Analysis of Genes Encoding Outer Membrane Protein of *Leptospira interrogans* and Its Modulation Due to Host Factors: An Approach for Understanding Host-Pathogen Crosstalk

A thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

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February, 2019

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by

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DEDICATION

I dedicate this work to my grandparents and parents for their selfless sacrifices and belief in my abilities. They are my inspiration and pillars of strength.

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

DECLARATION

I hereby declare that the matter embodied in this thesis entitled **"Analysis of Genes Encoding Outer Membrane Protein of *Leptospira interrogans* and Its Modulation Due to Host Factors: An Approach for Understanding Host-Pathogen Crosstalk"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India under the supervision of Dr. Manish Kumar.

In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work of other investigators are referred.

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CERTIFICATE

This is to certify that work described in the thesis entitled "**Analysis of Genes Encoding Outer Membrane Protein of *Leptospira interrogans* and Its Modulation Due to Host Factors: An Approach for Understanding Host-Pathogen Crosstalk**" by Ms. Karukriti Kaushik Ghosh (Roll No: 136106014), submitted to the Indian Institute of Technology Guwahati, India for the award of the degree of Doctor of Philosophy, is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Dr. Manish Kumar

(Thesis supervisor)

ACKNOWLEDGMENTS

I would like to express my heartfelt gratitude and sincere regards to Dr. Manish Kumar, for providing me the opportunity to work in his laboratory. I feel privileged to be the first graduate student of his lab and will cherish the memories of working with him in the lab during the initial years of my research. I would also like to thank him for his valuable guidance, suggestions and his patience throughout the duration of this study. His organized way of working has always inspired me and I hope to look upon him as a guide throughout my life.

I would like to convey my sincere thanks to the esteemed members of my doctoral committee, Dr. Anil Mukund Limaye, Dr. Sachin Kumar, Dr. Debasis Manna, and Prof. Vikash Kumar Dubey, for their valuable inputs and timely suggestions during the course of this study.

I would like to thank Dr. Vinayagamurthy Balamurugan for providing us with the precious serum samples which helped us a lot in our studies. My thanks are also due to RMRC (ICMR), Port Blair for providing us with the *Leptospira* cultures to pursue our studies. I am indebted to Dr. Rajeev Kumar Sharma and the Department of Microbiology, College of Veterinary Science, Guwahati for their help in the generation of the polyclonal antibodies. I would also like to thank Dr. David Haake (Division of Infectious Diseases, Los Angeles, California, USA) for his generous gift of the antibodies used in this study. I also acknowledge the DBT program support facility available in the Department of Biosciences and Bioengineering, IIT Guwahati, for generating the Circular dichroism data.

I owe my heartfelt gratitude to Prof. Latha Rangan, Head of the Department and former Heads of the Department, Prof. K. Pakshirajan and Prof. V. V. Dasu, for providing me the departmental facilities to carry out my research work. My thanks are also due to the technical and non-technical staffs of my department for their warm and generous behavior towards me throughout my study tenure.

Among the many people to whom I owe my gratitude, I would like to record my heartfelt thanks to Aman Prakash for his helping hand during my research work and thesis writing. I also record my sincere thanks to Prateek Shrivastav, Gary Fernandes, Saurabh Bora, Rishabh Singh, Bhuvan Dixit, Vineet Anand, Anusua Dhara, Md. Saddam Hussain, Juhi Rajesh Rathod, and all the past and present lab members for their constant help and support during the entire research period.

I am also thankful to my friends Reshmi Das, Neha Arora, Amrendra Kumar, Rakesh Kumar, Ashish Prabhu A. and Ankit Gangrade for making the past five years of stay a pleasant and joyful experience.

I shall always be indebted to my family members for their love, continuous support and innumerable sacrifices. I would like to thank my parents for their constant motivation. I would also like to express my gratitude to my grandparents, whom I lost during the course of my study, for believing in me from childhood.

Last but not the least; I am grateful to the Almighty, who gave me the courage and support to complete this study.

Karukriti Kaushik Ghosh

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

SR NO.	ABBREVIATION	FULL FORM
1	5 FU	5 Fluorouracil
2	bp	Base pair
3	BSA	Bovine serum albumin
4	CSF	Cerebrospinal fluid
5	DNA	Deoxyribonucleic acid
6	Epi	Epinephrine
7	ECM	Extracellular matrix
8	EDTA	Ethylenediaminetetraacetic acid
9	ELISA	Enzyme-linked immunosorbent assay
10	EMJH medium	Ellinghausen McCullough/Johnson-Harris medium
11	g	Gram
12	His Tag	Histidine tag
13	ICMR	Indian Council of Medical Research
14	IgG	Immunoglobulin G
15	IgM	Immunoglobulin M
16	IPTG	Isopropyl β-D-1-thiogalactopyranoside
17	kb	Kilobase pairs
18	kDa	Kilodalton
19	L	Litre
20	LB	Luria Bertani
21	*LIC*	*Leptospira interrogans* serovar Copenhageni
22	LPS	Lipopolysaccharide
23	µg	Microgram
24	µM	Micromolar
25	MAT	Microscopic agglutination test
26	mg	Milligram
27	mm	Millimeter
28	mM	Millimolar
29	NE	Norepinephrine
30	ng	Nanogram
31	nm	Nanometer
32	nM	Nanomolar
33	OD	Optical density
34	OM	Outer membrane
35	OMP	Outer membrane protein
36	ORF	Open reading frame
37	PO	Propranolol
38	PAGE	Polyacrylamide gel electrophoresis

39	PBS	Phosphate buffered saline
40	PBST	Phosphate buffered saline-Tween 20
41	PCR	Polymerase chain reaction
42	pmol	Picomoles
43	qRT-PCR	Quantitative Reverse transcription PCR
44	RNA	Ribonucleic acid
45	rpm	Rotation per minute
46	RT PCR	Reverse transcription PCR
47	SDS	Sodium dodecyl sulfate
48	SGB	Spirochete genome browser
49	TAE	Tris-acetate EDTA
50	TBST	Tris-buffered saline-Tween 20
51	TM	Transmembrane
52	TMB	3,3',5,5'-Tetramethylbenzidine
53	UV	Ultraviolet

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Pathogens including *Leptospira interrogans* have evolved to sense different host factors such as temperature, catecholamines, osmolarity, oxidative stress etc. in the human or animal body upon infection. This study is aimed at understanding the transcriptional modulation of genes encoding membrane proteins in *Leptospira interrogans* on exposure to host factors under *in vitro* condition. Based on bioinformatics analysis, a total of 18 genes encoding hypothetical membrane proteins of *Leptospira* were selected in this study. The reliability of selection criteria of these 18 genes was bolstered by including 26 additional leptospiral genes with known subcellular location and functions. The genes of pathogenic *Leptospira* responding to one or more host factor(s) were then analyzed using a molecular approach to speculate the role of these proteins in the pathogenesis of leptospirosis disease in the host. We initially characterized the *L. interrogans* response to host factor catecholamines (epinephrine or norepinephrine) by measuring the change in transcription of these genes (n=44) using the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique. A total of 7 genes including *LA0616*, *LA3961*, *LB186*, *LB047*, *LA3307*, *LB191*, and *LA3263* were found to be differentially transcribed in the presence of catecholamines and the effect of which was restored to the basal level (control) in the presence of its antagonist. The effect of other host factors including oxidative stress and temperature were studied on these 7 catecholamine modulated genes. Notably, all these 7 genes were also getting differentially transcribed in the presence of physiological oxidative stress condition. However, among these 7 genes, transcripts of *LA0616*, *LA3961*, *LB191* and *LB047* were found to be differentially modulated on a shift in temperature from 29ºC to 37ºC in the *Leptospira* grown under *in vitro* condition. Among the genes modulated in response to

host factors, *LB047* was found to be downregulated in the presence of epinephrine, oxidative stress and elevated temperature of 37ºC. Subcellular localization and immunoassay demonstrate LIC20035 (LB047 ortholog in *L. interrogans* serovar Copenhageni) to be a surface exposed adhesin which binds to host extracellular matrix components and is recognized by the host during infection. Out of the selected genes (n=18) encoding hypothetical membrane proteins, LIC13341 (LA4185 ortholog in *L. interrogans* serovar Copenhageni) gene transcript was not detected by RT-PCR or qRT-PCR from the total RNA obtained from *Leptospira* cultured in presence or absence of host factors under *in vitro* condition. This suggests that there can be other unknown host factor(s) or transcription factor(s) which regulate(s) *LIC13341* gene transcription. Attenuation of many virulent genes of *Leptospira* on frequent subculturing under *in vitro* culture condition is previously reported. Interestingly, *LIC13341* transcript expression was detected in low passage *Leptospira* which explains the attenuation of *LIC13341* in *Leptospira* on high passaging under *in vitro* condition. The protease accessibility assay and phase-separation experiment of *Leptospira* proteome suggested LIC13341 to be a surface exposed outer membrane protein. The r-LIC13341 can bind to a wide spectrum of host extracellular matrices (ECM), with a higher preference for laminin and hyaluronic acid. In addition, immunoassay with humans or bovine sera tested positive for leptospirosis recognized r-LIC13341 suggesting that *Leptospira* expresses LIC13341 in the various host(s) during infection.

The protein LIC11966 (LA1939 ortholog in *L. interrogans* serovar Copenhageni), from the selected genes encoding hypothetical membrane proteins (n=18), is annotated as putative lipoprotein and was found to be downregulated at a physiological osmolarity of the host. In this study, the protein LIC11966 was found to be a surface exposed outer membrane

lipoprotein which has the ability to bind to a diverse group of host ECM and with higher preference to fibrinogen. The r-LIC11966 protein can be serologically detected in diverse hosts infected with *Leptospira*. Among the selected previously characterized leptospiral proteins (n=26), an outer membrane efflux protein LIC12693 (ortholog of LA0957 in *L. interrogans* serovar Copenhageni) was previously reported to be downregulated in mammalian host-adapted *Leptospira*. In this study, LIC12693 was found predominantly in the detergent-insoluble phase during Triton X-114 phase-separation experiment, directing its possibility to be localized as a transmembrane protein. In addition, LIC12693 showed its potential to bind to host laminin and fibronectin component of ECM. Taken together, this study demonstrates that the *L. interrogans* surface proteins have the ability to respond to various host factors under *in vitro* condition and plays a critical role in interacting with host ECM components. We have characterized four novel outer membrane proteins of *L. interrogans* namely LIC20035, LIC13341, LIC11966, and LIC12693 which may serve as adhesins during host tissue dissemination and can be utilized as diagnostic marker for leptospirosis across a wide range of host.

Chapter 1

Introduction and literature review

CHAPTER 1

Introduction and literature review

Leptospirosis is one of the most widespread zoonotic diseases in the world caused by *Leptospira interrogans* 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 favor environmental survival of the pathogen. Leptospirosis is primarily an occupational hazard affecting those who work in close contact with animals. Recent studies have showed that leptospirosis is nowadays more common in urban slum communities (Binder and Mermel, 1998; Maciel et al., 2008). The disease is under-diagnosed due to lack of awareness and poor diagnosis; resulting in a major burden on populations from developing countries and tropical regions (McBride et al., 2005). The severity of leptospirosis ranges from a mild influenza-like illness to a severe form called the Weil's disease which is characterized by the involvement of the liver, kidney, and lungs, either alone or in combination (Levett, 2001; Plank and 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 (Plank and Dean, 2000). In recent decades, with the advent of the genome sequence of pathogenic leptospires, considerable efforts have been put forth in identifying novel diagnostic markers, unraveling 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, further efforts are needed in developing diagnostic kits for the precise detection of

Leptospira and mining diagnostic and vaccine candidates from the large pool of hypothetical conserved proteins of *Leptospira*.

1.1 Historical aspects

The history of leptospirosis began when Adolf Weil described about the severe form of leptospirosis which was subsequently named as Weil's disease (Weil, 1886). This disease is also called as "rice field jaundice" in China, whereas it is called as "autumn fever" or "seven-day fever" in Japan, "cane-cutter's disease', "swineherd disease' and "mud fever" in Europe and Australia (Alston et al., 1958; Kitamura and Hara, 1918; van Thiel, 1948). *Leptospira* was first observed in the kidney tissues of a leptospirosis victim who was thought of having died of yellow fever (Stimson, 1907). The first isolation of *Leptospira* was done in Japan by Inada in which he was able to reproduce leptospirosis in the hamsters using the blood of Weil's disease patients (Inada et al., 1916). Inada and his colleagues then subsequently succeeded in the propagation of leptospires *in vitro* in a medium made from emulsified guinea-pig kidney and showed a preference in growth at 25ºC. This group was also the first to conduct the vaccination studies. Finally, Inada and his colleagues demonstrated immune lysis of leptospires by patient serum within the guinea pig peritoneal cavity. The role of the rat as renal carriers of *Leptospira* followed within a year and hence this disease was also termed as rat fever (Ido et al., 1917). Noguchi created a new genus; *Leptospira* in 1918, for this organism based on its morphology and included it in the order Spirochaetales, along with *Borrelia* and *Treponema* (Noguchi, 1918). Other potential sources of human infection, i.e. domesticated or wild animals, livestock etc. were documented many years later (Alston et al., 1958).

1.2 Leptospirosis

Leptospirosis is an infectious disease of global importance. Human infection can occur either through direct contact with infected animals or, much more commonly through indirect

contact with water or soil contaminated by the urine of infected rodents or animals. In contrast, leptospires can survive for long periods in the renal tubules of infected animals without causing illness. The brown rat (*Rattus norvegicus*) is the primary source of human infections. Most human infections occur in young adult men and children that result from occupational or environmental exposure (Faine and Organization, 1982).

1.2.1 Epidemiology

Leptospirosis outbreaks have been reported globally including United States (Katz et al., 2011), Korea (Chang et al., 1989), Nicaragua (Zaki et al., 1996), El Salvador and Brazil (Ko et al., 1999), India (Vijayachari et al., 2008) and Mexico (Vado-Solis et al., 2002). The incidence of human infections is rampant in tropical regions with high rainfall where the human population gets exposed to water contaminated with the urine of infected animals (Everard and Everard, 1993). Populations living in developing countries with poor sanitary conditions have high chances of getting infected through contaminated water or soil. In India, leptospirosis poses a significant problem in the low lying areas which are highly populated and face excessive water-logging or floods during monsoon (WHO, 2007). Several occupational risk groups like farmers, sewage workers, butchers, etc. are at a high risk of contracting the disease. Recreational activities such as swimming, sailing, water sports, etc. are also considered as risk factors for leptospirosis (Kumar, 2012). The different states that are endemic for leptospirosis in India include Andaman and Nicobar Islands, Kerala, Gujarat, Tamil Nadu, Maharashtra, and Karnataka. Some sporadic cases have also been reported from Goa, Andhra Pradesh, Assam and Odisha (WHO, 2007). This disease is most prevalent in the coastal areas. A study in India showed that leptospirosis accounts for about 12.7% of cases of acute febrile illness attending hospitals (Sehgal et al., 2003). Karnataka and southern part

of Gujarat revealed 130 deaths within a period of two months due to leptospirosis in 2011. In October 2012, 16 deaths were reported from Gujarat. The Brihanmumbai Municipal Corporation (BMC) had reported 15 leptospirosis fatalities in Mumbai within 10 days in July 2015 (Akhilanand, 2016). After the massive Chennai floods in 2015, 1204 cases were reported (Akhilanand, 2016). In 2015, 43 people died of leptospirosis and in the later years, the number of deaths were found to be 35 in 2016 and 80 in 2017 in Kerala (James et al., 2018). From the month of January till July 2018, 28 deaths were reported due to leptospirosis in Kerala (James et al., 2018). In September 2018, there was an outbreak of human leptospirosis in Kerala post floods that resulted into the loss of approximately 70 lives (James et al., 2018). The predominant *Leptospira* serovars causing leptospirosis in India include Copenhageni, Autumnalis, Canicola, Pyrogenes, Grippotyphosa, Australis, Javanica, Sejroe, Louisiana, Valbuzzi and Pomona (WHO, 2007). The estimated annual morbidity of leptospirosis globally has been shown in Fig. 1.1.

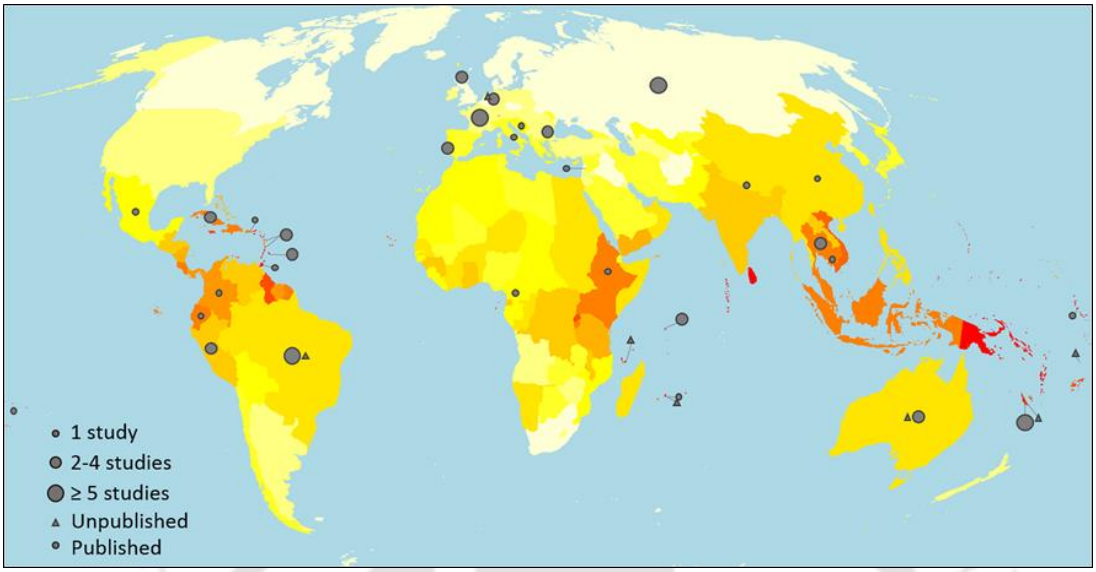

Figure 1.1. Estimated annual morbidity of leptospirosis by country or territory. Annual disease incidence is represented as a color gradient from white (0–3), yellow (7–10), orange (20–25) to red (over 100), in cases per 100,000 population. The symbols used in the map, circles, and triangles indicate the countries of origin for published and grey literature quality-assured studies, respectively (Costa et al., 2015).

1.2.2 Modes of transmission of *Leptospira*

Humans are accidental hosts to leptospirosis whereas wild and domestic animals serve as reservoir hosts with rodents playing a major role in disease transmission. The reservoir hosts remain symptom-free and shed leptospires via urine into the surrounding environment (Faine et al., 1999a). Humans get infected upon exposure to infected reservoir hosts, either directly or indirectly (Fig. 1.2). 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 shed on dairy farms, abattoir workers, butchers, hunters, and animal handlers (Tangkanakul et al., 2005). Indirect contact is more common and is responsible for disease following exposure to the 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 leptospirosis outbreaks (Sejvar et al., 2003).

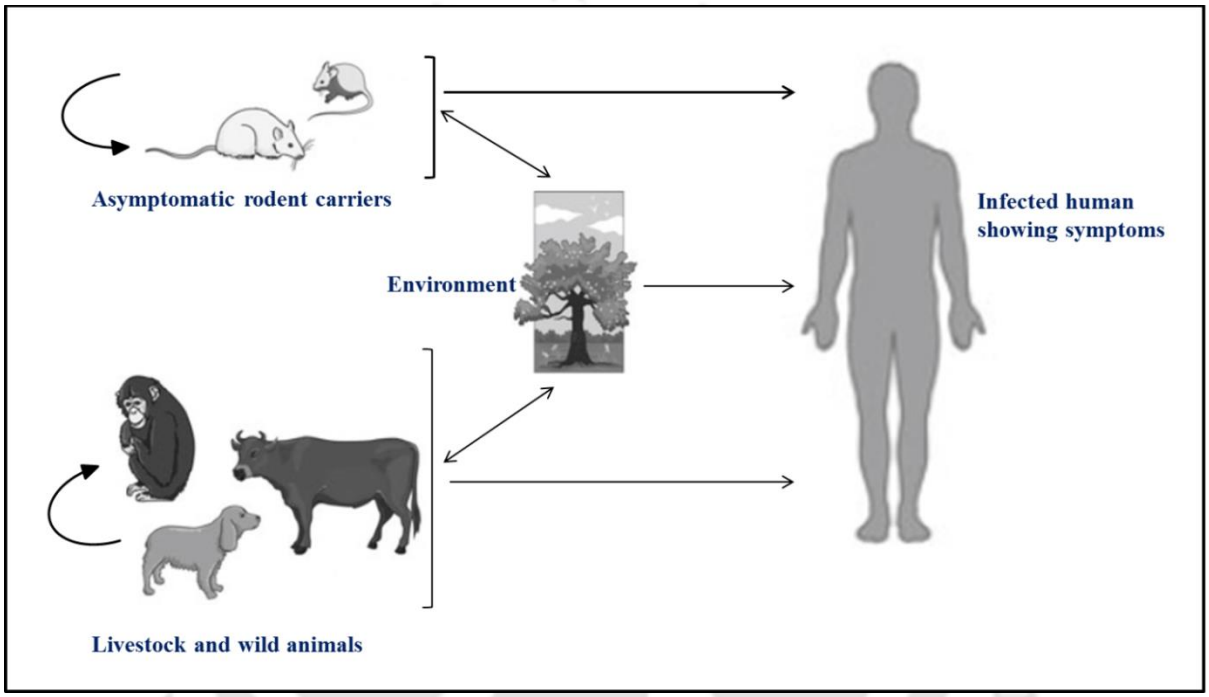

Figure 1.2. Leptospirosis transmission cycle. Leptospires are maintained in the environment by the rodents which are the main asymptomatic carriers. In these rodents, the infection is chronic and asymptomatic. Livestock and wild animals can either be asymptomatic or susceptible depending on the infecting serogroup and the health condition of the host. In these animals, the infection produces a range of clinical manifestations like miscarriages and/or uveitis. Leptospirosis is transmitted to humans by direct contact with infected animals or by exposure to environmental water or soil that is contaminated with their urine. *Leptospira* penetrate the skin and mucous membrane through cuts or wounds, enter the bloodstream and spread throughout the body. This infection causes an acute febrile illness in the early phase followed by severe multi-organ manifestations such as uveitis, meningitis, myocarditis, pulmonary hemorrhage, hepatic dysfunction, and renal dysfunction during the late phase. Humans are accidental hosts and do not shed enough leptospires to act as reservoirs for leptospirosis transmission (Ko et al., 2009).

