one or more of the sphingomyelinases were up - regulated by iron limitation. We studied not only iron limitation but also the simultaneous shift in temperature, as HbpA

expression was up - regulated under these conditions. We identified the 42 kDa protein, previously reported from our lab as SphH. This protein, released via OMVs by low iron serovar Lai was reported as sphingomyelinase (Velineni *et al.*, 2009); however, it was not known which of the sphingomyelinases it represented because it was detected with an antibody that recognised all the sphingomyelinases. The detection of a smaller protein, instead of the 63 kDa full length SphH seen in the whole cell lysates clearly indicates that the toxin is cleaved; the functionality of the 42 kDa cleaved product however remains to be ascertained. The processing of the protein has been indicated in earlier studies but however was not identified as a cleavage product of SphH (del Real *et al.*, 1989; Segers *et al.*, 1990). Similar post - translational processing of proteins have been reported previously for the immunoglobulin A protease from *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987), serine protease from *Serratia marcescens* (Miyazaki *et al.*, 1989), and activation of aerolysin from *Aeromonas hydrophila* (Howard & Buckley, 1985). The iron - regulated expression of a leptospiral protease that acts on the full length SphH warrants further studies. SphH expression upon iron limitation is an interesting observation as SphH, a pore - forming hemolysin (Lee *et al.*, 2002) could possibly account for the hemorrhage and cell damage seen in the severe form of the disease.

In addition to acquisition of iron discussed above, other environmental conditions that influence the expression of leptospiral proteins include osmolarity, serum, temperature and oxidative stress. The pathogen faces a shift in osmolarity upon entering the mammalian host. Rat and human tissues have an osmolarity of ~ 300 mOsM (Kratz *et al.*, 2004; Suckow *et al.*, 2005) as compared to the lower levels in non – host environments. Leptospires, exposed to physiological osmolarity conditions (~ 300 mOsM) showed increased expression of Sph2 when compared to EMJH medium that was ~ 67 mOsM (Matsunaga *et al.*, 2005). In their study on the transcriptional profiling by microarray analysis, they observed that several proteins, altered in many bacterial systems were found to be unaffected. None of the osmotic - stress induced genes like *kdpA, kdpA* and *kdpA* (associated with transport of potassium), *betB* (NAD^+ - dependent aldehyde dehydrogenase), *betA* (LA3998 / choline dehydrogenase), *betA* (LA3999 / choline dehydrogenase), *treC* (glcosidase), *gyrA* (DNA gyrase subunit A), *gyrB* (DNA gyrase subunit B), *topA* (DNA topoisomerase), *mscS* (LA0992 / mechano - sensitive ion channel), *mscS* (LB087 / mechano - sensitive ion channel) and general stress genes like *xthA* (exo -

deoxyribonuclease III), *dps* (DNA - binding ferritin - like protein), *katE* (catalase), *uspA* (universal stress protein UspA), *ftsQ* (cell division protein), *ftsA* (cell division protein, actin - like ATPase), *ftsZ* (cell division GTPase), *acs* (LA4254 / acetyl - CoA synthetase), *acs* (LA1173 / acetyl - CoA synthetase), *radA* (ATP - dependent serine protease) were induced in *L. interrogans* serovar Copenhageni upon addition of salt (Matsunaga *et al.*, 2005). While 6% of the total genes were affected by the presence of salt, 124 among them were up – regulated and 94 genes were down – regulated. *sph2* (LA1029) was among the up - regulated genes in addition to LA1400, LA3781, LA3778 (*ligB*), LA1691 (*lipL53*), LA2473 (*crp*) and LA2241 (3.6 fold). Among these, sph2, *lipL53* and *crp* (encoding cAMP regulatory protein) genes were induced both by salt and temperature. Remarkably, almost half of *L. interrogans* genes that were down - regulated by salt were pseudogenes or absent in the obligate parasite *L. borgpetersenii*. The effect of serum on leptospiral protein expression was studied by Patarakul & Adler (2010). While none of the sphingomyelinase genes were altered, 11 of 55 (20%) up - regulated genes are unique to *L. interrogans* and are not present in the genome of *L. biflexa*. With the exception of LigB, other known or potential virulence determinants that play a role in motility, chemotaxis, colonization or adhesin were not found to be up - regulated after exposure to serum. These include extracellular matrix binding proteins, enzymes capable of host cell membrane degradation such as sphingomyelinase, phosphatase, and hemolysin, as well as surface proteins previously shown to be expressed *in vivo*, including OmpL1, LipL41, LipL32, LipL21, LipL46, Loa22, and Lsa2.