1.2.3 Pathogenesis of *Leptospira*

Leptospires can enter the susceptible host via small cuts on the integument, conjunctiva, mucous membrane or genital tract. This requires chemotaxis mechanisms for adhesion. They may settle in the convoluted tubules of the kidneys can be shed in the urine for a period of a few weeks to several months and occasionally even longer. After the number of leptospires in the blood and tissues reaches to a critical level, lesions are seen due to the action of undefined leptospiral toxins or toxic cellular components and consequently, symptoms appear. Endotoxin activity has been reported in several serovars of *Leptospira*. Hemolysins of *Leptospira* have been proposed to be phospholipases which act on host erythrocytes (Narayanavari et al., 2012; Thompson and Manktelow, 1986) and other cell membranes containing phospholipids leading to their cytolysis (Lee et al., 2002). The primary symptoms include damage to the endothelium of small blood vessels leading to localized ischemia in organs, resulting in renal tubular necrosis, hepatocellular-pulmonary damage, meningitis, myositis, and placentitis. The incubation period depends on the infective dose, the growth rate of organisms, their toxicity, and immunity.

Until recently, the molecular basis for virulence remained unknown due to the absence of genetic tools for the manipulation of *Leptospira*. The recent availability of genome sequences from pathogenic and saprophytic *Leptospira* spp. (Bulach et al., 2006) coupled with the recent development of mutagenesis systems (Bourhy et al., 2005) has allowed for a more detailed and genetically defined investigation of cellular and molecular pathogenic mechanisms in leptospirosis. The humoral immune response appears in the first week of *Leptospira* infection which activates the process of phagocytosis by neutrophils and macrophages. Complement activation is also involved in lysis of the leptospires. In

susceptible hosts such as humans, the systemic infection can produce severe multi-organ manifestations. Initial symptoms, which may include chills, fever, headache (severe and persistent), diarrhea, or a rash, myalgia, malaise, prostration, retro-orbital pain, conjunctival suffusion, muscle tenderness and lung involvement, appear quite abruptly after an incubation period of about 10 days (Haraji et al., 2011). Cases that also have other symptoms such as meningitis, hemorrhage into the skin and mucous membranes, jaundice, hepatorenal failure, and myocarditis may be misdiagnosed (Daoud et al., 2007).

1.2.4 Host factors implicated in the pathogenesis of the bacteria

Host specificity is defined by the ability of a microbial pathogen to infect a host (Kirzinger and Stavrinides, 2012). While bacteria like *Borrelia borgdorferi* and *Yersinia pestis* can infect a wide range of hosts, certain bacteria such as *Haemophilus influenzae, Neisseria gonorrhoeae, Treponema pallidum etc.* have strict host selectivity for humans as obligate human pathogens (Pan et al., 2014). Understanding the genetic and molecular basis of host specificity in pathogenic bacteria is important for understanding pathogenic mechanisms, developing better animal models and designing new strategies and therapeutics for the control of microbial diseases. The host specificity of bacterial pathogens is determined by multiple molecular interactions between the pathogens and their hosts (Pan et al., 2014). The host specificity of viruses is predominantly defined by the interactions of viral proteins with their cognate cellular receptors (Medina and García-Sastre, 2011). However, the molecular mechanisms of host specificity for bacterial pathogens are much less understood (Pan et al., 2014). For pathogenic *Neisseria*, a number of surface exposed proteins appear to be associated with the human specificity of this bacterium. In the context of their host, several human proteins have also been implicated as host specificity determinants (Pan et al., 2014).

For *Streptococcus pneumoniae*, the molecular mechanisms behind the strict host specificity are unclear, however, several human-specific pathogen-host interactions have been recently showed using biochemical approaches (Pan et al., 2014). The major factors that affect the host specificity of *Salmonella* serovars include differences in the bacteria (e.g., the ability to survive host immunity, to grow in a given host environment, and to transmit to other individuals of the same host species) and host environments (e.g., pH, temperature, immune recognition and response, and the microbiota of a given host) (Foley et al., 2011; Torres et al., 2006). In the case of *E. coli*, the host specificity can be influenced by the molecular interactions of bacterial surface-exposed proteins with host cellular receptors (Babai et al., 2000; Ron, 2006).

Host factor refers to the traits of an individual person or animal that makes them susceptible to any disease, especially in comparison to other individuals. Host factors generally include temperature, pH, osmolarity, hormones, oxidative stress, serum etc. (Fig. 1.3). The review of these host factors studied till date has been assembled to provide a clear picture of its importance on bacterial pathogenesis.

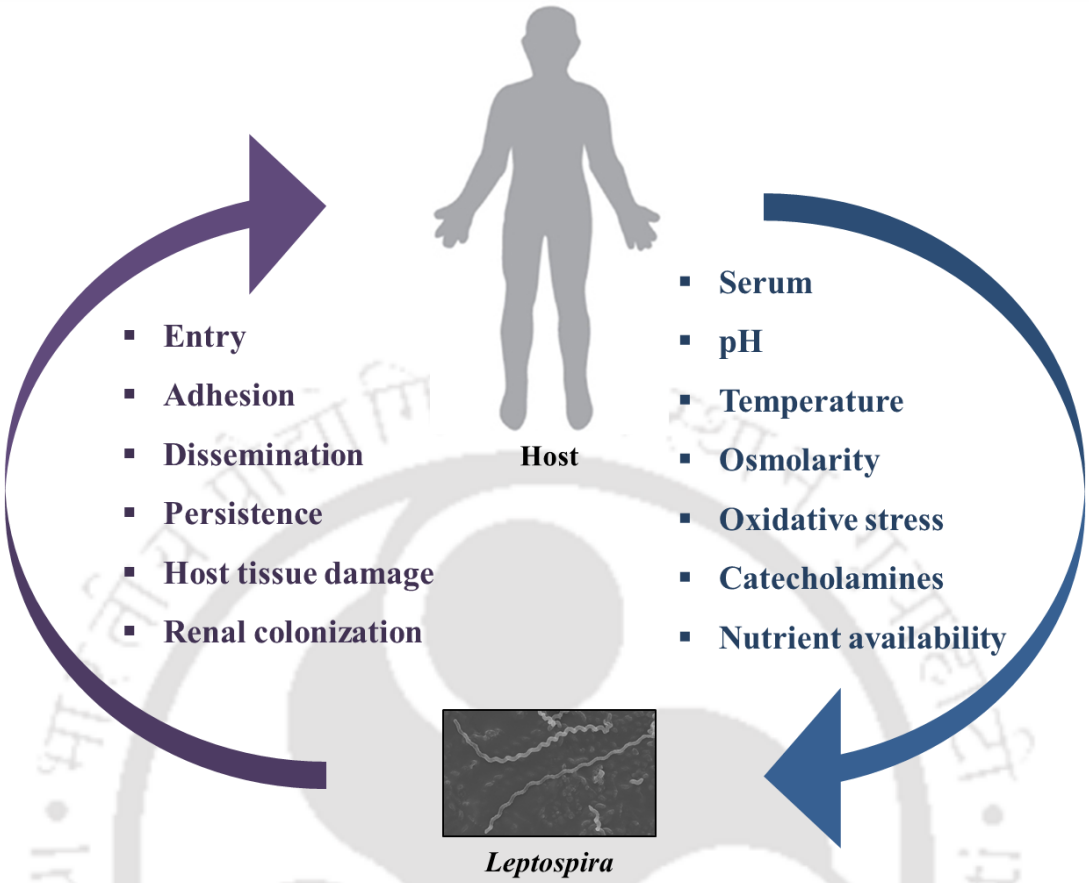

Figure 1.3. Host- *Leptospira* crosstalk via different host factors. The different host factors which are sensed by *Leptospira* are used as environmental cues. This causes marked changes in the expression of different virulent genes in the bacteria and helps it to successfully establish infection in the host.

1.2.4.1 Catecholamines (stress hormones)

Catecholamines are a large group of amine hormones derived from tyrosine and include epinephrine (Epi; adrenaline), Norepinephrine (NE; noradrenaline) and dopamine (Fig. 1.4). Catecholamines especially Epi and NE are sympathetic neuro-endocrine mediators of "Fight and Flight" response of the host. Another source of these mediators is adrenal medulla, an inner part of the adrenal gland (Peterson et al., 2011).

Catecholamines act exclusively by activating G-protein-coupled receptors. Norepinephrine and epinephrine act on α- and β-adrenergic receptors, respectively. Two subclasses of α-adrenergic receptors and three subtypes of β-adrenergic receptors are known. Alpha receptors, when activated, generally produce excitatory responses of smooth muscle in which they are located. Beta-receptors, when activated, generally produce inhibitory responses of smooth muscle in which they are located. Adrenergic antagonists refer to a pharmaceutical substance which acts by inhibiting the action of catecholamines at the adrenergic receptors. Thus, two types of antagonists are used: alpha-blockers (α Adrenergic receptor blocker) include phentolamine and beta-blockers (β-adrenergic blocker) include propranolol primarily (Peterson et al., 2011).

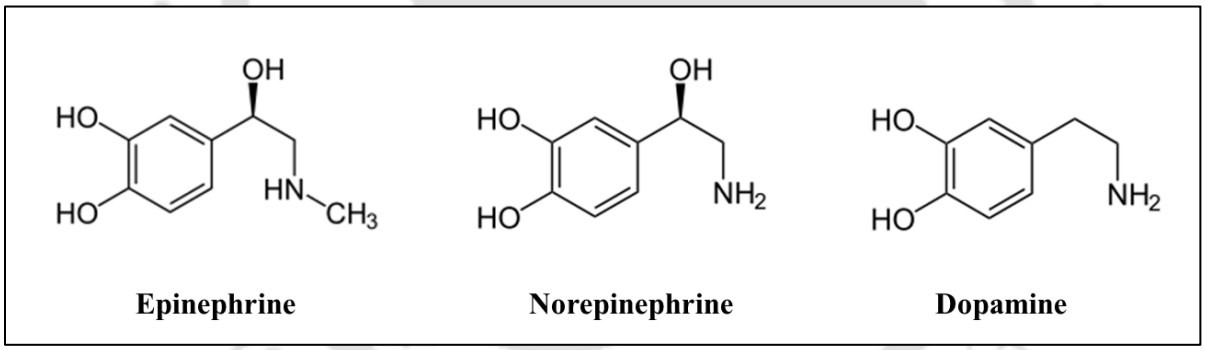

Figure 1.4. Chemical structure of the catecholamines. Catecholamines are monoamine compounds that have the distinct structure of a benzene ring with two hydroxyl groups, an intermediate ethyl chain, and a terminal amine group. Phenylethanolamines such as norepinephrine have a hydroxyl group on the ethyl chain. Catecholamines are derived from the amino acid tyrosine. Catecholamines include epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine. The release of the hormones epinephrine and norepinephrine from the adrenal medulla of the adrenal glands is part of the fight-or-flight response of the body (Source: https://en.wikipedia.org/wiki/Catecholamine).

The primary hormones synthesized under stress conditions are the catecholamines. In the last few years, a lot of studies have been done in regard to host-bacteria communication mediated

by catecholamines (Freestone, 2013). Pathogens have evolved sensor mechanisms to sense the presence of stress hormones in the human body upon infection (Lyte, 2004). Stress-induced hormones elicit pathogenic responses as well. In *Escherichia coli,* catecholamines can increase adhesion to host cells by upregulating adherence associated genes (Chen et al., 2006; Hendrickson et al., 1999; Lyte et al., 1997; Vlisidou et al., 2004). In *E. coli*, catecholamines can also affect chemotaxis, colonization to Hela cells (Bansal et al., 2007) and enhance Shiga toxin production (Lyte et al., 1996; Voigt et al., 2006).

Belay *et al.* (Belay and Sonnenfeld, 2002) found out that the different bacterial cultures did not respond uniformly in the presence of catecholamines; it enhanced the growth rate in case of *E. coli, Klebsiella pneumonae,* and *Pseudomonas aeruginosa* however, it had very less enhancement effect on the rate of growth of *Staphylococcus aureus*. Peterson *et al*. (Peterson et al., 2011), presented evidence that conjugative transfer of plasmids from clinical isolates of *Salmonella enterica* serovar Typhimurium to an *E. coli* recipient was increased in the presence of Norepinephrine hormone. Lyte *et al.* (Lyte et al., 1996) demonstrated that the growth of *E. coli* O157: H7 and the production of Shiga-like toxins are greatly increased in the presence of a neuroendocrine hormone. Scheckelhoff *et al*. demonstrated that OspA expression was upregulated in *B. burgdorferi* the presence of host catecholamines. When the β adrenergic blocker, propranolol was used along with the stress hormones, it resulted in reduced expression of OspA in *B. burgdorferi* (Scheckelhoff et al., 2007).

Li *et al.* were working extensively with *Actinobacillus pleuropneumoniae* which is a porcine pathogen. It was concluded by them that *A. pleuropneumoniae* could actively respond to the two stress hormones, epinephrine & norepinephrine which regulated many virulence genes as well. It was also inferred stress hormones can influence the infection process of the bacteria

& that it may use more than one response system for different catecholamines (Li et al., 2012).

1.2.4.2 Bacterial catecholamine-response system

1.2.4.2.1 Role of catecholamines in facilitating iron uptake by bacterial species

Catechols are a type of siderophores which are defined as small, high-affinity iron-chelating compounds secreted by microorganisms. Catechols are also present as catecholamines in neurotransmitters (Salton, 1964; Shearer and Walton, 2016). The most common catechol produced by bacteria to promote sequestration and uptake of iron is 2,3-dihydroxybenzoic acid which is also called as enterobactin (Neilands, 1984). Enterobactin and other siderophores including petrobactin are produced by various enteric bacteria, including *E. coli*, *Marinobacter hydrocarbonoclasticus*, and *Bacillus anthracis* (Hickford et al., 2004; Koppisch et al., 2005; Lee et al., 2007). Till date, there is no evidence of synthesis of siderophore by *Leptospira*. However, previous studies have demonstrated that *Leptospira* can use exogenous siderophores like aerobactin and ferrichrome which are produced by other microorganisms as iron source (Louvel et al., 2006). Studies have demonstrated that the ability to stimulate microbial growth in the host could be enhanced by epinephrine, norepinephrine, and dopamine through sequestration of the iron-containing medium, typically unavailable due to iron-transferrin/iron-lactoferrin complexes (Freestone et al., 2003). Thus, growth-stimulating effects of catecholamines have been mostly attributed to the catechol-containing moiety forming a complex with the iron within transferrin (Tf) or lactoferrin (Lf). This complex weakens the iron binding and enables the bacteria to acquire the normally inaccessible complexed iron (Fig. 1.5).

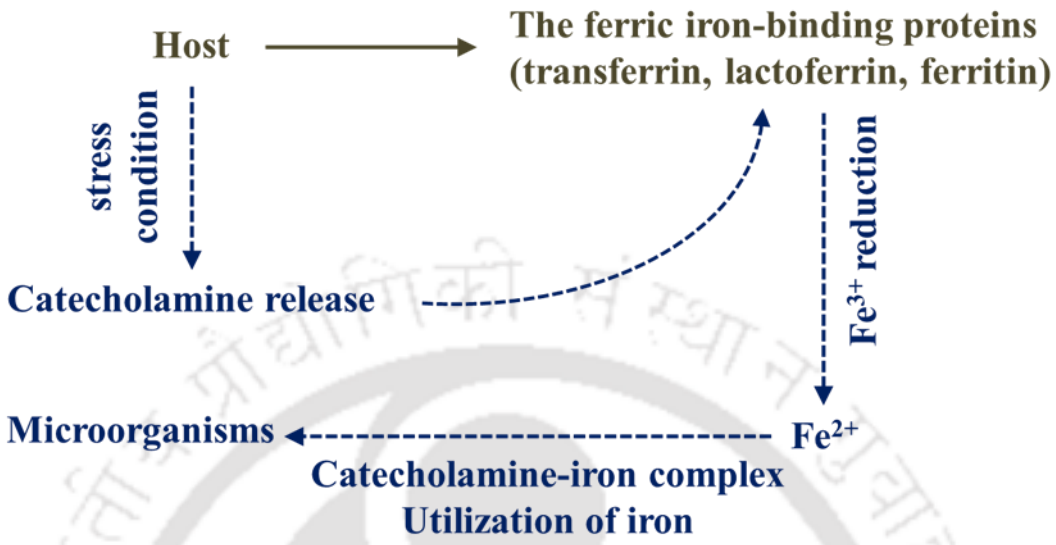

Figure 1.5. Catecholamine mediated iron uptake in bacteria. High-affinity ferric-binding proteins such as lactoferrin and transferrin are present in the blood. However, when catecholamines are present, this bound iron can become available to the bacteria. Catecholamines bind to these ferric-binding proteins resulting in the coordinated reduction of Fe (III) to Fe (II), an iron valency for which the ferric-binding proteins have low affinity (Adapted from Ngo Ndjom et al., 2018).

1.2.4.2.2 Receptor-based sensing of catecholamines by bacteria

The ability of catecholamines to stimulate bacterial growth in higher organisms has been known for long (Lyte, 2004), however very little is known about the nature of the putative bacterial adrenoceptors or dopamine receptor(s) to which norepinephrine, epinephrine, and dopamine might bind and exert their effects (Freestone, 2013). Although there is no evidence for the existence of mammalian-like catecholamine response systems in bacteria, the aromatic amino acid decarboxylases that are responsible for the sequential production of the catecholamine chemical messengers, are present in prokaryotes (Iyer et al., 2004). A recent report suggests that norepinephrine and epinephrine have been shown to bind to the *E. coli*

O157: H7 two-component regulator sensor kinase QseC *in vitro*, leading to the proposal that this might be the bacterial receptor for these catecholamines (Clarke et al., 2006). QseC also recognizes a novel autoinducer termed AI-3 (Reading and Sperandio, 2006; Sperandio et al., 2003), which suggests a possible intersection of microbial endocrinology and quorum sensing signaling pathways (Clarke et al., 2006; Reading and Sperandio, 2006; Sperandio et al., 2003; Walters and Sperandio, 2006). These studies indicate that bacterial pathogens use molecular sensors to facilitate their adaptation in the mammalian host.

1.2.4.3 Temperature, pH, osmolarity, oxidative stress and serum

It has been found that temperature plays a crucial role in on the gene expression profile of *Leptospira.* The transcription of virulence genes is induced at higher temperatures (37-39ºC) which is typical for the body cavities and host tissues (Lo et al., 2006). Several studies have shown that a shift in the culture temperature from 28°C to 37°C, similar to that encountered during infection in a host, is associated with differential synthesis of several proteins of the outer membrane, periplasm, and cytoplasm (Lo et al., 2006; Qin et al., 2006). The effect of temperature on protein synthesis in *Leptospira interrogans* was studied and it was concluded that different proteins like LipL36, GroEL, DnaK, Hsp15 were differentially synthesized (Nally et al., 2001a; Stamm et al., 1991b). LipL53, a novel surface leptospiral adhesin was found to be upregulated by elevated temperature and it may play a role in pathogenesis (Oliveira et al., 2010).

The effect of pH has not yet been studied in the role of pathogenesis in *Leptospira*. However, it has been ascertained that these bacteria survive longer in alkaline water than acidic water roughly around pH 7.3 (Khairani-Bejo et al., 2004; Smith and Turner, 1961).

Osmolarity is the measure of the solute concentration and is usually expressed as osmol/L. Invasion of host tissues by *Leptospira interrogans* involves a transition from a low osmolar environment outside the host to a higher physiologic osmolar environment within the host. Expression of the lipoprotein LigA and LigB adhesions is strongly induced by an upshift in osmolarity to the level found in mammalian host tissues. This suggests that *Leptospira* utilizes changes in osmolarity to regulate virulence characteristics and thus osmolarity is an important signal for regulation of gene expression by pathogenic leptospires during the transition from ambient conditions to the host tissue environment. Similar studies proved that osmolarity not only affects leptospiral gene expression by affecting transcript levels of putative virulence determinants but also affects the release of LigA, LigB and Sph2 into the surroundings (Khairani-Bejo et al., 2004; Matsunaga et al., 2007a; Matsunaga et al., 2007b; Matsunaga et al., 2005; Smith and Turner, 1961). In another study, the motility of *Leptospira* was analyzed under various osmotic conditions. The results showed that pathogenic *L. interrogans* maintained vigorous motility near physiological osmotic conditions. This suggests that active motility in physiological conditions is advantageous when *Leptospira* enters hosts and when it migrates toward target tissues (Takabe et al., 2013).

Oxidative stress in pathogens is observed when there is an imbalance between the production of reactive species such as peroxides and oxygen radicals and its scavenging ability. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell. Oxidative stress plays a dual role in infections. Free radicals protect against invading microorganisms and they can also cause tissue damage during the resulting inflammation (Pohanka, 2013). Oxidative stress can arise in the body both as an actively initiated process and an unwanted process. In the

actively initiated processes, there is the production of reactive species via enzymes such as myeloperoxidase (EC 1.11.2.2), NADPH oxidase (EC 1.6.3.1), and nitric oxide synthase (EC 1.14.13.39). Expression of these enzymes serves as a suitable marker for inflammation which involves oxidative stress (Sánchez-López et al., 2012). Phagocytosis is an important immune control mechanism during leptospirosis (Faine et al., 1999b). Hence for the persistence of the bacteria in the host, certain genes like *katE* and *clpB* play an important role. Catalase (KatE) found in pathogenic leptospires is required for resistance to hydrogen peroxide (Eshghi et al., 2012a). While the role of KatE in survival in macrophages has not been directly tested, hamsters infected with *katE* mutants of *L. interrogans* serovars Pomona or Manilae survived a challenge without signs of disease, indicating that oxidative stress resistance is essential for virulence. Another mediator of resistance to oxidative stress is the molecular chaperone ClpB; this protein is also required for growth under nutrient restriction and heat stress (Lourdault et al., 2011). A *clpB* mutant was also highly attenuated; gerbils receiving a very high dose survived infection with no clinical signs of leptospirosis and no macroscopic lesions normally associated with the disease. Restoration of growth under oxidative, heat, and nutrient stress conditions was achieved by complementation with an intact copy of *clpB*, along with partial restoration of virulence (Lourdault et al., 2011).

A recent study performed a transcriptional analysis of *L. interrogans* serovar Copenhageni upon exposure to serum in comparison with EMJH medium. This study showed that there was a global transcriptional change in pathogenic *Leptospira* upon exposure to guinea pig serum, representing a specific host environmental cue present in the bloodstream. This may provide a key insight into the pathogenesis of leptospirosis during the early bacteremic phase of leptospiral infection (Patarakul et al., 2010).

1.2.5 Clinical manifestations

Symptoms of leptospirosis begin abruptly 2 to 10 days after infection and include fever, headache, and myalgia. The most common clinical syndrome is anicteric leptospirosis, which is a self-limiting illness consisting of a septicaemic stage, with high fever and severe headache that lasts 3 to 7 days, followed by the immune stage (Fig. 1.6) (Haake and Levett, 2015). Symptoms associated with the immune stage (onset coincides with the appearance of IgM) are varied, but in general are milder than the septicemic stage. The hallmark of the immune stage is aseptic meningitis. Weil's disease, or icteric leptospirosis, is generally the most severe illness, with symptoms caused by liver, kidney, and/or vascular dysfunction with lethal pulmonary hemorrhage; death can occur in up to 10% of cases (Haake and Levett, 2015). Unfortunately, the clinical presentations of leptospirosis mimic those of many other unrelated infections like influenza, meningitis, hepatitis, dengue, and other viral hemorrhagic fevers.

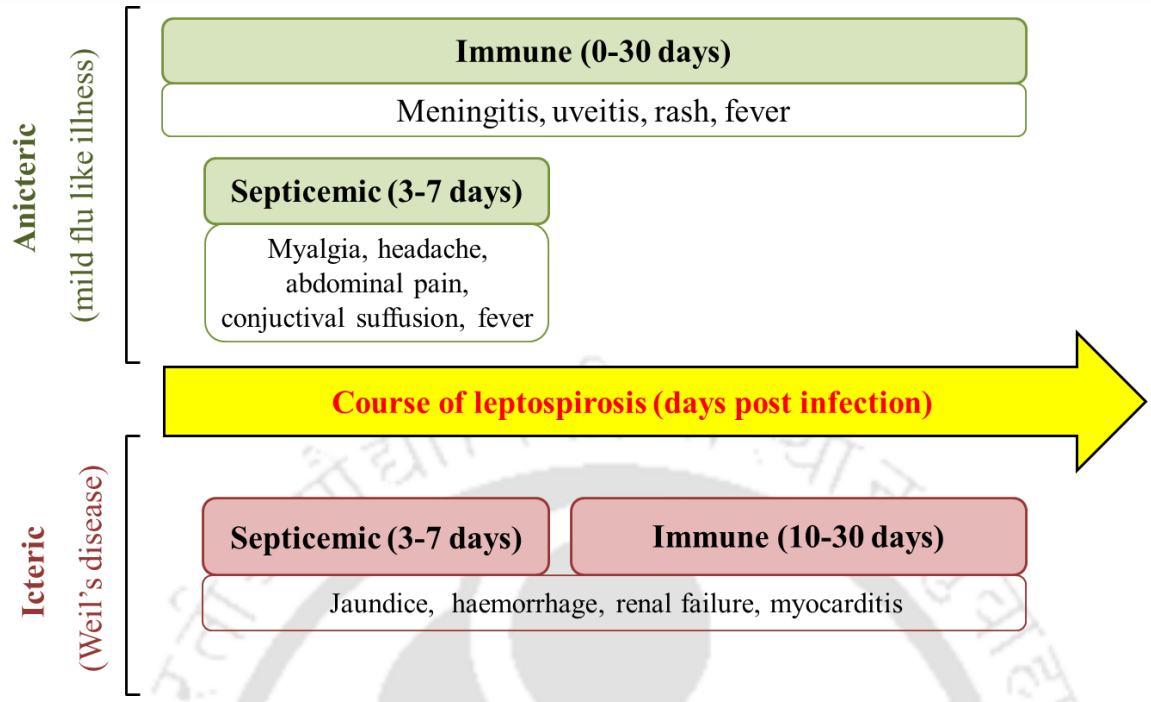

Figure 1.6. A typical course of leptospirosis infection. The infection starts with an initial incubation phase of around 2 to 10 days. This phase is then followed by an acute septicaemic phase of 4 to 7 days which is characterized by fever and nausea. The symptoms disappear briefly in the interphase which is then followed by an immune phase of around 30 days in which the bacteria is cleared from the body by the humoral immune response.

1.2.6 Laboratory diagnosis of leptospirosis

Microscopic agglutination test (MAT) is often considered as the gold standard for the diagnosis of leptospirosis (McBride et al., 2005). As the symptoms of leptospirosis mimic many others, it becomes a challenge to diagnose it accurately. A lot of laboratory tests are available which may be broadly classified into two categories: Direct and Indirect techniques. Direct techniques include either the demonstration of leptospires or its DNA and isolation of the organism from the clinical specimens. Detection of specific antibodies to *Leptospira* is an indirect method of detection as shown in Fig. 1.7.

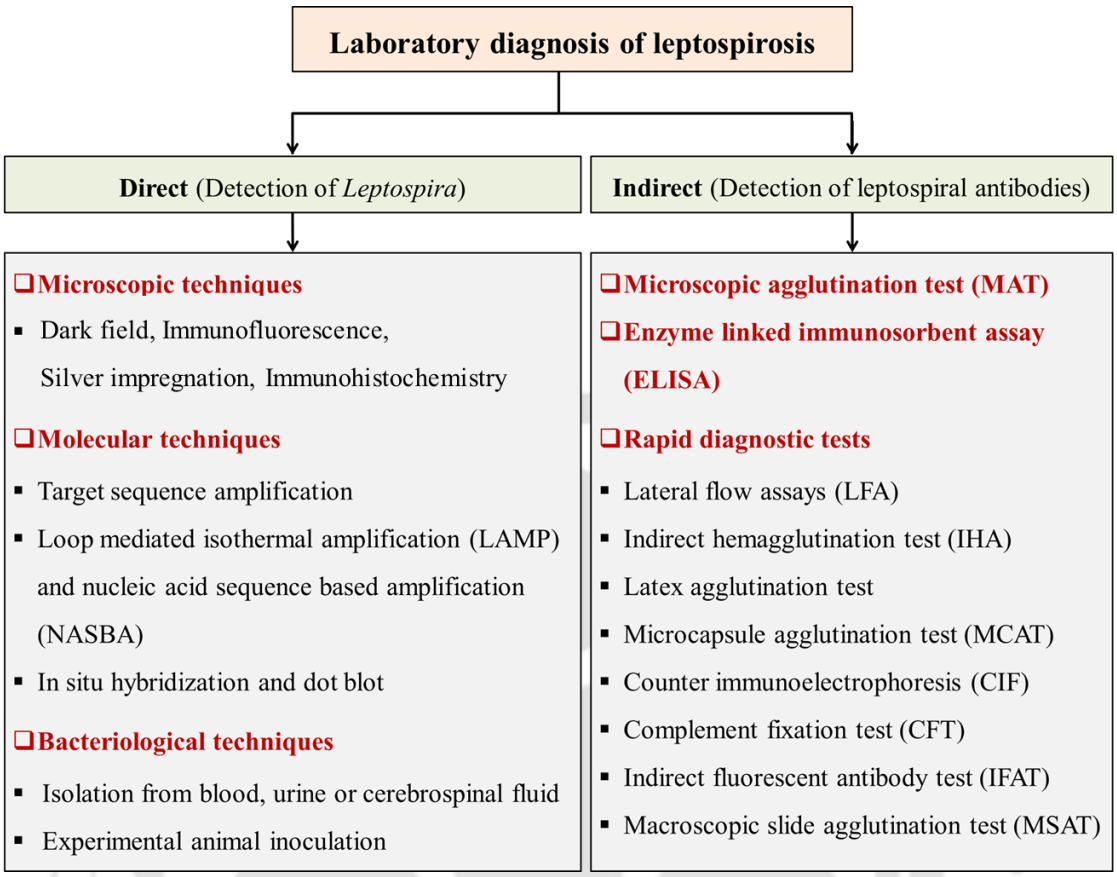

Figure 1.7. Different approaches to the laboratory diagnosis of leptospirosis. The laboratory diagnosis of leptospirosis can be broadly classified as the direct and indirect diagnosis of the etiologic agent. MAT is the gold standard for the diagnosis of the disease.

Direct diagnosis includes the demonstration of the organism in body fluids by dark-field microscopy, isolation of the bacteria from blood, urine or tissues, direct staining of the organisms, Immuno fluorescence, PCR, animal inoculation studies etc. However, dark-field microscopy is not recommended as the sole diagnostic tool in case of body fluids as cell remnants and other artifacts can resemble leptospires making the results unreliable (Vijayachari et al., 2001). Indirect diagnosis, however, relies on the demonstration of antibodies to leptospires by various serological tests, namely, Microscopic agglutination test (MAT), Enzyme-linked immunosorbent assay (ELISA), Macroscopic slide agglutination test

(MSAT), Lateral Flow assays, Indirect Haemagglutination test (IHA), Counter immunoelectrophoresis (CIE), Complement fixation test (CFT), Latex agglutination tests (LA), Indirect fluorescent antibody test (IFAT), etc. However, MAT and ELISA are the commonly used serodiagnostic techniques (Picardeau et al., 2014). Some of the major diagnostic techniques for *Leptospira* are detailed briefly in the following section.

1.2.6.1 MAT

The MAT titer is determined by incubating different serum dilutions with a panel of leptospires. The agglutination is visualized by the dark field microscopy and the endpoint is considered as the serum dilution that gives 50% agglutination. This test is often considered as the gold standard method by the World Health Organization (WHO) for the diagnosis of leptospirosis (WHO, 2009). The difficulties associated with this test include the routine maintenance and updating of newly isolated serovars on a regular basis (Fraga et al., 2015).

1.2.6.2 ELISA

The most common ELISA used for the diagnosis of leptospirosis is IgM-ELISA. A number of recombinant proteins like LipL32, LigA, OmpL1 have been used as antigen preparations (Adler and de la Peña Moctezuma, 2010). Although the overall sensitivity of IgM-ELISA is low in the first week of symptoms, it is a very important diagnostic tool during the second week when the levels of IgM increases in the body (Grassmann et al., 2017a). Another advantage of ELISA is that it may help to differentiate between current and old cases because antibodies from the old infection may not be detected in the test (Fraga et al., 2015). However, the ELISA results should always be confirmed by MAT.

The IgM-ELISA can usually detect the disease earlier than MAT (Niloofa et al., 2015). The antigens used in commercialized ELISA are usually total protein extract of the saprophyte *L. biflexa*. ELISA and MAT are generally used 1 week after the onset of symptoms as the antibodies re not detected earlier (Picardeau et al., 2014).

1.2.6.3 PCR

The samples which are routinely used for the amplification of leptospiral DNA include blood, urine, aqueous humor, cerebrospinal fluid, and dialysate fluid. Most of the quantitative PCR assays for diagnosis target either the housekeeping genes like *rrs, gyrB* or pathogen-specific marker genes like *lipL32* or *lig* genes (Ahmed, 2012). Many times the bacterial load can be quantified through real-time PCR assays (Agampodi et al., 2012). A major drawback of such PCR-based diagnosis is that it is unable to identify the infecting serovar.

Leptospirosis usually presents itself in the form of acute phase which is followed by the immune phase in which the antibodies are produced by the host to eliminate the organisms from the body (Levett, 2001). During the acute phase, generally, the culturing of the bacteria and PCR is conducted. Sometimes, however, an IgM-ELISA may prove helpful which gives a positive result if the patient is towards the end of the acute phase and beginning of the immune phase (Milner et al., 1985). However, once the immune phase sets in, this diagnosis predominantly relies on the MAT (Faine et al., 1999a). The serological confirmations generally require a fourfold or greater rise in the agglutination titer between the acute and immune phase serum (WHO, 2007).

1.2.7 Treatment

Leptospirosis is treated with antibiotics such as penicillin, ampicillin, ceftriaxone or cefotaxime. In acute cases, benzylpenicillin is given by injections. Delayed or inadequate treatment can lead to a severe form of the disease thereby emphasizing the need for timely diagnosis (Vijayachari et al., 2008). Chemoprophylaxis with doxycycline and azithromycin has been shown to be effective for short-term prophylaxis in high-risk environments (Grassmann et al., 2017a). However, the antibiotic treatment can cause a Jarisch-Herxheimer reaction (JHR) which is characterized by the release of endotoxins from the dying leptospires and production of IL-6, IL-8, and TNF-α (Bryceson, 1976). As JHR is fatal, anti-inflammatory, including anti-TNF-α, inhibitors should be co-administered to prevent the JHR effects on the patients (Fekade et al., 1996). In severe cases of leptospirosis, hospitalization is needed. Dialysis and/or administration of intravenous fluids may help to prevent the renal injuries (Andrade et al., 2008). Mechanical ventilation proves beneficial to patients suffering from lung damages (Amato et al., 1998).

1.2.8 Prevention

Prevention of leptospirosis is essentially done by identifying the source and interrupting the transmission. However, preventive measures that block transmission can be practiced by the maintenance of hygiene and prevention of infection by using protective clothing and footwear [12]. Implementation of rodent control measures is also important in limiting the extent of contamination.

1.2.9 Vaccines against leptospirosis

The first report of a vaccine for leptospirosis prophylaxis was published in 1916 which was composed of whole inactivated *Leptospira* cells (bacterin) (Ido et al., 1916). The development of an effective vaccine against leptospirosis, however, remains a challenge. The ability to rapidly colonize multiple organs poses a high threat to the host and is the main reason for the need to develop a safe and efficient leptospirosis vaccine (Koizumi and Watanabe, 2005). Although the immunity conferred is restricted to serovars with closely related lipopolysaccharide antigen, certain vaccines have remained useful, especially in endemic regions (Bashiru and Bahaman, 2018). In the present scenario, with the recent breakthrough and availability of complete genome sequences of *Leptospira,* development of novel vaccine including recombinant protein vaccine using reverse vaccinology approaches has yielded encouraging results (Bashiru and Bahaman, 2018). This has significantly contributed to the identification and selection of conserved vaccine candidates based on *in silico* sequence analysis (Fig. 1.8).

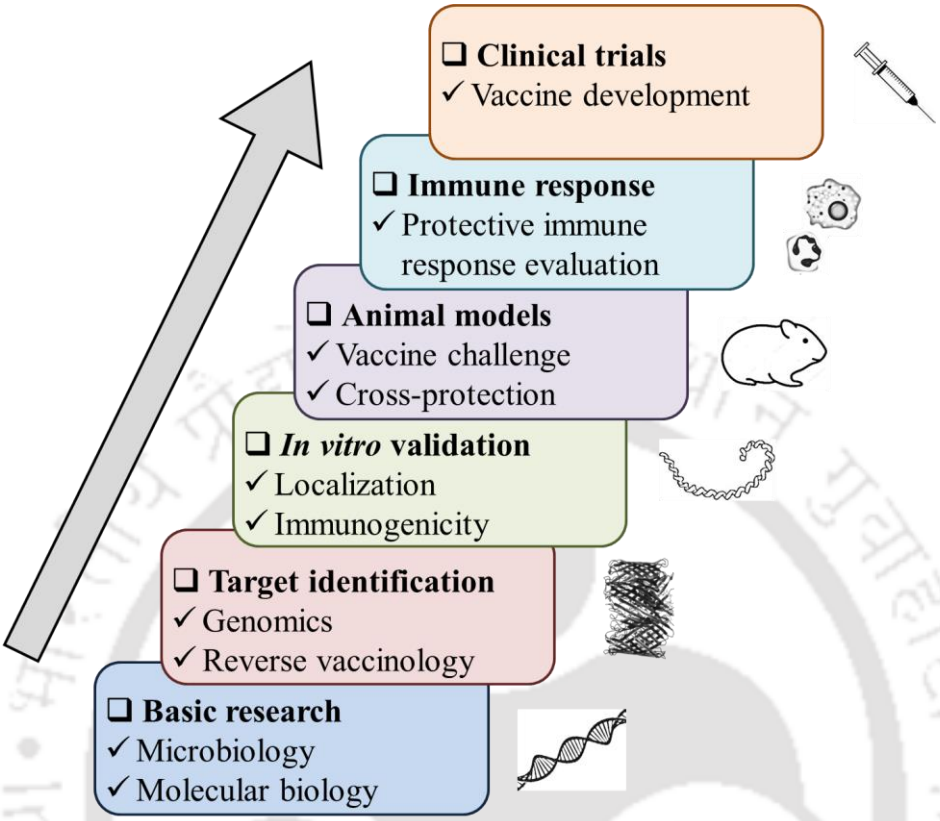

Figure 1.8. A schematic representation of vaccine development against leptospirosis. Vaccine development essentially relies on the basic research on *Leptospira* microbiology. Genomics and reverse vaccinology studies are of utmost importance for the development of leptospirosis vaccines, among different *Leptospira* spp. After the potential vaccine candidates are identified, an *in vitro* validation is required to confirm the localization of these antigens on the surface of the leptospiral cell. At this stage, surface-exposed, conserved and immunogenic leptospiral antigens must be evaluated in vaccine challenge experiments using animal models. The protective immune response is evaluated and finally, the experimental vaccine candidates are tested in clinical trials (Adapted from Grassmann et al., 2017c).

Three major types of leptospirosis vaccines are currently available. They are the live vaccines, bacterin vaccines, and the lipopolysaccharide vaccines. Till date, bacterin vaccines have been used in humans, cattle, dogs, and swine and remain the only vaccine licensed currently (Adler, 2015). To effectively protect against multiple serovar infections,

recombinant technology is being used (Dellagostin et al., 2011). Recombinant protein vaccines (e.g. r-OmpL1 and r-LipL41) and DNA vaccines (e.g. OmpL1 plasmid DNA, Lig) are proving to be a successful approach for vaccine development using animal models (Faisal et al., 2008; Haake et al., 1999; Maneewatch et al., 2007). However, the same success could not be achieved in the human clinical trials owing to the low immunogenicity of these vaccine candidates. The major drawback with recombinant proteins is the use of inadequate challenge doses and improper statistical analysis which invalidates many of the claims of protection. Further studies are warranted to elucidate the molecular mechanisms of pathogenesis that will contribute to the development of novel vaccines for the treatment of leptospirosis in humans and animals (Bashiru and Bahaman, 2018).