In this study, we performed identical experiments (120 mM NaCl is equivalent to ~ 300 mOsM) and studied not only the expression of Sph2 but also the other sphingomyelinases, both at the transcript and protein levels. We studied the influence of osmolarity and serum, effected individually and in combination on the expression of sphingomyelinases. In this study also, we observed elevated Sph2 levels upon addition of 120 mM NaCl. This was reflected at the levels of mRNA transcripts and the protein. While both the serovars Pomona and Manilae showed significant up - regulation, it was interesting to note that Sph2 expression in the serovar Pomona was observed even in the absence of added salt as against the almost negligible levels in serovar Manilae. The Sph2 protein has an apparent molecular mass of 89 kDa protein in serovars Manilae, Lai and Copenhageni while it was 95 kDa in the serovar Pomona; they were larger than the calculated molecular

mass of 71.7 kDa in Manilae, Lai, Copenhageni and 73.5 kDa in Pomona indicating post – translational modifications. The serovar Pomona also secreted out the protein in addition to the 95 kDa protein, two proteins of 75 and 85 kDa molecular mass were detected in the spent growth medium. Another notable feature was the large number of bands in serovar Pomona in the whole cell lysate recognized by anti - Sph2 antibodies. While we are unable to explain this observation, they do not appear to be degradation products. These bands appear to represent processed forms of Sph2. Protein processing such as cleavage or post – translational modifications may be possible due to the varying sizes of bands. Further work is required to understand the presence of these up - regulated protein bands. Addition of serum had no effect on the expression of these proteins but a combination of salt and serum amplified the changes seen with a 184 – fold increase in *sph2* mRNA level that was also seen in the serovar Manilae. Serum must contain components that triggered Sph2. This must be heat - stable as heat inactivation of serum at 56°C for 30 min did not result in a decrease in Sph2 level. It is proposed that a hormone(s), growth factor(s), salt or other low molecular weight compound could be responsible for this.

Hemolytic and sphingomyelinase activities were also detected in the spent growth medium in cultures with added NaCl; serovar Pomona exhibited higher biological activity compared to Manilae. But serum addition abolished the activity. This non – specific inhibition of hemolytic activity by serum has been reported previously (Carvalho *et al.*, 2010; Yanagihara *et al.*, 1982).

Not all serovars appear to respond to external stimuli in the same manner. Serovar Pomona showed basal levels of Sph2 expression even in normal medium that was however negligible in Manilae, Copenhageni and Lai. Hence we compared the upstream region of the *sph2* gene in both serovars Pomona and Copenhageni. The *sph2* gene along with the respective up – stream and downstream regions presuming to contain the control elements regulating the expression of the gene were amplified from the genomes of the serovars Pomona and Copenhageni and transformed into the non - pathogenic *L. biflexa* via a shuttle vector. The high levels of the 95 kDa Sph2 protein in serovar Pomona clearly indicated that the upstream region possibly contained additional control elements. 5’RACE showed that the there was no difference in the transcriptional start site in both the serovars. The presence of an insertion sequence like - element in the upstream region in serovar Pomona led us to presume that this could possibly account for the expression observed in this

serovar. But a deletion of this region did not show any change in the expression of Sph2. Further experimental studies are required to prove differences in Sph2 expression. It was reported that Sph2 could not be detected in cell lysates of *L. interrogans* serovar Pomona type Kennewicki (Artiushin *et al.*, 2004). A comparison of the upstream region of the latter with the strain *L. interrogans* serovar Pomona strain LC82 - 25 used in this study did not reveal any changes in the upstream region. Whether this indicates the role of any trans - acting factor is a possibility and further work is required to understand the regulation of expression of Sph2.