1.3 *Leptospira*, the etiological agent of leptospirosis

1.3.1 Taxonomy and Classification

The genus *Leptospira* belongs to the family of Leptospiraceae. The genus *Leptospira* was initially divided into pathogenic and non-pathogenic species, and then further classified based on the structural diversity in the carbohydrate component of their lipopolysaccharide layer, into more than 300 serovars (Picardeau, 2017). To date, 35 species of *Leptospira* have been identified as shown in Table 1.1. These 35 species have been classified as pathogens (n=13), intermediates (n=11) and saprophytes (n=11). The pathogenic species are responsible for causing leptospirosis

Table 1.1. The taxonomic groups of the *Leptospira* spp. among pathogenic, intermediate and saprophytic species

Taxonomic division	Species	Reference
Pathogens	*L. alexanderi*	(Brenner et al., 1999)
	L. alstonii	(Smythe et al., 2013)
	L. borgpetersenii	(Yasuda et al., 1987)
	L. interrogans	(Faine and Stallman, 1982)
	L. kirschneri	(Ramadass et al., 1992)
	L. kmetyi	(Slack et al., 2009)
	L. mayottensis	(Bourhy et al., 2014)
	L. noguchii	(Yasuda et al., 1987)
	L. santarosai	(Yasuda et al., 1987)
	L. weilii	(Yasuda et al., 1987)
	L. adleri	(Thibeaux et al., 2018)
	L. ellisii	(Thibeaux et al., 2018)
	L. barantonii	(Thibeaux et al., 2018)
Intermediates	*L. broomii*	(Levett et al., 2006)
	L. fainei	(Perolat et al., 1998)
	L. inadai	(Yasuda et al., 1987)
	L. licerasiae	(Matthias et al., 2008)
	L. wolffii	(Slack et al., 2008)
	L. venezuelensis	(Puche et al., 2017)
	L. perolatii	(Thibeaux et al., 2018)
	L. neocaledonica	(Thibeaux et al., 2018)
	L. saintgironsiae	(Thibeaux et al., 2018)
	L. haakeii	(Thibeaux et al., 2018)
	L. hartskeerlii	(Thibeaux et al., 2018)
Saprophytes	*L. biflexa*	(Faine and Stallman, 1982)
	L. idonii	(Saito et al., 2013)
	L. meyeri	(Yasuda et al., 1987)
	L. terpstrae	(Smythe et al., 2013)
	L. vanthielli	(Smythe et al., 2013)
	L. wolbachii	(Yasuda et al., 1987)
	L. yanagawae	(Smythe et al., 2013)
	L. levettii	(Thibeaux et al., 2018)
	L. macculloughii	(Thibeaux et al., 2018)
	L. brenneri	(Thibeaux et al., 2018)
	L. harrisiae	(Thibeaux et al., 2018)

whereas the intermediate species are able to cause a very mild form of the disease in humans and animals (Bharti et al., 2003).

1.3.2 Nutritional requirements, growth, and cultivation of *Leptospira*

Leptospires require special nutritional conditions for their growth. Nevertheless, they are able to sustain itself in alkaline soil, mud, swamps, streams, stagnant water reservoirs, organs, and tissues of live or dead animals (Al-orry et al., 2016). In general, they are sensitive to dryness, heat, acids and basic disinfectants. In the environment, they require high humidity for survival and are killed by dehydration or temperatures greater than 50°C. The primary nutritional requirements for growth of *Leptospira* are sources of nitrogen, carbon and select vitamins and supplements. The optimal growth of the *Leptospira* is observed at temperatures between 28ºC and 30°C in a media supplemented with long-chain fatty acids, vitamins (B1 and B12), and ammonium salts. Long-chain fatty acids are utilized as the carbon source and are metabolized by the β-oxidation pathway (Levett, 2001). Ammonium ions are the only known nitrogen source for *Leptospira*. Nutritional supplements generally include thiamin, phosphate, calcium, magnesium, and iron (Faine et al., 1999b; Shenberg, 1967).

The most frequently used liquid medium for culturing *Leptospira* under *in vitro* condition in the laboratory is the Johnson and Harris modification of Ellinghausen McCullough (EMJH) medium, which contains oleic acid, bovine serum albumin, and polysorbate (Tween). The doubling time for pathogenic leptospires *in vivo* and *in vitro* (provided they have had prior adaptation to *in vitro* culture) is estimated at 6–8 hours. However, the doubling time for pathogenic species freshly isolated from the host and cultured *in vitro* is estimated at 14-18 hours (Cameron, 2015). Leptospires grow under aerobic conditions within a pH range of 7.2-7.6. The growth of cultures is best monitored via darkfield microscopy, with cell counting

accomplished using a Petroff-Hausser counting chamber (Adler and de la Peña Moctezuma, 2010; Faine, 1994; Levett, 2001).

1.3.3 Cell Biology

Leptospira spp. are thin, right-handed helical, motile organisms that have a diameter of 0.15 μm and a length of 10–20 μm (Cameron, 2015). The hooked ends of this bacterium give its distinctive question-mark shape (interrogative remark) as shown in Fig. 1.1. The leptospires have two periplasmic flagella, one originating at each end of the cell and the free ends of the periplasmic flagella extend toward the center of the cell but do not overlap as they do in other spirochetes (Cameron, 2015). They have a Gram-negative-like cell envelope, however; the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique to spirochetes. The peptidoglycan layer and cytoskeletal proteins are key contributors to the spiral shape of *Leptospira* (Cameron, 2015).

1.3.4 Genome of *Leptospira*

The first two *Leptospira* genomes to be sequenced were that of *L. interrogans* serovar Lai and *L. interrogans* serovar Copenhageni (Nascimento et al., 2004a; Ren et al., 2003). The genome comprises of a large circular chromosome of 4277 kb and a smaller replicon of 350 kb (Nascimento et al., 2004a; Ren et al., 2003). The larger chromosome (cI) has a gene density of approximately 75-92% and is found to encode mostly the housekeeping genes. The smaller replicon (cII) is known to encode the essential genes (Bourhy and Saint Girons, 2000; Zuerner et al., 1993). A third circular replicon (p74) is of 74 kb and is found only in the saprophytic *L. biflexa* (Picardeau et al., 2008). The genome has a G+C content ranging between 35-42% in different species with the genome size varying between 3.9 to 4.6 Mbp

(Picardeau, 2015). Leptospires contain two sets of 16S (*rrs*) rRNA and 23S (*rrl*) rRNA genes but only one 5S (*rrf*) rRNA gene (Fukunaga and Mifuchi, 1989a). These rRNA genes are widely scattered on the large chromosome (Baril et al., 1992; Fukunaga and Mifuchi, 1989b). Many insertion sequences (IS) coding for transposases such as IS*1500*, IS*1502*, IS*1533* etc. have been identified in *Leptospira* (Picardeau, 2015) .

1.3.5 Gene content in *Leptospira*

L. interrogans serovar Lai is found to contain a total of 3718 coding sequences (Zhong et al., 2011). DNA microarray hybridization analysis has shown that among the 11 *L. interrogans* strains from different serovars, the gene content is highly similar (He et al., 2007). The genome sequences of *L. interrogans, L. borgpetersenii, L. santarosai*, L. *licerasiae*, and *L. biflexa* were found to contain around 35 % of putative protein coding genes with no functions (Picardeau, 2015). A peculiar feature of the leptospiral genome, in contrast to the other spirochetes, is that it contains a locus for the genes necessary for the synthesis of lipopolysaccharide (LPS) (de la Peña-Moctezuma et al., 2001). It is also noteworthy that the pathogenic *L. interrogans* has a sequence similarity in 2708 genes with the saprophyte *L. biflexa* (Picardeau et al., 2008). A comparison among the genome sequences of the pathogenic *L. interrogans* and *L. borgpetersenii* with the saprophytic *L. biflexa* has revealed 893 pathogen associated genes. Nearly 78% of these pathogen specific genes encode hypothetical proteins, indicating the presence of pathogen associated mechanisms in *Leptospira* (Adler et al., 2011; Picardeau et al., 2008).

1.3.6 Outer membrane proteins (OMPs) of *Leptospira*

Adaptation to a wide variety of environmental conditions has required leptospires to acquire a large genome and a complex outer membrane with features that are unique among bacteria. The leptospiral outer membrane also includes lipopolysaccharide and many homologs of well-known beta-barrel transmembrane outer membrane proteins (Fig. 1.10). Outer membrane proteins constitute about half of the mass of the bacterial outer membrane and play a significant role in adhesion, immunity, and pathogenicity.

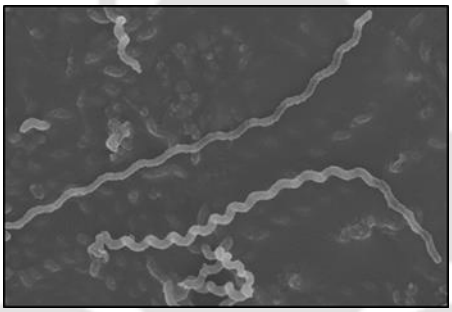

Figure 1.9. Scanning electron micrograph of *Leptospira interrogans* (20,000X magnification). The electron micrograph shows the elongated structure and helically coiled shape of the bacteria. The characteristic hooked ends can also be seen in the micrograph.

Three classes of leptospiral OMPs have been identified thus far namely: transmembrane proteins, lipoproteins, and peripheral proteins. The cell wall surface of *Leptospira* constitutes a variety of proteins like non-specific porins, specific channels for nutrient acquisition, efflux channels, lipoproteins, adhesins, S layer glycoproteins, peripheral membrane proteins or surface-maintenance proteins. The major reasons why researchers focus on the OMPs as a target for diagnosis and vaccine development are; LPS is serovar specific, where more than 200 serovars of pathogenic *Leptospira* are reported so far and the immunity elicited will be against only homologous strains; therefore the conserved protein antigens of OMPs may play

a major role due to their wide cross-reactivity apart from the serovar specificity (Raja and Natarajaseenivasan, 2015).

(I) Transmembrane proteins

The transmembrane protein is a polytopic protein that spans an entire biological membrane and has a significant role in maintaining the bacterial cell structure, attachment to various substrates, importing nutrients, and exporting bactericidal and toxic agent. Immunoprecipitation studies have reported OmpL1; a 31kDa protein with 320 amino acid residues is the heat modifiable porin, present in the OM in small amounts. It is the first transmembrane outer membrane to be described from a pathogenic spirochete (Haake et al., 1993). *In silico* study using PRED-TMBB or TMBETA-NET program has put forth a growing list of OmpL proteins namely OmpL36, OmpL37, OmpL47, and OmpL54 (Pinne and Haake, 2009). Additionally, Triton X-114 fractionation, surface proteolysis, surface immunofluorescence, surface biotinylation, and membrane affinity analysis results showed these proteins to be surface exposed. A recent report highlights OmpL37 protein to have an ability to bind human skin and other host factors (Pinne et al., 2010). Through this, it can be hypothesized that OmpL37 may play a role in the invasion of leptospires in the skin of humans. Iron is an essential nutrient for pathogenic leptospires and the transport of heme or other iron-containing molecules by Gram-negative bacteria often relies on the active transport through TonB dependent OM receptors (Asuthkar et al., 2007; Louvel et al., 2006).

(II) Lipoproteins

The interest in lipoprotein search is sparked by its abundant quantity on the surface of *Leptospira* and its application in developing the vaccine and diagnostic antigens for leptospirosis. LipL32, the most abundant antigen that accounts for 75% of the OM proteome,

is the field of intense research for the past two decades (Haake et al., 2000b). Second most abundant protein is Loa22. Loa22 has an OmpA domain located in the OM, whereas a small portion is exposed on the cell surface (93% periplasmic space, 25% OM) (Koizumi and Watanabe, 2003). Since it has the OmpA domain, the major function of this protein is to play a role in structural integrity (Picardeau et al., 2008). LipL40, a 40-kDa leptospiral OM lipoprotein, was reported to interact with plasminogen (PLG) and generate plasmin, in the presence of an activator (Gamberini et al., 2005; Vieira et al., 2010a). The identification of this protein showed the ability of *Leptospira* to bind PLG, which may have a role in pathogenesis. Lig proteins are the first leptospiral proteins described to be expressed only during infection. Three classes of leptospiral Lig proteins have been described until now namely LigA, LigB and LigC (Palaniappan et al., 2002; Vieira et al., 2010b).

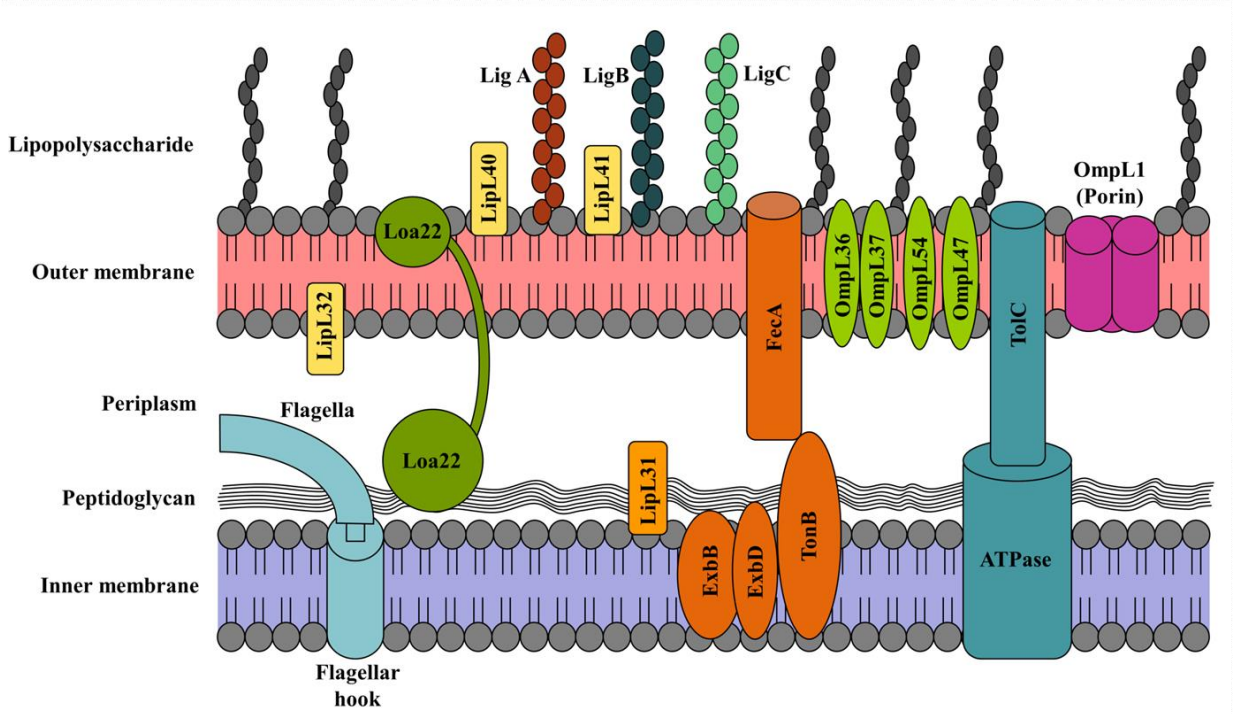

Figure 1.10. The membrane architecture of *L. interrogans*. The outer membrane contains the lipopolysaccharide layer along with lipoproteins such as LipL40, LipL41, LipL32, Loa22, and Lig. Several transmembrane proteins such as FecA, OmpL1, TolC, OmpL36, OmpL37, OmpL54 and OmpL47 are also present in the outer membrane. Peripheral membrane proteins like LipL45 are also found associated with the outer membrane. The inner membrane contains several transport systems like TonB dependent receptors and LipL31 among many others. The peptidoglycan layer and the periplasmic flagella are located in the periplasm of *Leptospira*.

(III) Peripheral membrane proteins

LipL45, an inner membrane protein has been found associated at its C terminus to a 31-kDa peripheral membrane protein, P31LipL45. The exact mechanism of its transport and its role in infection is not well understood, however, the high-level expression of P31LipL45 in stationary phase indicates the protein to be involved in environmental functioning and regulation (Matsunaga et al., 2002).

1.3.7 Hypothetical membrane proteins

Genome sequencing of *Leptospira* has shown that there are some proteins whose functions are unknown and they need to be characterized. Hypothetical proteins are predicted from nucleic acid sequences that have not been shown to exist by experimental protein chemical evidence. Moreover, these proteins are characterized by low identity to known, annotated proteins (Lubec et al., 2005). "Conserved hypothetical proteins'' form a large fraction of genes in sequenced genomes encoding those that are found in organisms from several phylogenetic lineages but have not been functionally characterized (Galperin, 2001; Galperin and Koonin, 2004). A general prediction of a conserved hypothetical protein can be based upon several factors like conserved sequence motif, sequence similarity to a protein which has been characterized previously, the presence of diagnostic structural features etc. (Galperin and Koonin, 2000). Various approaches have been exploited in order to predict the function of a conserved hypothetical protein. Protein function can be predicted by *in silico* methods which include comparative genomics, protein-protein interactions, 3D structure determination, etc. (Sivashankari and Shanmughavel, 2006). There is a need to study and classify these hypothetical proteins which can open a new way to design drug molecules against infectious organisms. Functional annotation of these hypothetical proteins involved in infection, drug resistance, and essential biosynthetic pathways is important for the development of the potent antibacterial against infectious agents. Improved understanding of these proteins may make them potential targets of antimicrobial drugs. These hypothetical proteins await experimentation to show their existence at the protein level and subsequent bioinformatics analysis to assign these proteins a tentative function.

1.3.8 Role of OMPs in the pathogenesis

Outer membrane proteins of *Leptospira* are the potential key players during infection and has diverse roles in pathogenesis like iron uptake, toxin production, adhesion, invasion, serum resistance, hemolysins, maintaining the bacterial cell structure, attachment to various substrates, importing nutrients, exporting bactericidal and toxic agents etc. Several leptospiral OMPs like OmpL37, OmpL1, Lsa21, Lsa63 etc. are reported to have the binding capacity with ECM components like fibronectin, fibrinogen, collagen, laminin, and elastin (Raja and Natarajaseenivasan, 2015).

1.3.9 Role of OMPs in diagnosis

Presently available laboratory diagnostic methods for leptospirosis are isolation of the causative organism from body fluids include dark field microscopy or to determine elevation of IgG and IgM in the serum, staining techniques, microscopic agglutination test (MAT) and whole cell-killed leptospiral antigen-based tests like macroscopic slide agglutination test, microcapsule agglutination test, ELISA, lepto dipstick, lepto dridot and polymerase chain reaction (PCR) reference (Brandão et al., 1998; Sehgal et al., 1999; Suputtamongkol et al., 1998). All these techniques were found to have very low sensitivity during the acute phase infection. Hence research is being pursued to use OMP based assays. For that several OMPs like OmpL1, HbpA, LipL32, LipL21, LipL36, LipL41, Loa22, LigA, LigB, MPL17, and MPL21 have been used. However, these seem ineffective in the detection of leptospires in the early phase of infection (Raja and Natarajaseenivasan, 2015).

From the literature review it is evident that the pathogens can sense the host factors like catecholamines, physiological temperature, oxidative stress etc. during infection. A lot of work has been done in regard to the effect of temperature and oxidative stress on the gene transcription of pathogenic *L. interrogans*. Although the effect of catecholamines on the modulation of genes in pathogenic bacteria has been reported, as of date, no work has been done on the effect of catecholamines on the gene modulation in pathogenic *L. interrogans*. Hence, based on the current literature on the effect of various host factors on the differential gene expression of *Leptospira* and their pathogenesis, the work presented in the thesis is divided into three experimental chapters, each comprising of the following objectives:

1. *In silico* based selection of genes of *Leptospira interrogans* encoding hypothetical membrane proteins and extracellular proteins
2. Analysis of *L. interrogans* selective gene transcripts in the presence of host factors under *in vitro* condition
3. Cloning, expression, and characterization of selected membrane proteins for the serological diagnosis of leptospirosis

Chapter 2

In silico based selection of genes of Leptospira interrogans encoding hypothetical membrane proteins and extracellular proteins

CHAPTER 2

In silico based selection of genes of *Leptospira interrogans* encoding hypothetical membrane proteins and extracellular proteins

2.1 Abstract

An indispensable hub of the current leptospiral research is the pinpointing of outer membrane proteins (OMPs). As OMPs form the interface between pathogens and the host, they are likely to be relevant in host-pathogen cross-talk and their potential ability to interact with various host factors and immune system. This chapter highlights the selection of the genes encoding hypothetical membrane or extracellular proteins of *L. interrogans* for investigating the effect of individual host factors like catecholamines, oxidative stress, and temperature on the transcription of these genes under *in vitro* condition. Bioinformatics analysis of *Leptospira* genome revealed several genes which encode hypothetical proteins (n=18) and were predicted to be surface exposed or membrane-bound. Moreover, selective genes of *Leptospira* encoding proteins with known cellular localization and functions (n=26) were included to brace our bioinformatics analysis on genes encoding hypothetical proteins. The signal peptide and the antigenicity of all these selected genes were predicted through *in silico* analysis. The overall results derived from the combined computational analysis performed for the genes encoding hypothetical proteins could be correlated with the available experimental data of the proteins with known localization and functions, thus indicating the reliability of these servers. This *in silico* approach of screening immunogenic membrane proteins of *Leptospira* may help in elucidating new virulence genes, or serodiagnostic candidates for leptospirosis.

2.2 Introduction

Leptospira spp. have a larger genome (3.9 Mb) when compared to the other spirochetes like *Borrelia burgdorferi* (1.5 Mb) (Fraser et al., 1997) and *Treponema pallidum* (1.1 Mb) (Fraser et al., 1998a). All the members of the *Leptospira* genus carry two circular replicons (Zuerner, 1991). The larger chromosome (cI, >3.6 Mb) has a gene density of 75 - 92% and encode mostly housekeeping genes. The smaller replicon cII ranges from 278 – 350 kb and carry essential genes (Bourhy and Saint Girons, 2000; Zuerner et al., 1993). In the last two decades, classical research approach is being used to identify and characterize the bacterial genes encoding membrane proteins, virulence factors or vaccine and diagnostic candidates. This progress has been facilitated by the availability of whole genome sequences with improvements in bioinformatics, genome analysis, proteomic studies and the development of mutagenesis systems for pathogenic *Leptospira*. In bacterial pathogens, both cells surface-exposed outer membrane proteins and proteins secreted into the extracellular environment play crucial roles in host-pathogen interaction and pathogenesis (Viratyosin et al., 2008). Thus, the candidates for diagnostics or vaccine purpose include outer membrane and extracellular proteins, several of which have been implicated in chemotaxis, adherence and other aspects of pathogenesis (Viratyosin et al., 2008). A lot of efforts have been made to identify hypothetical membrane proteins, lipoproteins and extracellular proteins produced by *L. interrogans*, which may be used as novel targets for the development of infection markers and leptospirosis vaccines. However, till date, the success rate of these studies to reach the translational or grass-roots level is at the rock-bottom. Therefore, we intended to pinpoint the hypothetical protein candidate of *L. interrogans* in a novel approach by mimicking host stress condition that is sensed by bacteria by recognizing host stress hormone concentration and

responding by differential expression of membrane proteins required for pathogenesis and dissemination in the host tissues. The outer membrane proteins of pathogenic bacteria are mostly involved in cross-talk with the host components for their survival and pathogenesis (Cullen et al., 2004). Hence, these genes have attracted the attention of researchers for finding better diagnostics and vaccine candidates for diseases of microbial origin. The genome sequence of the pathogenic serovars of *L. interrogans* is available, and thus mining of the genes encoding membrane proteins have become easier. Bioinformatics coupled with molecular characterization of these predicted membrane proteins may help to identify the potential ligands of the pathogen which help in mediating the attachment of the bacteria to the host (Haake and Matsunaga, 2010). The pinpointing of the hypothetical protein subcellular location based on bioinformatics tools will narrow down the universe to be tested. In addition, this approach has the advantage of revealing proteins independently of their abundance and without the need of growing the microorganism *in vitro*.

In this chapter, the proteome of *L. interrogans* serovar Lai strain 56601 was screened using *in silico* approach. The genes predicted to encode hypothetical membrane proteins were selected along with some other genes encoding proteins with known sub-cellular location and function in the *L. interrogans*. The rationale for the choice of these predicted coding sequences is that the membrane and extracellular proteins are potential targets for inducing immune responses in hosts and may serve as vaccines against leptospirosis and/or for use in diagnostic tests. Subsequently, regulation of these genes was analyzed for their transcription in the presence of different host factors.

2.3 Materials and Methods

2.3.1 *Leptospira* nucleic acid and protein sequence retrieval

The Spirochete Genome Browser (SGB) and the National Center for Biotechnology Information (NCBI) databases were used to get the details of the gene sequences of *L. interrogans* serovar Lai strain 56601 (accession number NC_004342 (chromosome I) and NC_004343 (chromosome II) (Ren et al., 2003) and *L. interrogans* Copenhageni strain L1-130 (accession number AEO16823 (chromosome I) and AEO16824 (chromosome II) (Ren et al., 2003). Both these serovars of *L. interrogans* are pathogenic in nature. The SGB presents analysis of genomic data from spirochetes with an emphasis on genome comparison (http://sgb.leibniz-fli.de/cgi/index.pl?ssi=free) whereas NCBI provides access to genomic information (https://www.ncbi.nlm.nih.gov/). The protein sequences of *Leptospira* were retrieved from the UniProt database (https://www.uniprot.org/). The obtained gene sequences encoding hypothetical proteins of *Leptospira* were utilized for designing specific oligomers for quantitative reverse transcription PCR (qRT-PCR) and cloning.

2.3.2 Identification of hypothetical proteins of *L. interrogans* serovar Lai strain 56601

The selection of the *Leptospira* hypothetical proteins was done using databases such as SGB and UniProt. The subcellular localization of *Leptospira* hypothetical proteins was predicted using a CELLO (http://cello.life.nctu.edu.tw/) (Yu et al., 2006), ProtCompB (http://linux1.softberry.com/berry.phtml?topic=pcompb&group=programs&subgroup=proloc (Viratyosin et al., 2008) and SOSUIGramN (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuigramn/sosuigramn_submit.html) (Imai et al., 2008) web-based programs. The consensus prediction for each sequence was calculated using majority votes. This implies that out of the three different servers, the output result given by any two servers

(majority) was considered for the final localization of the protein. The presence and location of signal peptide cleavage sites in amino acid sequences of the predicted hypothetical and extracellular proteins of *Leptospira* was determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011) and PrediSi (http://www.predisi.de/) (Hiller et al., 2004) programs. These programs predict the presence and location of signal peptide cleavage sites in amino acid sequences in Gram-negative bacteria. The consensus voting was performed for each protein sequence using both the servers for the presence of the signal peptide. This implies that if both the servers agree for the presence of signal peptide, it was counted as a consensus vote. The characterization of the lipoprotein signal peptide region was performed manually as described previously (Setubal et al., 2006). Briefly, there are three regions of the spirochaetal lipoprotein signal peptide which are the carboxy-terminal region (C-region or lipobox), the hydrophobic (H-) region and amino-terminal (N-) region.

(I) Characterization of the lipobox/carboxyl terminal region (C-region)

The lipobox consists of 4 or 5 positions. The C-terminal region, or lipobox, of the spirochaetal lipoprotein signal peptide is defined as the four positions (−1, −2, −3 and −4) upstream of the cleavage site (position −5 is also considered for possible lipoproteins). The lipobox differs for probable and possible lipoproteins. A cysteine residue is always found in +1 position. The lipobox rules for probable lipoproteins include;

(a) Positions -1: only Alanine, Glycine, Serine, Asparagine or Cysteine are allowed.

(b) Positions -3 or -4: at least one of these positions should contain at least one of Leucine, Isoleucine, Valine or Phenylalanine;

(c) The charged amino acids Lysine, Arginine, Aspartic acid, Glutamic acid and Histidine are forbidden anywhere in the lipobox.

The lipobox rules for possible lipoproteins include;

(a) Position -1: in addition to Alanine, Glycine, Serine, Asparagine and Cysteine, the related amino acids such as Glutamaine and Threonine are also allowed.

(b) Position –5 is also considered to be part of the lipobox. This position should contain at least one of Leucine, Isoleucine, Valine or Phenylalanine amino acids.

(c) In addition to Leucine, Isoleucine, Valine and Phenylalanine, the hydrophobic amino acids Tyrosine and Methione are also included as possible amino acids required in positions -3,-4 or -5.

(II) Characterization of the hydrophobic region (H-region)

The charged residues like Lysine, Arginine, Aspartic acid, Glutamic acid and Histidine are forbidden in the H-region. The H-region is at least 7 amino acids long for probable lipoproteins and 6 amino acids long for possible lipoproteins.

(III) Characterization of the amino-terminal region (N-region)

In a lipoprotein signal peptide, the N-terminal region should be always positively charged. It extends from the first residue to the last charged residue (i.e. Lysine, Arginine, Aspartic acid, Glutamic acid or Histidine).

The genes encoding such lipoproteins should have 50 amino acids downstream to the +1 position.

2.3.3 Identification of orthologs in the different serovars of *L. interrogans*

The orthologs of the shortlisted genes of *L. interrogans* Lai were identified in other pathogenic serovars of *Leptospira* using Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) (Kanehisa and Goto, 2000) and OrthoDB program (https://www.orthodb.org/) (Waterhouse et al., 2012). KEGG is a large collection of databases having entries of genes, proteins, pathways in metabolism and diseases, drug and ligands for different organisms whereas OrthoDB program is a comprehensive catalog of orthologs.

2.3.4 Prediction of antigenicity of the selected proteins of *L. interrogans*

To determine the ability of the selected proteins to induce the immune response in the host (antigenicity), all the selected leptospiral protein sequences were submitted to the VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with the default threshold set at 0.4 and the target organism set as Bacteria (Doytchinova and Flower, 2007). The VaxiJen is a reliable and consistent tool for the prediction of protective antigens and subunit vaccines. Thus, it identifies the probable antigens which could induce immune response in the host.

2.4 RESULTS

2.4.1 Identification of hypothetical proteins in *L. interrogans* serovar Lai strain 56601

In silico analysis was performed to identify selective gene transcripts used in the present study to analyze the effect of different host factors on their transcription. The bioinformatics workflow followed for this selection procedure is shown in Fig. 2.1.

The proteome of the *L. interrogans* serovar Lai strain 56601 was screened using the SGB and NCBI database to identify the potential genes encoding hypothetical/putative proteins in the present study. The hypothetical/putative proteins were shortlisted by screening the proteome of *L. interrogans* serovar Lai strain 56601 in the databases. Along with this, for the screening of diagnostic markers of leptospirosis, the prediction of subcellular-localization of the leptospiral proteins was performed. The hypothetical proteins were shortlisted by screening the proteome of *L. interrogans* serovar Lai strain 56601 in the databases. A total of 18 genes encoding hypothetical lipoproteins were shortlisted based on the subcellular localization. The different subcellular localization programs (CELLO, SOSUI-GramN, and ProtCompB) predicted 10 hypothetical proteins/lipoproteins (i.e. LA3276, LA3064, LA0426, LA1569, LA3501, LA3210, LA3200b, LA3394, LA3340, and LB047) to be secreted in the extracellular space of *L. interrogans*. Three lipoproteins (i.e. LA3440, LA4185 and LA1939) were predicted to be located in the outer membrane and 2 hypothetical proteins (i.e. LA3263 and LA3230) were predicted to be located in the inner membrane of *L. interrogans*. A total of 3 lipoproteins (i.e. LA3446, LA3262 and LA3017) were identified to be located in periplasmic space of *L. interrogans*. All these hypothetical proteins were identified to contain a signal peptide for translocation from the cytoplasm to the membrane by both the SignalP and PrediSi servers as shown in Table 2.1.

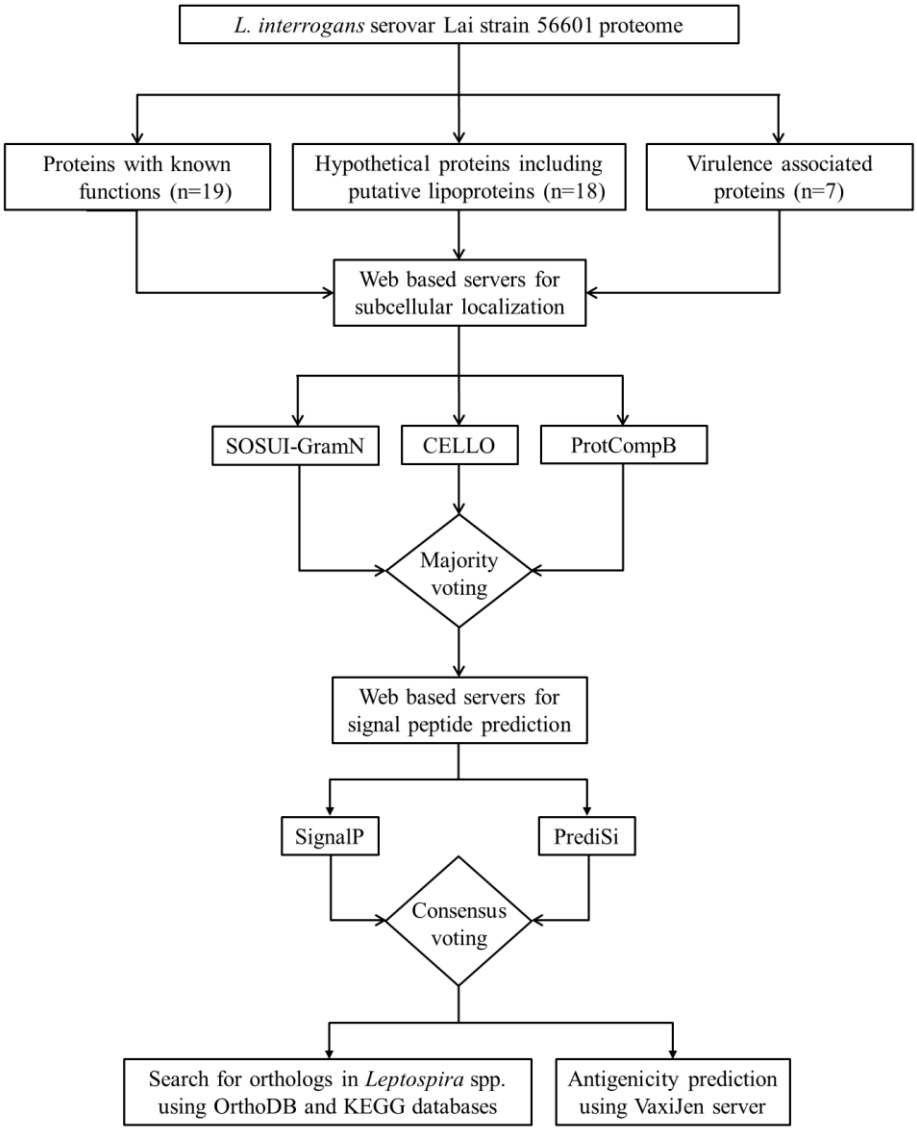

Figure 2.1. Bioinformatics workflow for the selection of the leptospiral proteins used in the present study. The complete proteome of *L. interrogans* serovar Lai strain 56601 was analyzed. Proteins with known functions and associated with virulence were shortlisted based on previous reports. The subcellular localization of the hypothetical proteins was predicted using CELLO, SOSUIGramN, and ProtCompB online servers. If 2 of the 3 servers agreed for the localization, it was assigned as a majority vote. The signal peptide for the hypothetical proteins was predicted by SignalP and PrediSi servers. If both the servers agreed for the presence of signal peptide, it was assigned as a consensus vote. The prediction of the antigenicity of all the 44 proteins selected through *in silico* analysis was predicted by VaxiJen server.

Table 2.1. *In silico* analysis of the genes encoding hypothetical proteins in *L. interrogans* Lai along with their subcellular localization

Sr. No	Type of protein	Lai locus	UniProt ID	Ortholog in Copenhageni	UniProt ID	Subcellular localization[a]	Signal peptide[b]	Antigenic protein
1	**Hypothetical proteins**	LA3276	Q8F161	LIC10868	Q72TZ6	EX	Yes	Yes
2		LA3263	Q8F174	LIC10878	Q72TY6	IM	Yes	Yes
3		LA3230	Q8F1A6	LIC10906	Q72TV8	IM	Yes	Yes
4	**Hypothetical lipoproteins**	LA3064	Q8F1R3	LIC11030	Q72TJ1	EX	Yes	Yes
5		LA0426	Q8F8X3	LIC10373	Q72VC8	EX	Yes	Yes
6		LA1569	Q8F5U8	LIC12209	Q72QA3	EX	Yes	Yes
7		LA3446	Q8F0P5	LIC10730	Q72UC9	P	Yes	Yes
8		LA3501	Q8F0J2	LIC10686	Q72UH1	EX/OM	Yes	Yes
9		LA3440	Q8F0Q2	LIC10734	Q72UC5	OM	Yes	No
10		LA3262	Q8F175	LIC10879	Q72TY5	P	Yes	No
11		LA3210	Q8F1C6	LIC10920	Q72TU6	EX	Yes	Yes
12		LA3200b	D4YVY7	LIC10927	Q72TT9	EX	Yes	Yes
13		LA3017 (LemA)	Q8F1W1	LIC11058	Q72TG3	P	Yes	Yes
14		LA3394	Q8F0U7	LIC10774	Q72U87	EX	Yes	Yes
15		LA3340	Q8F100	LIC10821	Q72U43	EX	Yes	Yes
16		LB047	Q8EY10	LIC20035	Q75G29	EX	Yes	Yes
17		LA4185	Q8EYM8	LIC13341	Q72M50	OM	Yes	No
18		LA1939	Q8F4V0	LIC11966	Q72QY9	OM/ EX	Yes	Yes

EX: Extracellular, OM: Outer membrane, P: Periplasm and IM: Inner membrane

[a] Majority voting among CELLO, SOSUI-GramN and ProtCompB servers.

[b] Consensus voting among SignalP and PrediSi servers.

To identify *L. interrogans* serovar Lai orthologs in the other pathogenic serovar of *L. interrogans*, i.e. *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, OrthoDB, and KEGG database were used, the results of which are summarized in Table 2.1. The antigenicity of the shortlisted hypothetical proteins was determined by analyzing the protein sequences of each hypothetical protein using VaxiJen server. An overall score depicting their antigenicity indicated their potential to evoke the immune response of the host. Among the 18 selected hypothetical lipoproteins, only 3 proteins (i.e. LA3440, LA3262 and LA4185) were predicted to be non-antigenic, having scores of 0.3047, 0.3602 and 0.2656 respectively (Table 2.1).

In spirochetes, lipoproteins form the most significant proteins in the entire membrane protein profile. It has been shown in the previous reports that in *Leptospira* and *Borrelia*, the differential expression of lipoproteins is an important step in the transition of the pathogen from free-living form to the mammalian host conditions (Chamberlain et al., 1988; Haake, 2000; Howe et al., 1985). Large numbers of spirochetal lipoproteins have been shown to the candidates of the protective immune response highlighting the importance of these proteins in the pathogenesis of spirochetal diseases. To characterize these selected lipoproteins, the lipobox along with the lipoprotein signal peptide region was predicted manually as based on the guidelines previously reported (Setubal et al., 2006). As per the criteria set forth by Setubal, there are two types of lipoproteins which can be predicted in spirochetes. Probable lipoproteins are those which strictly follow the rules set for predicting the protein to be a lipoprotein. Possible lipoproteins are additional lipoproteins predicted by altering the rules slightly to accommodate these lipoproteins as detailed in section 2.3.2 (Fig. 2.2).

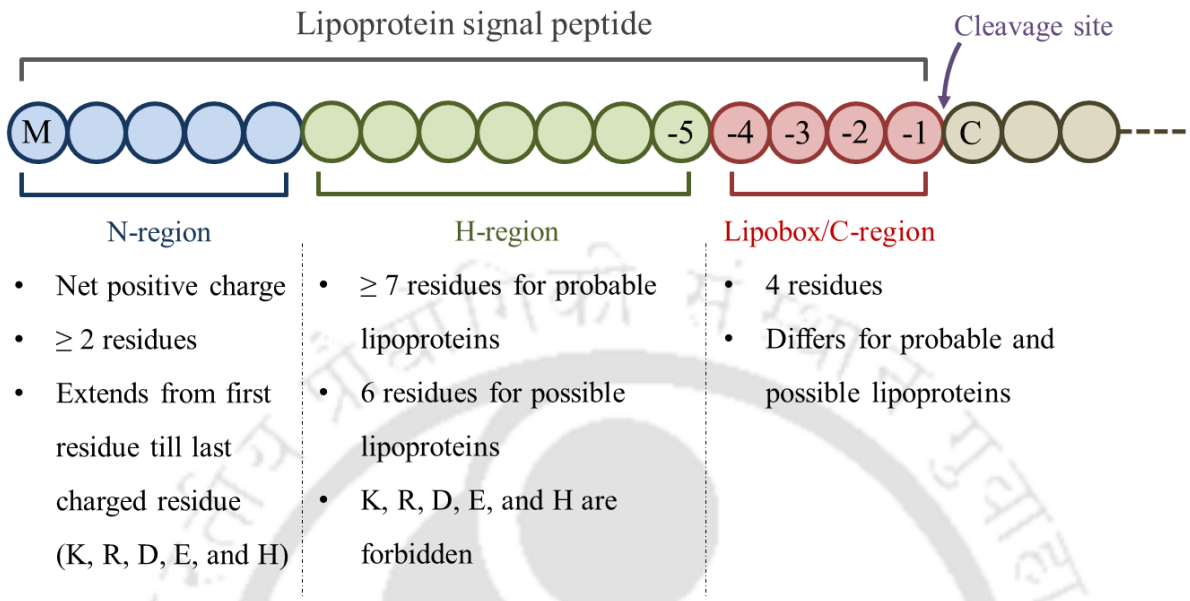

Figure 2.2. Determination of the spirochetal lipoprotein signal peptide regions. The amino-terminal region (N-region) extends from the methionine (M) to the last charged residue. The hydrophobic region (H-region) starts from the last charged residue to the –5 position and is at least 6 residues long. The lipobox or the carboxyl-terminus region (C-region) is 4 residues long. Charged amino acids such as lysine (K), arginine (R), aspartic acid (D), glutamic acid (E) and histidine (H) are prohibited in the H and C-regions. The cysteine (C) becomes the amino-terminal residue after the lipoprotein signal peptide is removed by the lipoprotein signal peptidase (Adapted from Setubal et al., 2006).

Based on the above guidelines, the 15 selected hypothetical lipoproteins of *L. interrogans* serovar Lai were characterized for lipoprotein signal peptide (Table 2.2). Interestingly, the genes *LA3340* and *LA4185* are designated to encode hypothetical lipoproteins by both NCBI and UniProt databases, however, the protein sequences could not be predicted as lipoproteins as per the previously set criteria (Setubal et al., 2006). It is also worth mentioning that the orthologs of *LA3340* and *LA4185* in *L. interrogans* serovar Copenhageni which were

designated to encode hypothetical lipoproteins by both NCBI and UniProt databases could be manually predicted as lipoproteins (Table 2.3).

Table 2.2. The determination of N, H, and C (lipobox) regions of the predicted hypothetical lipoproteins of *L. interrogans* serovar Lai

Sr No.	Lai locus	N-region sequence	H-region sequence	Lipobox (C-region)
1	LA3064	MTR	FLLLCLSIIL	LLGGC
2	LA0426	MIR	LIILIFII	FFQAC
3	LA1569	MK	YLYILLFI	FIQAC
4	LA3446	MDSKFVVR	FFLICFFCASI	ITTNC
5	LA3501	MILFKILTSEISKYISFRNGYKKQKGFNFANQMKLK	YIFPILLIL	FFFSC
6	LA3440	MKLILTFANKFRKRILTLIQSLR	FFLKLALIVF	GVSSC
7	LA3262	MKR	NITLVVLSIGIAL	LLGAC
8	LA3210	MKR	FYIGFLSSIFFAA	IFLNC
9	LA3200b	MKNQKWKKEKGLKKMKR	WILYITTICLFL	TFINC
10	LA3017 (LemA)	MKTKIDFK	LFLITTLTLL	VFSNC
11	LA3394	MK	QILSITLFM	FLVGC
12	LA3340	-[a]	-[a]	-[a]
13	LB047	MKR	FCLAIVTV	AISYC
14	LA4185	-[a]	-[a]	-[a]
15	LA1939	MKKHSISK	IIITACCIFL	LTYGC

[a] - applies for not being able to manually detect the spirochetal lipoprotein signal peptide regions.