SphH was elevated both upon addition of serum and oxidative stress. This effect was more pronounced in serovar Manilae with a moderate rise in serovar Pomona. As mentioned earlier, SphH was also up - regulated under iron - limiting conditions. Though this was demonstrated in serovar Lai, it is likely that the addition of serum perhaps results in iron limitation by virtue of the iron - chelating property of serum transferrin. Additionally, factors in serum could possibly influence the response of *Leptospira* to serum. The gene *hemO* (LIC20148 / LB186) encoding heme oxygenase was induced 2.47 fold in response to serum (Patarakul *et al.*, 2010) and by osmolarity (Matsunaga *et al.*, 2007a). Heme oxygenase, required for the utilisation of hemin acquired via the hemin - binding protein HbpA must be necessary for iron acquisition during iron limitation conditions. Indeed, HemO is required for disease pathogenesis in hamsters (Murray *et al.*, 2009b). It is interesting to note that a bacterioferritin - associated ferridoxin (LA4021), which may function in iron storage, was up – regulated under osmolarity conditions.

Further analysis is needed to understand the role of environmental factors on the other sphingomyelinases. While *sph1* transcripts were higher in the presence of salt and a mixture of salt and serum, *sph3* did not show marked change. Specific antibodies are needed to study their expression at the protein level. Though efforts were taken to raise peptide - specific antibodies, they were not found to be useful in differentiating the sphingomyelinases. There is a need to develop antibodies that can differentiate the different sphingomyelinases. Cloning and expression of the specific sphingomyelinases has met with difficulties due to the formation of inclusion bodies that necessitate the use of harsh detergents to solubilise the proteins making it biologically inactive. Also, the complex medium used for the growth of leptospires interferes with biological assays, with serum particularly interfering with the hemolytic assay.

SphH is secreted to the outside via outer membrane vesicles. This sphingomyelinase, Sph1 and Sph3 possess a cleavable amino - terminal signal peptide that is indicative of export out of the cytoplasm. SphB from *L. borgpetersenii* is also likely to be transported to the outside as it carries the signal peptide. Sph2 and SphA lacks the signal peptide and it is likely that they are transported by the TolC protein that we reported earlier (Velineni *et al.*, 2009). This TolC protein, a 63 kDa protein encoded by LA0957 was immunoprecipitated from an outer membrane preparation of *L. interrogans* with antiserum raised against the enzymatic domain of Sph3, indicating that one or more sphingomyelinase is secreted via this TolC - based Type I secretory pathway (Jenewein *et al.*, 2009). Another TolC homolog (LA3927) has also been implicated in the secretion of sphingomyelinase (Louvel *et al.*, 2006).

So, how do sphingomyelinases offer advantage to the invading pathogen in establishing itself within the new host? Sphingomyelinases must certainly play a role in nutrient acquisition, as also suggested in other reports (Bulach *et al.*, 2006a). These proteins, by lysing the host cells release the essential nutrients including iron. Heme released from damaged erythrocytes is a potential source of iron for *Leptospira* during infection. It is highly likely that the intracellular iron levels regulate both SphH and HbpA expression, the former serving to lyse the cells and the latter facilitating the uptake of iron from the released heme. While the host cell membrane serves as a rich source of fatty acids for the growth of leptospires that utilises lipids as a source of energy (Henneberry & Cox, 1970), the cleavage product ceramide cannot possibly be utilised as ceramidase is not encoded in the leptospiral genome; however the phospholipases expressed by these pathogens release fatty acid from the glycerophospholipids abundantly found in the host (Kasarov, 1970). In other bacteria, the choline released from host membranes by sphingomyelinase activity is converted to glycine betaine that serves to function as an osmoprotectant under osmotic stress (Shortridge *et al.*, 1992). However, *L. interrogans* lacks the *betT* gene that encodes the choline transporter. As *betA* gene encoding BetA that catalyzes the first step in the conversion of choline to glycine betaine is down – regulated, there is little role of sphingomyelinase in this pathway. Sphingomyelinases may play a role in immune evasion. Though *Leptospira* is primarily an extracellular pathogen, it is able to escape from the phagosome of cultured mouse macrophages (Toma *et al.*, 2011) and this escape, as observed in *Listeria* species requires the cooperation of lipases and sphingomyelinases (Gonzalez-Zorn *et al.*, 1999; Schnupf & Portnoy, 2007).