Table 2.3. The determination of N, H and C (lipobox) regions of the orthologs of LA3340 and LA4185 in *L. interrogans* serovar Copenhageni

Sr. No.	Lai locus	Ortholog in Copenhageni	N-region sequence	H-region sequence	Lipobox (C-region)
1	LA3340	LIC10821	MK	QIFSIWLAALL	LFSGC
2	LA4185	LIC13341	MNPFLFLLRKIFSASFIVGFKIQISRLKLCRYGR	FQTTCLALFILLQA	LFVAC

The amino acid residues encompassing the lipobox regions present in the lipoproteins of *L. interrogans* when compared with those of *E. coli*, showed significant differences between the two bacterial species (Gonnet et al., 2004). As compared to *E. coli*, a considerable variation in the amino acid residues in the leptospiral lipobox was observed (Setubal et al., 2006). Nevertheless, experimental evidence is essential for confirming a protein to be a lipoprotein which is covalently modified by fatty acids at its amino-terminal cysteine (Haake, 2000).

2.4.2 Selection of genes encoding proteins of known functions in *L. interrogans* serovar Lai strain 56601

In the present study, many genes encoding proteins of known functions based on previous reports were included to validate our bioinformatics analysis (Table 2.4). A total of 26 genes of *L. interrogans* serovar Lai proteins with known functions were selected. While selecting these genes, major emphasis was given to the genes encoding proteins which have direct or indirect role in regulating the iron uptake in *L. interrogans* (n=10). Catecholamines have been showed to act as siderophores which bind to the ferric iron binding proteins of the host like lactoferrin and transferrin, thereby liberating iron for uptake and helping the bacteria to survive in the media or inside the host (Lo et al., 2010). All these 10 iron uptake proteins (i.e. LB187, LB191, LB183, LA2579, LB186, LA4253, LB194, LA1005, LA1796 and LA0634) were previously established to help *Leptospira* to survive in the media or in the host (Lo et al., 2010). The other genes encoding proteins with known functions (n=9) were selected to understand if these essential proteins were getting differentially expressed in the presence of host factors. LA3371 (MdoC) is involved in the periplasmic glucans synthesis in Gram negative-bacteria (Lacroix et al., 1999). LA3294 (UppS) is involved in the polyprenol biosynthetic pathway which is essential for bacterial peptidoglycan synthesis (Lee and

Helmann, 2013). The protein LA3307 (Rfe) is important for the bacterial lipopolysaccharide (LPS) synthesis which is one of the virulent factor of *L. interrogans* (Murray et al., 2010). The protein LA3434 (AmiC) is responsible for the breakdown of peptidoglycan layer and its recycling during cell growth (Garcia and Dillard, 2006). LA3468, LA3247 and LA3258 are essential for transmembrane transport (Grassmann et al., 2017b; Louvel et al., 2006). The protein LA3444 is involved in penicillin binding (Fernandes et al., 2014) whereas LA0957 is an important TolC like efflux protein in *L. interrogans* (Caimano et al., 2014).

Several other genes encoding virulence proteins of *L. interrogans* were also included in the present study (n=7) based on previous reports as detailed in Table 2.5. *LA0616* (*lipL41*) encodes a hemin binding protein (Asuthkar et al., 2007) whereas *LA3961* (*ompL36*) is a flagellar component of *L. interrogans* (Pinne and Haake, 2009). The gene *LA2637* (*lipL32*) encodes the most abundant protein in pathogenic *Leptospira* which was found to bind to a wide range of extracellular matrix components (i.e. laminin, fibronectin, collagen type IV) and was expressed during infection (Haake et al., 2000b). Both the genes namely *LA3778* (*ligB*) and *LA1691* (*lipL53*) are found to interact with a variety of extracellular matrix components like fibronectin and collagen type IV (Choy, 2012; Oliveira et al., 2010). The *LA3138* encodes the porin, OmpL1 which interacts with laminin, fibronectin and plasminogen (Haake et al., 1999). The gene *LA0222* encodes for the Loa22 protein which is surface exposed and binds peptidoglycan through its OmpA domain. This protein is one of the most well characterized virulent factors of *L. interrogans* (Ristow et al., 2007). The differential transcription of these set of virulence genes in the presence of host factors was studied in the next chapter.

Table 2.4. *In silico* analysis of the genes encoding proteins of known functions in *L. interrogans* serovar Lai

Sr. No.	Lai locus	UniProt ID	Ortholog in Copenhageni	UniProt ID	Function	Reference	Subcellular localization*	Signal peptide	Antigenic protein
Iron uptake proteins									
1	LB187	Q8EXM1	LIC20149	Q75FN3	Multidrug efflux transporter	(Lo et al., 2010)	IM	Yes	Yes
2	LB191 (HbpA)	Q8EXL7	LIC20151	Q75FN1	TonB dependent outer membrane receptor/ Hemin-binding protein A	(Lo et al., 2010)	OM	Yes	Yes
3	LB183 (Fur)	Q8EXM5	LIC20147	Q75FN5	Ferric uptake regulator	(Lo et al., 2010)	CYT	No	Yes
4	LA2579 (FeoB)	Q8F332	LIC11402	Q72SI0	Ferrous transporter	(Lo et al., 2010)	IM	Yes	Yes
5	LB186 (Hol)	Q8EXM2	LIC20148	Q75FN4	Heme oxygenase	(Lo et al., 2010)	CYT	No	No
6	LA4253 (FepB)	Q8EYG3	LIC13403	Q72LZ0	Substrate binding protein of an ABC transporter complex	(Lo et al., 2010)	CYT	No	No
7	LB194	Q8EXL4	LIC20153	Q75FM9	Hypothetical lipoprotein	(Lo et al., 2010)	EX	Yes	Yes
8	LA1005	Q8F7E0	LIC12655	Q72P21	Aminopeptidase	(Lo et al., 2010)	CYT	No	Yes
9	LA1796	Q8F588	-	-	Hypothetical protein	(Lo et al., 2010)	CYT	No	Yes
10	LA0634 (DppC)	Q8F8C3	LIC12953	Q72N84	Dipeptide/ oligopeptide ABC transport system permease	(Lo et al., 2010)	IM	Yes	Yes

* EX: Extracellular, OM: Outer membrane, P: Periplasm and IM: Inner membrane

Continued…

Table 2.4. *In silico* analysis of the genes encoding proteins of known functions in *L. interrogans* serovar Lai

Sr. No.	Lai locus	UniProt ID	Ortholog in Copenhageni	UniProt ID	Function	Reference	Subcellular localization*	Signal peptide	Antigenic protein
Other selected proteins of known functions									
11	LA3371 (MdoC)	Q8F0X0	LIC10792	Q72U70	Glucans biosynthesis	(Lacroix et al., 1999)	IM	Yes	Yes
12	LA3294 (UppS)	Q8F144	LIC10854	Q72U10	Isoprenyl transferase	(Lee and Helmann, 2013)	CYT	No	No
13	LA3307 (Rfe)	Q8F131	LIC10841	Q72U23	Undecaprenyl-phosphate alpha-N-acetylglucosaminyl-transferase	(Murray et al., 2010)	IM	Yes	Yes
14	LA3434 (AmiC)	Q8F0Q8	LIC10739	Q72UC0	N-acetylmuramoyl-L-alanine amidase	(Boneca, 2005; Garcia and Dillard, 2006)	OM	Yes	Yes
15	LA3468 (Smc)	Q8F0M4	LIC10714	Q72UE3	Outer membrane receptor for Fe3+-dicitrate/TonB-dependent receptor	(Grassmann et al., 2017b)	OM	Yes	Yes
16	LA0957	Q8F7I8	LIC12693	Q72NY5	TolC like outer membrane efflux protein	(Caimano et al., 2014; Nally et al., 2007)	OM	Yes	Yes
17	LA3444	Q8F0P8	LIC10731	Q72UC8	Penicillin binding protein	(Fernandes et al., 2014)	CYT	No	Yes
18	LA3247	Q8F189	LIC10889	Q72TX5	TonB	(Louvel et al., 2006)	IM	Yes	Yes
19	LA3258	Q8F179	LIC10881	Q72TY3	TonB dependent receptor	(Louvel et al., 2006)	OM	Yes	Yes

* EX: Extracellular, OM: Outer membrane, P: Periplasm and IM: Inner membrane

Table 2.5. *In silico* analysis of the genes encoding proteins associated with virulence in *L. interrogans* serovar Lai

Sr. No.	Lai locus	UniProt ID	Ortholog in Copenhageni	UniProt ID	Function	Reference	Subcellular localization	Signal peptide	Antigenic protein
1	LA0616 (LipL41)	Q8F8E1	LIC12966	Q72N71	Hemin binding protein	(Asuthkar et al., 2007; King et al., 2013)	CYT	No	Yes
2	LA2637 (LipL32)	LA2637	LIC11352	Q72SM7	Binds calcium, laminin, fibronectin, collagen IV, and plasminogen	(Haake et al., 2000b)	P	Yes	Yes
3	LA3961 (OmpL36/ FcpA)	Q8EZ95	LIC13166	Q72MM7	Flagellar component	(Pinne and Haake, 2009; Wunder et al., 2016)	OM	Yes	Yes
4	LA3778 (LigB)	Q8EZS3	LIC10464	Q72V39	Binds elastin, fibronectin, fibrinogen, collagen I, and collagen IV	(Choy, 2012)	EX	Yes	Yes
5	LA3138 (OmpL1)	G1UB30	LIC10973	Q72TP4	Porin, binds laminin, fibronectin, and plasminogen	(Haake et al., 1999)	EX	Yes	Yes
6	LA1691 (LipL53)	Q8F5J0	LIC12099	Q72QL4	Binds laminin, fibronectin and collagen IV	(Oliveira et al., 2010)	EX	Yes	Yes
7	LA0222 (Loa22)	Q8F9H3	LIC10191	Q72VV5	Binds peptidoglycan through OmpA domain	(Ristow et al., 2007)	P	Yes	Yes

* EX: Extracellular, OM: Outer membrane, and P: Periplasm

2.5 DISCUSSION

One of the essential principles for any vaccine or diagnostic candidate development is the evaluation of all the potential candidates encoded in the genome of *L. interrogans* through *in silico* screening. In the present study, this initial screening was carried out using bioinformatics to identify the targets and was further validated using *in vitro* assays in the present study. Outer membrane proteins may evoke immune response during host infection and therefore, constitute targets for generating innate protection through mechanisms such as antibody-dependent phagocytosis and complement-mediated killing. It is also the need of the hour to annotate and characterize hypothetical proteins in *L. interrogans*. Interestingly, 78% of the *L. interrogans* specific genes have no defined function and have been classified as hypothetical in nature (Adler et al., 2011). These hypothetical proteins may have a significant role in producing many virulence factors which help to successfully cause leptospiral infection in the host. A proportion of the membrane/extracellular proteins might act as antigens expressed during infection and could be recognized by the host immune system. Therefore, they may serve as the basis to develop recombinant protein-based serological tests.

Development of potential bioinformatics tools and databases has opened a new platform for *in silico* study of pathogenic bacteria without the unwanted sacrifice of laboratory animals. These *in silico* screened hypothetical proteins may ease the identification of novel proteins, having an impact in the pathogenesis of leptospirosis. The computational prediction of the hypothetical proteins can now be easily performed using databases such as UniProt and NCBI (Viratyosin et al., 2008). Along with this, another key step for the recognition for the

potential diagnostic and vaccine targets for leptospirosis is the prediction of subcellular localization of the proteins. In this study, we have used three localization servers namely CELLO, SOSUIGram-N and ProtCompB server for the cellular prediction. We have used a majority voting for the identification of the localization of these proteins in a similar fashion reported in other studies (Grassmann et al., 2017b; Viratyosin et al., 2008). This majority voting among diverse servers available ensures to reduce false possible predictions. It is important to check for signal peptides to identify if the protein is an outer membrane or extracellular in nature. In this study, we have utilized two signal prediction servers namely SignalP and PrediSi to validate the output results. A consensus voting between the two servers indicated that the identification of signal peptides was reliable as described previously (Grassmann et al., 2017b). We have checked the antigenicity of the selected proteins in an attempt to scrutinize them for their use in the diagnostic or vaccine candidates in the near future. Using the VaxiJen server, out of the 44 genes selected, 38 genes were found to be encode antigenic proteins. Among the 7 genes (i.e. *LA0616*, *LA2637*, *LA3961*, *LA3778*, *LA3138*, *LA1691*, and *LA0222*) previously described to be associated with the virulence of *Leptospira*, all of these proteins encoded by these genes were detected as antigenic by the VaxiJen server indicating its reliability for the analysis.

Leptospiral lipoproteins were reported to be vaccine and diagnostic candidates for leptospirosis (Haake, 2000). The UniProt database revealed several hypothetical lipoproteins in *L. interrogans*. However, we have selected those lipoproteins which are predicted to be either extracellular or associated with the membrane of the bacteria (Grassmann et al., 2017b). A manual detection of the lipoprotein signal peptide was done based on previously laid criteria (Setubal et al., 2006). All the lipoproteins contained the requisite leptospiral

lipoprotein signal peptide which is cleaved by the lipoprotein signal peptidase to render the cysteine as the amino-terminal residue of the mature lipoprotein (Haake and Zückert, 2015). However, experimental validation of lipidation in the selected proteins is important before annotating a protein to be a lipoprotein.

Most of the proteins selected in the present study were found to be extracellular and antigenic in nature, indicating the possibility of these proteins to be interrogated as diagnostic or vaccine targets. Extracellular proteins are easily exposed to the host immune system, indicating the involvement of such proteins in the immunopathogenesis of leptospirosis or as potential vaccine and diagnostic candidates (Grassmann et al., 2017b). The *in silico* approach used in the present study is the consensus voting among all the servers used to correctly characterize a protein. However, it is important to note that no *in silico* analysis is accurate without the validation through experiments (Viratyosin et al., 2008).

Thus, the present chapter deals with the *in silico* analysis of the genes encoding hypothetical proteins with an emphasis on the subcellular localization and antigenicity of these proteins using various servers. A total of 11 different bioinformatics programs and databases were used to screen the *L. interrogans* serovar Lai proteome which resulted in the shortlisting of 44 genes encoding hypothetical proteins. It is now well documented that host factors like temperature, pH, osmolarity, oxidative stress, hormones, etc. influence the gene expression profile of *Leptospira* (Xue et al., 2010). Similarly, several studies have shown that catecholamines play a crucial role in triggering pathogenic responses in bacterial diseases by facilitating bacterial growth in the host through numerous mechanisms (Pan et al., 2014). The transcriptome analysis of the genes encoding the selected proteins in the presence of different host factors has been performed in the subsequent chapter using quantitative reverse qRT-

PCR. This is an attempt to use the *in silico* approach along with experimental evidence to shortlist proteins which could be important as vaccine and/or diagnostic candidates for leptospirosis.

Chapter 3

Analysis of L. interrogans selective gene transcripts in the presence of host factors under in vitro condition

CHAPTER 3

Analysis of *L. interrogans* selective gene transcripts in the presence of host factors under *in vitro* condition

3.1 Abstract

The host specificity of bacterial pathogens is determined by interactions between the pathogens and their host factors. It is established that host stress hormones like catecholamines are one of the host factors which stimulates the adaptation of various pathogenic microbes in the human or animal body upon infection. Temperature and oxidative stress are other known host factors which have been interrogated to quantify the transcription of several genes in pathogenic bacteria including *Leptospira*. In this chapter, the effect of host factors namely, catecholamines, temperature, and oxidative stress on *Leptospira* genes encoding OMPs was investigated. Supplementation of catecholamines to the growth bacteria had no impact on the *in vitro* growth pattern of *L. interrogans,* however, 7 genes namely *LA0616, LA3961, LB186, LB047, LA3307, LB191* and *LA3263* out of 44 were differentially transcribed and the effect of which was reversed to basal level in the presence of its antagonist propranolol. Notably, these 7 genes responding to catecholamines were also getting differentially expressed in the presence of oxidative stress. On the contrary, four of the seven genes namely *LA0616, LA3961, LB191* and *LB047* were found to respond to shift in temperature from 29ºC to 37ºC. Importantly, two of the genes *LA3307* and *LA3263* showed differential upregulation when individually grown in the presence of the host factors tested. Similar pattern of downregulation was observed for *LB047* in the presence of host factors tested individually suggesting that these host factors work in combination to modulate the genes important for successful infection. These findings highlight that host factors

including catecholamines are important signals for the modulation of genes encoding OMPs by *L. interrogans* during adaptation from the environment to host.

3.2 Introduction

Leptospira are classified as spirochetes which may be pathogenic or non-pathogenic with a variety of different habitats in nature. *L. interrogans* can infect most mammals throughout the world, as well as reptiles, amphibians, fish, birds, and invertebrates. The organism is maintained in nature by virtue of persistent colonization of renal tubules of carrier animals (Bharti et al., 2003).

Host specificity is defined by the ability of the pathogen to infect a host (Kirzinger and Stavrinides, 2012). The host specificity of bacterial pathogens is determined by multiple physical, chemical and biological interactions between the pathogens and their hosts (Pan et al., 2014). Some of these host factors which lead to adaptation of various pathogenic microbes include stress hormones (Bansal et al., 2007; Chen et al., 2006; Hendrickson et al., 1999; Lyte, 2004; Lyte et al., 1997; Lyte et al., 1996; Vlisidou et al., 2004; Voigt et al., 2006), temperature (Lo et al., 2006; Nally et al., 2001c), pH (Khairani-Bejo et al., 2004; Smith and Turner, 1961), osmolarity (Matsunaga et al., 2007a; Matsunaga et al., 2007b; Matsunaga et al., 2005; Takabe et al., 2013), and oxidative stress (Eshghi et al., 2012a; Pohanka, 2013; Sánchez-López et al., 2012).

Outer membrane proteins of *Leptospira* are core components by which pathogenic *Leptospira* interact with the host and play an essential role as adhesins, receptors for various host molecules, and key mediators for adaptation to change in the diverse environment. There are three classes of the outer membrane protein of *Leptospira* identified till date viz.

outer membrane lipoprotein, transmembrane protein, and the peripheral membrane protein (Cullen et al., 2004). Several leptospiral OMPs have been reported to have adhesion capacity with extracellular matrix component of host and are an essential requisite for tissue invasion. Several proteins of *Leptospira* bind to laminin and plasma fibronectin-which indicates that leptospires have a redundant pool of adhesion molecules which are probably part of their invasion strategies, however, the exact role of such proteins is still not clear (Vieira et al., 2014).

Taking consideration of above points, this study was aimed at understanding modulation of gene expression in *L. interrogans* mimicking host like environment under *in vitro* condition namely, catecholamines, oxidative stress in the form of hydrogen peroxide and elevated temperature of 37ºC. We report a selective transcript analysis of genes encoding outer membrane proteins of *L. interrogans* Copenhageni in response to epinephrine/norepinephrine and its antagonist propranolol using qRT PCR technique. This is an initial step towards the comprehensive understanding of the effect of catecholamines on the expression of membrane proteins of *L. interrogans*. We also demonstrate that these selective genes encoding membrane proteins and several extracellular proteins also respond to physiological temperatures and oxidative stress condition.

3.3 Materials and Methods

3.3.1 Bacterial strains, media and growth condition

The bacterial strains *L. interrogans* serovar Lai strain Lai and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were obtained from Indian Council of Medical Research (ICMR), Regional Medical Research Centre, Port Blair, Andaman, and Nicobar Island, India. Spirochetes were grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium

(Difco) maintained at 29°C with 10% heat-inactivated rabbit serum (56°C for 30 min) unless stated. To prevent the growth of contaminants in the leptospiral cultures, 5 Fluorouracil (100 µg/mL) was added to EMJH medium.

3.3.2 Leptospiral growth conditions for gene expression studies using different host factors

The host specificity of bacterial pathogens is determined by multiple molecular interactions between the pathogens and their hosts (Pan et al., 2014). Most pathogens are capable of infecting multiple hosts (Woolhouse et al., 2001). Host factors generally include temperature, pH, osmolarity, hormones, oxidative stress, etc.

3.3.2.1 Catecholamines as host factor

For evaluating *Leptospira* growth rate and gene transcription, 2×10^3 spirochetes were seeded per mL of EMJH medium. To this, catecholamines (Epinephrine [Epi] or Norepinephrine [NE]; Sigma, USA) and its antagonist propranolol (PO) were added in combination or alone to a final concentration of 500 µM until it reached log-phase as described before (Li et al., 2009). A control without the addition of the catecholamines and/or propranolol was also included in the study. Stock solutions of epinephrine (200 mM), norepinephrine (200 mM) and propranolol (150 mM) were prepared and stored at 4ºC for short-term use. All the hormones were dissolved in double autoclaved distilled water to prepare the stock solutions. The spirochetes were identified and counted every 24 h for five days under phase contrast microscopy (CX41, Olympus) using Petroff-Hausser cell counting chamber (Touff, Cat No. CCB100) as per manufacturer's protocol. Briefly, the frame of the counting chamber is composed of 9 large squares each with a 1 mm^2 area (Fig. 3.1). The large central square is subdivided into 25 medium squares of 0.2 mm sides. Each medium square consists of 16

small squares with 0.05 mm sides, each having an area of 0.0025 mm^2. The four large squares in the corners of the frame are subdivided into 16 medium squares with 0.25 mm sides. The depth of the counting chamber is 0.1 mm.