There are varied reports on the cytotoxic effect of Sph2. Recombinant Sph2 (Lk73.5) from a Pomona strain of *L. interrogans* was demonstrated to be cytotoxic to equine pulmonary endothelial cells (Artiushin *et al.*, 2004), while another study showed that disruption of the endothelial cell layer integrity by *L. interrogans* crossing the monolayer did not affect the viability of the cells (Martinez-Lopez *et al.*, 2010). The true relevance of sphingomyelinase in leptospiral pathogenesis may lie in sub - lytic effects that do not damage the host cell membrane. For example, alteration of vascular permeability is caused in part by generation of ceramide by acid sphingomyelinase (Goggel *et al.*, 2004), which may explain the ability of sphingomyelinase - producing *Leptospira* to cross the endothelial layer without cytolytic effects. Excessive ceramide production induced by leptospiral sphingomyelinase could also explain the pulmonary edema observed in some cases of severe leptospirosis. Alterations of sphingolipid homeostasis and lipid rafts have also been linked to altered renal function (Zager, 2000). The activity of the renal Na^+ / H^+ NH_3 transporter, whose levels are diminished in the proximal tubule of severe leptospirosis patients, depends on formation of lipid rafts (Araujo *et al.*, 2010; Murtazina *et al.*, 2006). The sphingomyelinases may play a role in biofilm formation as demonstrated in *S. aureus*, in which the β - toxin played a non - catalytic role in biofilm formation (Huseby *et al.*, 2010). Pathogenic leptospires have been shown to form biofilms *in vitro* (Ristow *et al.*, 2008), and biofilm formation may be essential for long term leptospiral survival in the renal tubules of the reservoir host. Therefore, leptospiral sphingomyelinases need to be explored further to understand the role of these molecules in the survival and perpetuation of these pathogens.

4.1. Conclusions and scope for future work

In conclusion, we have demonstrated some salient features of leptospiral sphingomyelinases, virulence factors expressed by pathogenic leptospires. They include the structural similarities and the unique features of these molecules, bioinformatic and experimental evidence to prove that Sph2 is a true sphingomyelinase and the factors influencing the expression of these molecules that is of clinical relevance. This work, in addition to providing considerable information has opened up avenues for future work. The mechanism of regulation of expression of Sph2 needs further work as we observed that the insertion sequence in the upstream region had no role in the higher levels of expression in the serovar

Pomona. It would be interesting to identify other factors, possibly transcriptional factors that possibly regulate the expression of Sph2. In addition, the roles of the other sphingomyelinases Sph1 and Sph3 need further analysis.

CHAPTER 5

SUMMARY

5. Summary

Sphingomyelinases are expressed by pathogenic *Leptospira* spp. There are five sphingomyelinases in the genome of *L. interrogans* serovar Lai, the roles of which remains to be elucidated. Leptospiral sphingomyelinases are unique and form a separate cluster as compared to other bacterial sphingomyelinases due to the presence of N – terminal and C – terminal extensions. All of them with the exception of Sph4 possess the exo - endo phosphatase domain; the latter showed similar three – dimensional folding as other bacterial sphingomyelinases. The Mg^{2+}- binding Glu53 and the two His residues (His151 and His28) are present exclusively in Sph2 and not in other leptospiral sphingomyelinases. Sph2 exhibited Mg^{2+} - dependent hemolytic and sphingomyelinase activities. The β – hairpin loop involved in membrane interaction is missing in all leptospiral sphingomyelinases but the latter possessed several surface exposed aromatic amino acids that can mediate membrane interaction. Expression of Sph2 was elevated upon addition of sodium chloride and rat serum. This probably reflects the adaptation of the pathogen to the immediate environment inside the renal tubules where the organism is presented with conditions of high osmolarity. SphH, unaffected by salt and serum is up - regulated by iron limitation. Limitation of iron by the mammalian host called 'nutritional immunity' is known in leptospirosis. As iron regulates expression of bacterial virulence determinants at the molecular level, the regulation of expression of this pore - forming sphingomyelinase is an interesting observation. Differential expression of Sph2 was seen in different serovars with notably high levels in the serovar Pomona as evident by the high transcript & protein levels. The molecular size of Sph2 is greater than the expected size possibly due to post – translational modifications. The spent growth medium retained the biological (hemolytic and sphingomyelinase) activities due to secreted form of the sphingomyelinase. The serovar Pomona possessed unique 330 nucleotide *IS* – like element in the up – stream region of *sph2,* which has no effect on Sph2 expression. It is possible that serovar Pomona has an activator that is missing in Copenhageni, Lai and Manilae or Pomona lacks a repressor which is present in other serovars. However the molecular basis for this observation requires further studies. All these observations indicate that pathogenic *Leptospira* regulates the expression of potential virulence factors like sphingomyelinases according to the environmental signals.

CHAPTER 6

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