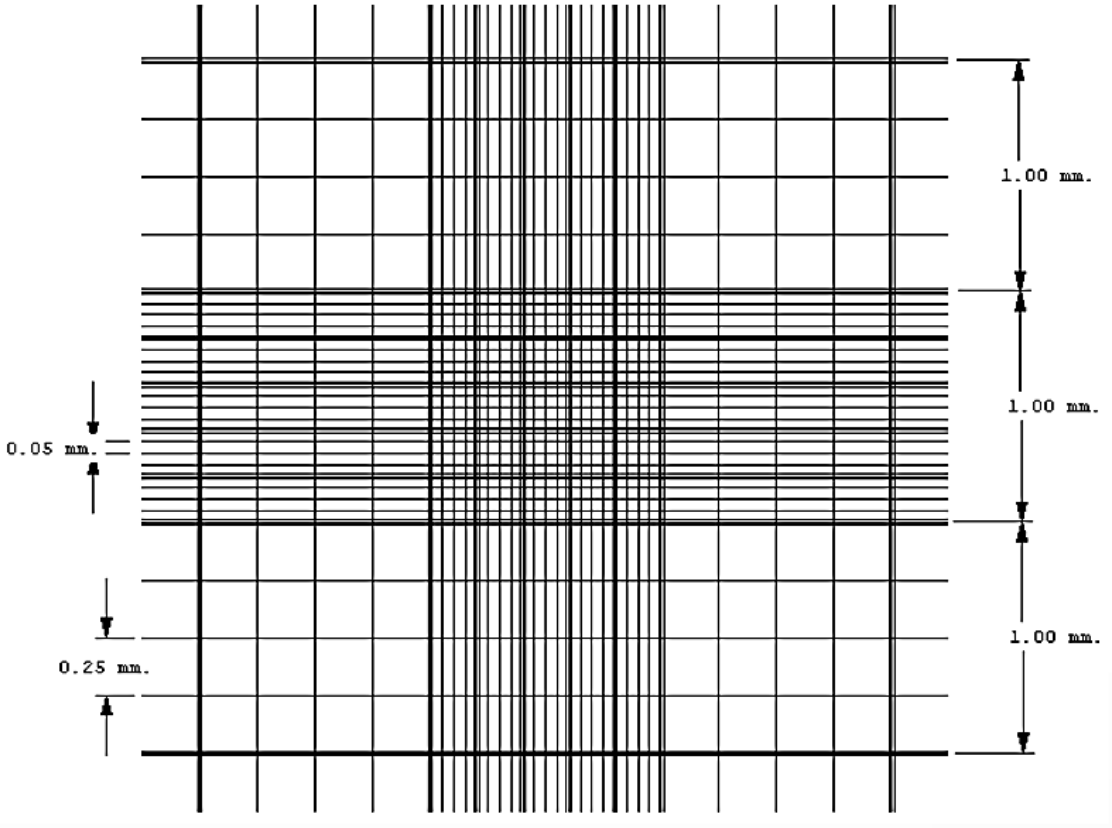

Figure 3.1. Petroff-Hausser Counting Chamber. This cell counter consists of 9 large squares each with an area of 1 mm^2. The four large squares in the corners are subdivided into 16 medium squares with 0.25 mm sides. The depth of the counting chamber is 0.1 mm. *L. interrogans* was counted in the corner squares and the average of the count was taken (Source: http://hausserscientific.com/products/petroff_hausser_counter.html).

The counting of *L. interrogans* was performed in the corner squares and the average of the bacterial count was taken. The total number of bacterial cells per milliliter of EMJH was determined by the average number of cells and the area counted. As the culture was diluted

before counting, the final count of *Leptospira* per milliliter of EMJH medium was achieved by multiplying the total count with the dilution factor used. The total RNA was isolated from the log-phase growing *L. interrogans* in the presence and absence of catecholamines or its antagonist, using TRIzol reagent (Invitrogen) according to the manufacturer's instructions (Chomczynski, 1993; Fraser and Brown, 2017).

3.3.2.2 Oxidative stress as host factor

The effect of oxidative stress using hydrogen peroxide on the transcription of selected genes of *L. interrogans* was investigated under *in vitro* growth condition. For gene expression profiling, spirochetes were grown to 4×10^8 spirochetes/mL at 29ºC. This culture was then diluted to 3×10^8 spirochetes/mL in fresh EMJH medium (without hydrogen peroxide) or EMJH medium containing hydrogen peroxide at a final concentration of either 1 mM or 10 mM for providing oxidative stress to *L. interrogans*. Culture without the addition of hydrogen peroxide was used as a control. All the cultures were incubated for 1 h at 37ºC. The cells were centrifuged after incubation and total RNA was isolated using the TRIzol method as per the manufacturer's protocol (Chomczynski, 1993; Fraser and Brown, 2017).

3.3.2.3 Temperature as host factor

L. interrogans was grown in EMJH medium at 29°C to a density of 1×10^8 spirochetes/mL. The culture was diluted 6×10^6 spirochetes/mL in fresh EMJH medium and incubated at 37°C till a count of 2×10^8 spirochetes/mL was achieved. A culture growing at 29°C was taken as the experimental control. These spirochetes were then harvested and the total RNA was isolated using TRIzol method (Chomczynski, 1993; Fraser and Brown, 2017).

The total RNA was reconstituted in DEPC treated nuclease free water and 1 µg of the RNA (DNase treated) was used to synthesize its complementary strand DNA (cDNA) using random hexamers of the Verso cDNA synthesis kit (Thermo Scientific) and as per manufacturer's protocol. The RT PCR reaction used in the study is described below.

Components	**Volume (Final concentration)**
5X cDNA synthesis buffer	4 µL (1X)
dNTP Mix	2 µL (500 µM each)
Random hexamers	1 µL
RT Enhancer	1 µL
Verso Enzyme mix	1 µL
Template (RNA)	Variable (50 ng/µL)
DEPC treated, nuclease free water	To 20 µL
Total volume	20 µL

Additionally, to rule out genomic DNA contamination, cDNA synthesis was performed using 1 µg of total RNA of *Leptospira* (DNase treated) without adding the reverse transcriptase which served as a no-enzyme control. For evaluating the effect of host factors on the spirochetes gene expression, the shortlisted genes in the previous chapter were analyzed for the differential transcription in the presence of host factors. The oligonucleotides used for qRT-PCR reaction were designed based on the available *L. interrogans* serovar Lai genomic sequence using OligoPerfect primer design software (Invitrogen) as detailed in Table 3.1. All oligonucleotide pairs for qRT-PCR were designed with a similar annealing temperature (60ºC) and amplicon size (100-200 base pairs). Each primer pair was tested for efficiency and non-specific amplification using genomic DNA of *Leptospira* as a template in a PCR reaction, the amplified products of which were run on 2% agarose gel. Controls with each reaction included a no-template control that contained all the reagents except the cDNA.

Table 3.1. Details of the *Leptospira* genes selected and its oligomers in this chapter

Gene ID of serovars (Lai/ Copenhageni)	Gene name/ function	Gene size (bp)	5' to 3' Forward/Reverse Sequence
LAr04/ LIC11508	*rrs2, 16S rRNA*	1512	TTATTGCTCGGAGATGAGCC
			TTCAGGGTTCCCCCCATT
LA2019/ LIC11889	Flagellin (*fla*B)	852	ATGATTATCAATCACAACCTGAGTG
			TCAGTATTCCTTTCCGCTTGA
LA1005/ LIC12655	Uncharacterized	459	GATTCACAGAGTTATCCTAACTTTCTG
			CCTGGTATCGAAATGTCAGTTG
LA1796	Uncharacterized	546	TAGGTGAAGGATTTTCATATCCAAA T
			AAATCCATCAGCATTACTTCTTAGA
LA0634/ LIC12953	*dppC*	762	TTCTTCTGTGTTTGTTTCCGTTC
			AAGCAAGTACAAATCCACTTCCA
LB187/ LIC20149	Multidrug efflux transporter	1209	TACTCTTTTTACTCTTTTATTTCTCCG
			CTACGGCCCCTCCTAAAGAA
LB191/ LIC20151	*Ton*B dependent receptor	2133	CGAAGTCATTTCCCGTAAAAAG
			GTCCGGAAATTCTTTCGC
LB183/ LIC20147	*fur*, transcription regulator	393	TACGAACTTTCCCGAAAGAATTT
			ACACGGTCGCATTGTTTACA
LA2579/ LIC11402	*feo*B, Ferrous transporter	2106	TTAGTTTGGGAGGGAGCTCA
			TTTCATCGTGACCGCGAC
LB186/ LIC20148	*Heme oxygenase*	678	TCAAGACTATGTGCAAAGAATTCG
			TAGGAAATTCGTAAAAAGAAATTCCTT
LA4253/ LIC13403	*fep*B	774	TCGGATTTTCAGATATACAATCGG
			CTTTCTTTTCCAACCTTCAATCA
LA3064/ LIC11030	Putative lipoprotein	1035	AAACCGATACGGAGTTGTCATC
			ATTTTGTGCATAAAGTCTCGTGTTT
LA0426/ LIC10373	Putative lipoprotein	1530	ATAGTTGGAACCTCTAATGGACCA
			CCGCGACATTCTTTGGGTA
LA0616/ LIC12966	*lipL41*, OM lipoprotein	1068	GTGAAGGTTCCAGCTTTATCGA
			CGCTCCAATCAGATTTCCG
LA2637/ LIC11352	*lipL32*, OM lipoprotein	819	GTGAAGGTTCCAGCTTTATCGA
			CGCTCCAATCAGATTTCCG

LA3961/ LIC13166	*ompL*36/ *fcpA*	921	AAGAGCAGAAGCGTCGTA
			TTGGAGAGTTGGTGGAGTT
LA3778/ LIC10464	*lig*B	1890	ACGGTACCAGTACAACCCTAGAAG
			TAGGCCGTTGTATTCTGCTTTT
LA1569/ LIC12209	Putative lipoprotein	1416	GGAGACGAGGATTGGGTCC
			GTGTTCCGATACTAGAGGGCTTATT
LA3446/ LIC10730	Putative lipoprotein	480	AAAAACGAATCGGACTTTCTCC
			ATCCATACATTGTCTTTGTTTTGC
LA3247/ LIC10889	*ton*B	591	TCATCCAAGAACCAAACGTTG
			GGCTTCATCGGGATAGTCTG
LA3258/ LIC10881	*ton*B dependent OM receptor	1674	ATTCTAAAGTGTTTTCTTTGTCGGA
			GGTTACCTTTTTTGTTTTTTGTACG
LA3478/ LIC10704	Putative lipoprotein	669	CAGACAAGGAACGAGAATCTATTG
			AGATTGACCTTTCAATTCCGC
LA3444/ LIC10731	Putative lipoprotein	1260	ACCGGAAGATGCCGATTTA
			TTTTGTGACCGAATAAACGCT
LA3501/ LIC10686	Putative lipoprotein	1272	GTATGTCTCCAGCAGATGCTCC
			GGGCGTTCGATTAGAAAAATT
LA3440/ LIC10734	Putative lipoprotein	795	TTCCGGATTTTGTACAAAATCTTC
			ATGATATTCTGCCCATATACGCA
LA3371/ LIC10792	*mdoC*, Glucans biosynthesis	1173	CCCTTTTTTTCTTTCTTTCGG
			AGAAATTTTCGTTCCAGCTTGT
LA3294/ LIC10854	*uppS*, Isoprenyl transferase	726	CATAGAGAAGGTGCCCAGG
			TGAATCGTATCTAATCGAGTTTCTATA
LA3276/ LIC10868	Uncharacterized	1395	GACTTTTAGGGGTAGCGGGA
			TCTTGTCCATTGTTTGTTTCCA
LA3263/ LIC10878	Uncharacterized	1224	CTTCAGCATAAAAAACCTCTTTTGA
			ACGATAAATCCTCCCGCC
LA3262/ LIC10879	Putative lipoprotein	480	GATCCTTCCTTACTCAATGCTTCT
			TCTACTTTTTCAGTTTTTACACGTACA
LA3230/ LIC10906	Uncharacterized	909	TAACGTTCCTCCCGATGTC
			AGTGTCCGGATCTCCCAGTA
LA3307/ LIC10841	*rfe*, transferase	915	GATCGTCTACGTTTTGTTTGTTGT
			AGTTAAAACCTAAAAAACCGAAAACA
LA3210/ LIC10920	Putative lipoprotein	756	AACCACTGCAGAATCTATGGTG
			TTCCGCTTGGATAAACTGGA

LA3200b/ LIC10927	Putative lipoprotein	1494	CGATTACTATTTCCGGAGCC
			TCCGAAGCAGTTATATCCACTC
LA3138/ LIC10973	*ompL1*	963	TAAATTGATTACCCTCGATAGAACTAC
			CTGCTTTTGTAATACCGCCAG
LA1691/ LIC12099	*lipL53*	1431	AATCAAACTTGGGTCAGACAACTT
			TCACGCCGAAGTCATTTATCT
LA3017/ LIC11058	*lem*A, Putative lipoprotein	597	TTCACAAGCGCAAGCACAG
			TCTGTTTCTGGCTACGGTAATTC
LA3434/ LIC10739	*ami*A, alanine amidase	1092	CGTTATGTGCGCTTTGAAGA
			TAAAGAATCGGAACGGAAATTTT
LA3394/ LIC10774	Putative lipoprotein	1107	CAAGAATCCGAAAAATCTTCTTCA
			TTGAAGGGTTGGTTTGACGT
LB194/ LIC20153	Putative lipoprotein	579	CTACTTCCATTCAAACTCTTGTTTACG
			AGTATTATAAATCAAAGGTGCATTCTC
LA3340/ LIC10821	Putative lipoprotein	783	GCGATTCCAATGCTGGTAC
			ACTGTCCCCATATAGATTGACACC
LB047/ LIC20035	Hypothetical protein	1323	TGTAACTCCACTCCTAATGTGGAG
			CGATTTTAAACCATCTAACTGTTCAG
LA0222/ LIC10191	*Loa22*, OmpA lipoprotein	588	GATAGTTACGCTCTTGAA
			GATACGATTTGCTGGAAT
LA4185/ LIC13341	Putative lipoprotein	1230	CTAGCTAGCAAAACACCTCCTGATTCCAAA
			CCTTTTTGCTTTTTAAAAATTCCA
LA1939/ LIC11966	Putative lipoprotein	480	GTTTTAAAAATGCAAGCGTCC
			AGCATTGAGAGTATTTACAGCTTTTTC
LA0957/ LIC12693	TolC like outer membrane efflux protein	1674	CCACCGTCCGCTTTTACATA
			CTAGCTAGCGAGGACATACTTCCGGAAGAAA
LA1662/ LIC12123	*rfbA*	885	TGTTAGGAGATGGAAAACAATGG
			GTTTTCTAAAAGAGAAGCAAGATTATG
LA1661/ LIC12124	*rfbB*	1050	TATCTTCGATCTTTCAAGAGCATAAA
			TTTTTTTCCTTCATAAGATCCATTC

LA1659/ LIC12126	*rfbC*	561	ACGATCAAGGAAAGTTAGTCCG
			ATCTTCTAAGGTTAAAAATCCATGAGC
LA1660/ LIC12125	*rfbD*	921	CCGACTCAGAGAACGCTTACA
			CGGAGAAAGTGTAGAATCCGG
LA1664/ LIC12121	*rfbF*	912	TGGGGTTTGCCCTAAATAGAG
			AGGACCATAAGAAGCAACTTTCTC
LA1632/ LIC12151	*rfbG*	1092	ATCGAGCTTCGCTTTCTCAA
			GGCTTCTACAAACGATTTAGTACGG

Note: OM stands for outer membrane.

The qRT-PCR was performed in a 7500 Real Time PCR System (Applied Biosystems) which was programmed with the following cycling conditions: 50ºC, 2 min; 95ºC, 10 min; 40 cycles of 95ºC, 15 s; 60ºC, 1 min followed by melt curve analysis of the PCR products. Briefly, qRT-PCR reaction was set up in a 20 µL reaction volume containing 10 µL of 2X SYBR Green, 1 µL of primers (Forward and Reverse, 10 µM each), 8 µL of DEPC treated nuclease free water and 1 µL of cDNA. Expression of each gene was quantified using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) and normalized with *LAr04*/*LIC11508* (*rrs2*, *16S rRNA2*), one of the housekeeping genes of *L. interrogans* not responding to any of the host factors at the transcriptional level (Matsui et al., 2012). Initially, two constitutive genes *LA2019*/*LIC11889* (*flaB*) and *16S rRNA* (*rrs2*) of *Leptospira* were included in the study to select for the best gene that can be implemented for normalizing gene transcription data obtained through qRT-PCR. Since there was no significant change in the transcription of *16S rRNA*, it was chosen for normalization. Two independent experiments were performed to obtain statistically significant results.

3.4 RESULTS

3.4.1 Checking of the primers designed for qRT-PCR reaction for transcriptional analysis

The primers which were designed for the qRT-PCR experiments were first checked for efficiency and non-specific amplification using *Leptospira* genomic DNA as a template in a PCR reaction as shown in Fig. 3.2. All the sets of primers gave a specific amplification between 100-200 bp.

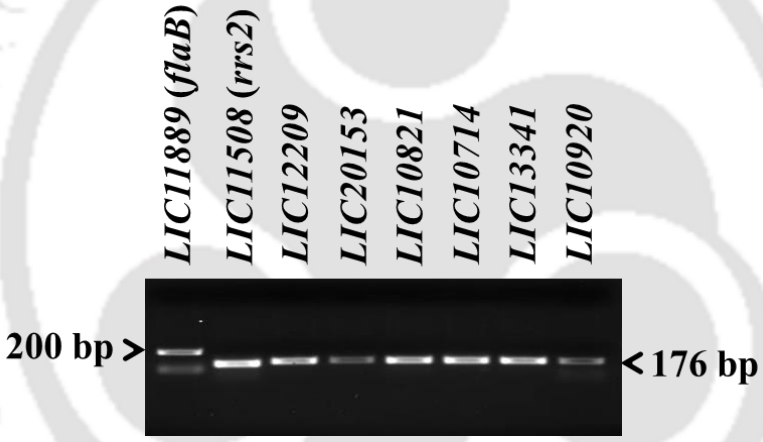

Figure 3.2. Quality check of the primers designed for qRT-PCR experiment for transcriptional analysis. A PCR was performed using the genomic DNA of *L. interrogans* serovar Copenhageni as template and the primers designed for the qRT-PCR experiments. A representative image of the 8 genes tested in the PCR reaction namely *LIC11889*, *LIC11508*, *LIC12209*, *LIC20153*, *LIC10821*, *LIC10714*, *LIC13341,* and *LIC10920* is shown. The amplified products were run on 2% agarose for visualization. All the amplicon sizes ranged between 100-200 bp. The primer dimer can be visualized for *LIC11889* (*flab*) gene, however it is not visible for the other genes tested.

3.4.2 Effect of catecholamine supplementation on the growth of *Leptospira*

Human adrenergic agonists can affect the growth of several medically important bacteria due to its ability to act as siderophores providing elemental iron essential for metabolic activity

(O'Donnell et al., 2006). A growth rate analysis of *L. interrogans* serovar Lai was performed in the presence and absence of Epi/NE (500 μM) and its antagonist PO (500 μM) to examine any effect on the growth of spirochetes in EMJH medium under *in vitro* culture condition at 29ºC. There was no statistical difference in the growth rate of the spirochetes at any time point (0-120 hrs) in the presence of catecholamines or its antagonist (Fig. 3.3). These results show that the working concentration of catecholamines (500 μM) used for the experiments was not affecting the growth of the bacterium under the given *in vitro* condition. Since, the catecholamines have been previously shown to be involved in modulating bacterial virulence (Li et al., 2012; Scheckelhoff et al., 2007), it was interesting to study their effects on the selective transcripts of the spirochete genes predicted to encode a membrane protein.

3.4.3 Effect of catecholamines on spirochetes selective gene transcripts

A total of 44 genes including those encoding hypothetical membrane proteins (n=18) and proteins with known functions (n=26) of *L. interrogans* serovar Lai were evaluated for *in vitro* differential transcription pattern in the presence of Epi/NE and its antagonist through qRT-PCR. Iron is one of the essential nutrients required for the growth of *Leptospira*. Pathogenic *Leptospira* have evolved complex iron acquisition mechanisms to harness the available iron of the host during establishing infection (Lo et al., 2010). The constitutive genes *fla*B and *16S rRNA* (*rrs2*) of *Leptospira* were included in the study to select for the best gene that can be implemented for normalizing gene transcription data obtained through qRT-PCR as per previous reports (Matsui et al., 2012).

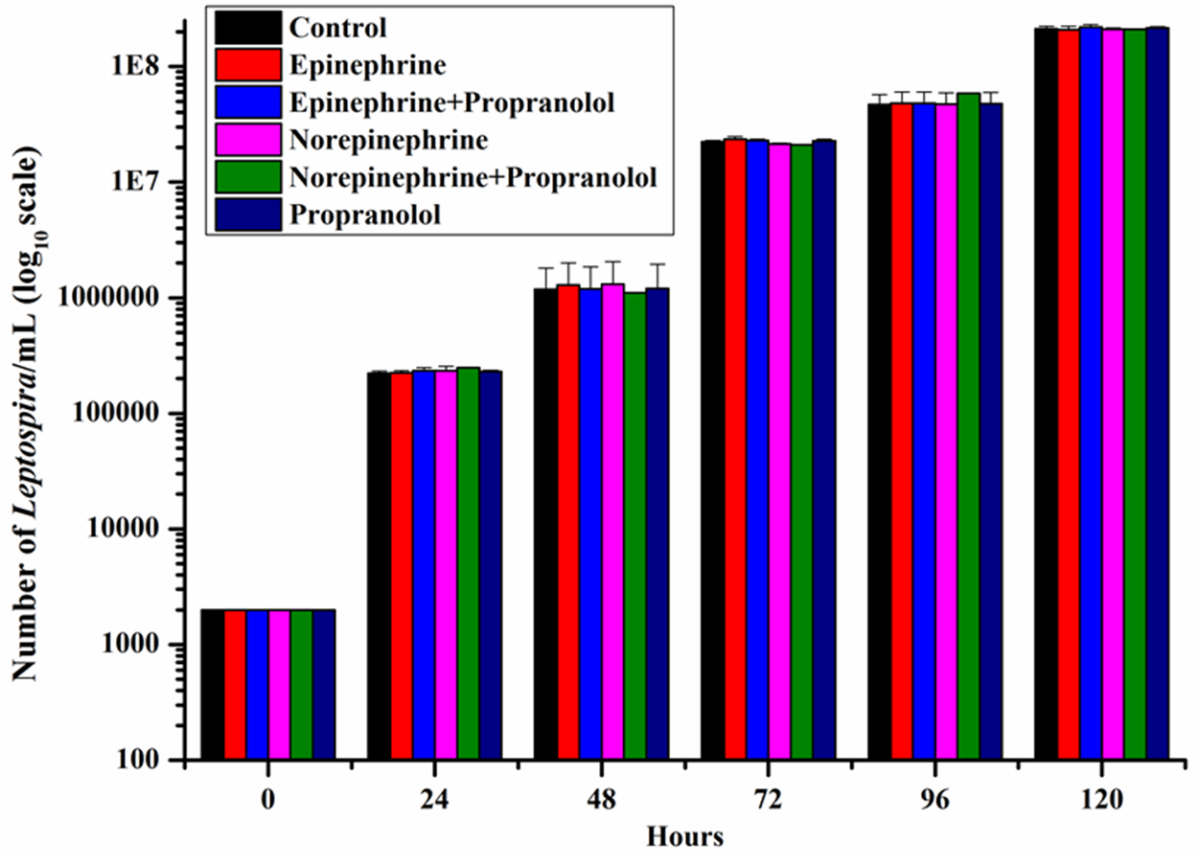

Figure 3.3. Effect of catecholamines and its inhibitor on the growth of *Leptospira.* *L. interrogans* Lai cultures were incubated and grown in the presence of Epi, NE, the *β*-antagonist propranolol (PO), or with the catecholamines and PO combined. Spirochete culture was monitored daily and its number was determined by counting under 40X Phase contrast microscopy. Each treatment showed similar growth to untreated control cultures, and at no time point was a significant difference detected among any group. Results are indicative of two independent experiments performed.

There was no significant change in the transcription of *16S rRNA* (threshold cycle, Ct values) in comparison to *fla*B in all the experimental samples analyzed by qRT-PCR. Therefore, the transcripts of target genes of *Leptospira* were normalized with *16S rRNA* transcripts using $2^{-\Delta\Delta Ct}$ method (Carrillo-Casas et al., 2008; Matsui et al., 2012; Schmittgen and Livak, 2008).

The transcripts of each normalized gene are represented as the number of gene transcripts per 1000 copies of *16S rRNA*. There were 7 genes (*LB047*, *LB186*, *LB191*, *LA0616*, *LA3263*, *LA3307*, and *LA3961*) out of 44 of *L. interrogans* serovar Lai, that showed significant (P-value < 0.05) differential transcription in the presence of Epi/NE and the effect of which was restored to the basal level (control) in the presence of its antagonist (Fig. 3.4). Such findings indicate the differential gene transcription was specifically due to the catecholamines as its antagonist; propranolol was able to restore the gene transcription to the basal level. The gene transcripts (normalized) data that showed statistically insignificant differential transcription (33 genes) of the spirochetes were segregated in three-independent clusters based on number of gene transcripts (Fig. 3.5 to 3.7). Among the 44 selected genes, 4 genes were excluded from the present study. A total of 2 genes (*LB194* and *LA3340*) were excluded as they showed high fluctuations in their Ct values in two independent experiments. The other 2 genes (*LA3468* and *LA4185*), which were amplifying from the genomic DNA of *L. interrogans*, were not amplifying from the cDNA of the same.

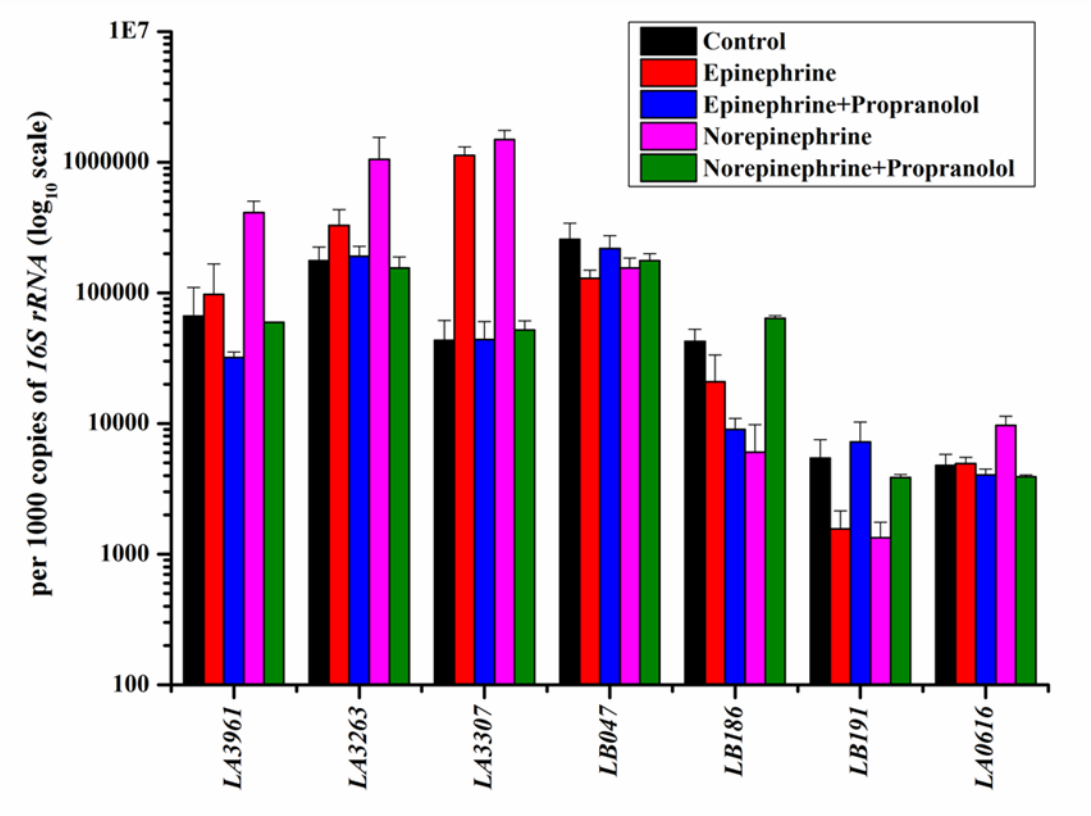

Figure 3.4. Effect of catecholamines and its inhibitor, propranolol on the selective gene transcripts of *L. interrogans* serovar Lai. Transcript analyses of the 7 genes encoding OMPs out of 44 by qRT-PCR of the cDNA synthesized from *Leptospira* grown in the presence of catecholamines and inhibitors (500 µM). The gene transcription was calculated based on threshold cycle (Ct) values using $2^{-\Delta\Delta Ct}$ method and normalized against *16S rRNA* values. The number of transcripts of 7 genes in the presence of catecholamines was restored to the basal level on the addition of its inhibitor propranolol (PO). Student's paired *t*-test was used to determine the significance of the gene transcription and $P<0.05$ was considered statistically significant. The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

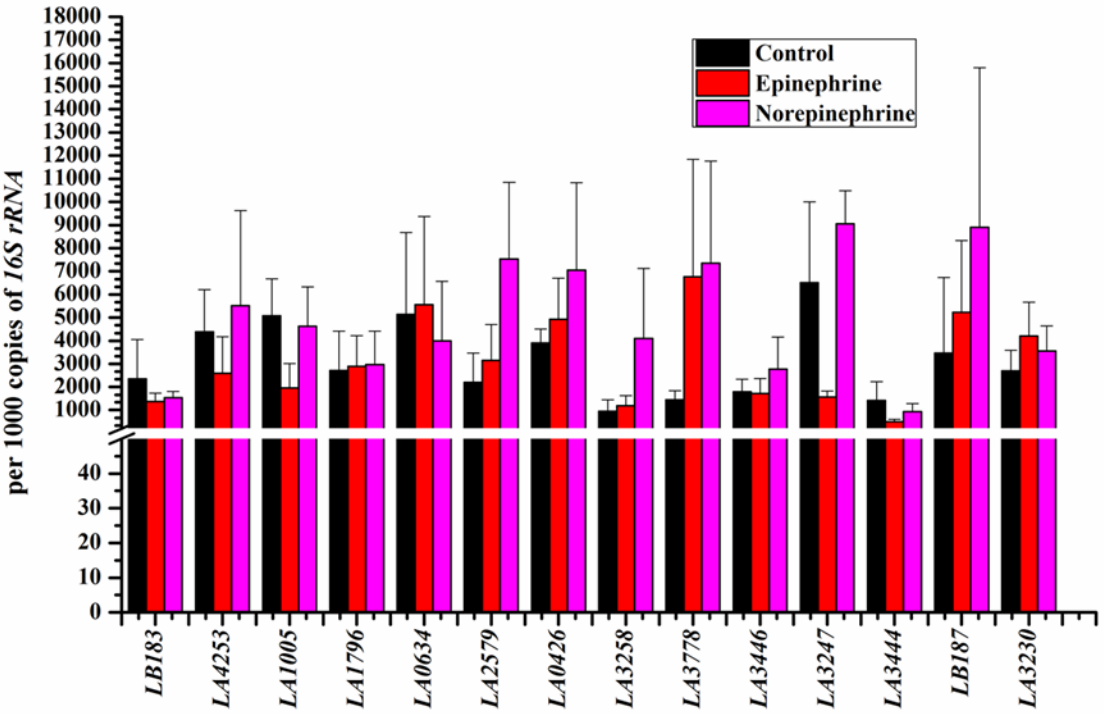

Figure 3.5. The insignificant differential gene transcripts of spirochetes due to presence of catecholamines. Transcript analyses of the 33 out of 40 genes by qRT-PCR of the cDNA synthesized from *Leptospira* grown in the presence of catecholamines. The gene transcription were calculated based on threshold cycle (Ct) values using $2^{-\Delta\Delta Ct}$ method and normalized against *16S rRNA* Ct values. The gene transcripts of spirochetes have been segregated in three-independent clusters on number of copies of gene transcripts per 1000 copies of *16S rRNA*. The list of genes having the lowest number of copies of gene transcripts per 1000 copies of *16S rRNA* is represented. Student's paired t-test was used to determine the significance of the gene transcription and P>0.05 was considered statistically insignificant. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

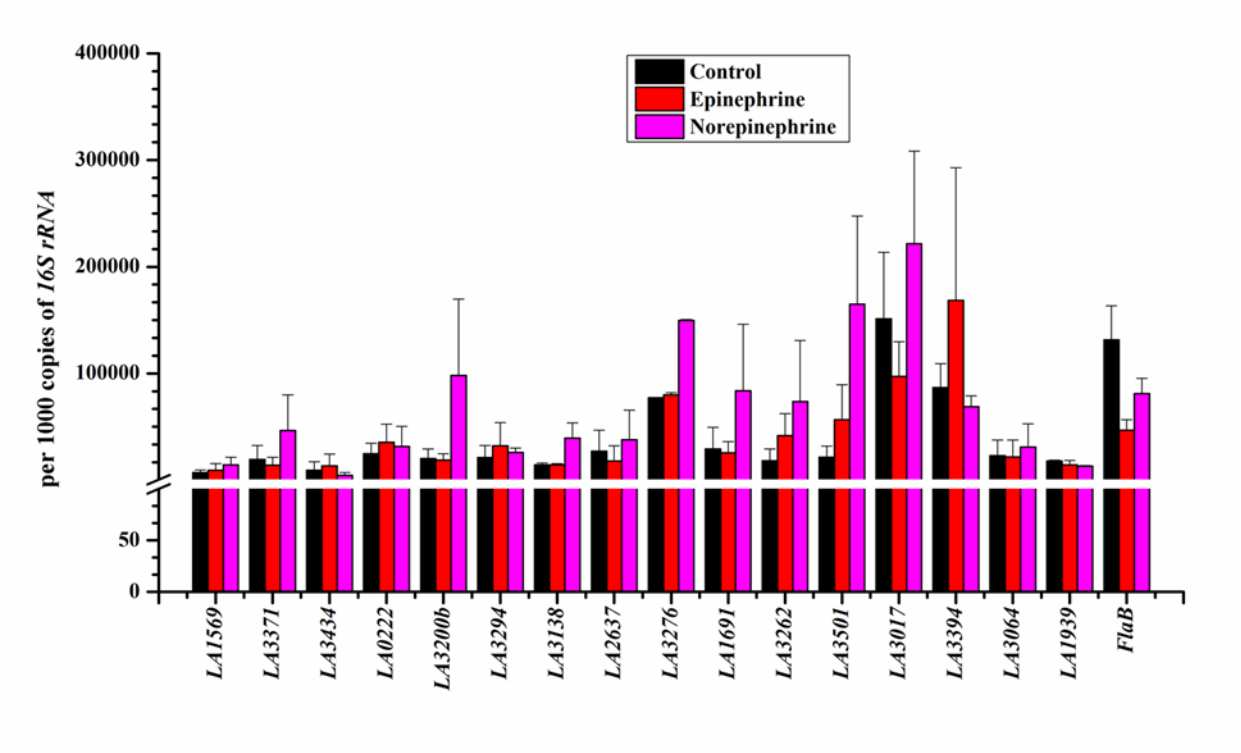

Figure 3.6. The gene transcripts of spirochetes responding insignificantly to catecholamines. The gene transcripts of spirochetes have been segregated in three-independent clusters on number of copies of gene transcripts per 1000 copies of *16S rRNA*. The list of genes with intermediate copies of gene transcripts per 1000 copies of 16S rRNA is represented. Student's paired t-test was used to determine the significance of the gene transcription and P>0.05 was considered statistically insignificant. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

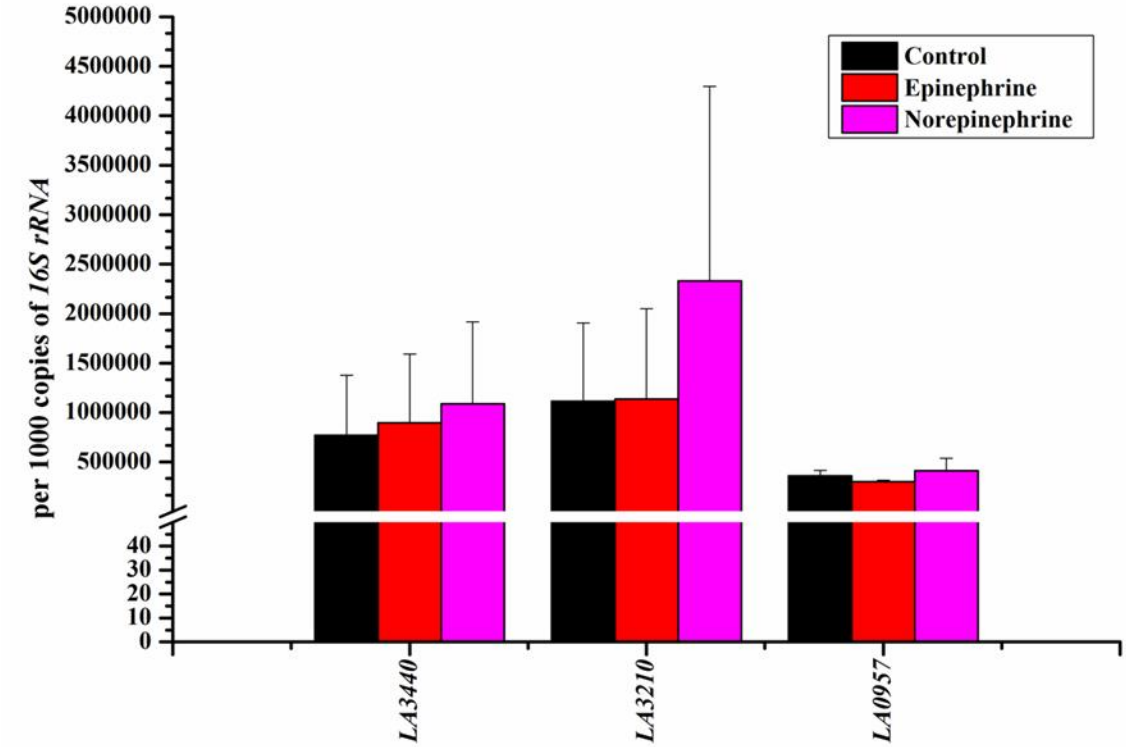

Figure 3.7. The gene transcripts of spirochetes responding insignificantly to catecholamines. The gene transcripts of spirochetes have been segregated in three-independent clusters on number of copies of gene transcripts per 1000 copies of *16S rRNA*. This represents the list of genes having the highest number of copies of gene transcripts per 1000 copies of *16S rRNA.* Student's paired t-test was used to determine the significance of the gene transcription and P>0.05 was considered statistically insignificant. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

Moreover, it was interesting to evaluate if the effect of catecholamine was universal to other pathogenic serovars of *Leptospira*. A nucleotide basic local alignment search tool (BLAST) was used to determine the sequence identity of the genes in pathogenic serovars Lai and Copenhageni using the KEGG database. The genes of *Leptospira* which were significantly up-regulated in serovar Lai were found to be present in serovar Copenhageni with 99% gene sequence identity as shown in Table 3.2.

Table 3.2. Comparative analyses of the gene sequence identity of the differentially regulated genes of serovars Lai and Copenhageni in the presence of catecholamines

***Leptospira* serovars**	**Locus**	**Gene**	**Coding DNA sequence (CDS)**	**Identity (%)**
Lai	*LA0616*	*lipL41*	1068	1065/1068 (99%)
Copenhageni	*LIC12966*		1068	
Lai	*LA3961*	*ompl36/ fcpA*	912	910/912 (99%)
Copenhageni	*LIC13166*		921	
Lai	*LB186*	*hol*	678	676/678 (99%)
Copenhageni	*LIC20148*		678	
Lai	*LB191*	*hbpA*	2133	2131/2133 (99%)
Copenhageni	*LIC20151*		2133	
Lai	*LA3307*	*rfe*	1047	912/915 (99%)
Copenhageni	*LIC10841*		915	
Lai	*LA3263*	Hypothetical	1224	1221/1224 (99%)
Copenhageni	*LIC10878*		1224	
Lai	*LB047*	Hypothetical	1320	1313/1320 (99%)
Copenhageni	*LIC20035*		1323	

Therefore, under similar experimental conditions, effect of catecholamines on *L. interrogans* serovar Copenhageni orthologous gene transcripts was analyzed. As expected, orthologous gene transcripts of these selective 7 genes (*LIC20035*, *LIC20148*, *LIC20151*, *LIC12966*, *LIC10878*, *LIC10841*, and *LIC13166*) in serovar Copenhageni also showed a similar trend of differential transcription in the presence of catecholamines or its antagonist (Fig. 3.8). The fold changes of transcripts of these 7 genes in the presence of Epi/NE were calculated with respect to basal level expression (control) for both the serovars of *Leptospira* (Fig. 3.9A and Fig. 3.9B). It was observed that the two genes (Lai/Copenhageni: *LB191/LIC20151*, *LA3307/LIC10841*) differentially transcribed in the presence of both Epi and NE whereas 5 genes responded either in presence of Epi (*LB047/LIC20035*) or NE (*LA0616/LIC12966*, *LA3961/LIC13166*, *LA3263/LIC10878*, and *LB186/LIC20148*). Additionally, three genes (*LB047/LIC20035*, *LB186/LIC20148*, and *LB191/LIC20151)* in the presence of Epi/NE showed down-regulation in its transcription and four genes (*LA0616/LIC12966*, *LA3961/LIC13166*, *LA3263/LIC10878*, and *LA3307/LIC10841*) were upregulated. The impact of gene transcription represented here was by growing spirochetes in the presence of catecholamine at a concentration of 500 µM. In contrast, numerous studies on other bacteria have been performed by supplementing catecholamine at physiological concentration (50 µM) of host gastrointestinal tract stressful conditions (Freestone et al., 2003). Therefore, another independent experiment was performed to analyze the effect of catecholamine at physiological concentration (50 µM) on the 7 differentially transcribed genes of spirochetes. The gene transcript results indicated a similar pattern of response in *L. interrogans* serovar Lai in the presence of catecholamine at physiological concentration (Fig. 3.10). It has been previously reported that the pattern of gene transcription remains invariant over a

catecholamine concentration range of 50-2000 µM (Freestone et al., 2008a; Freestone et al., 2008b; Sandrini et al., 2015) and this was further bolstered by our observation using 50 µM and 500 µM concentration of catecholamines.

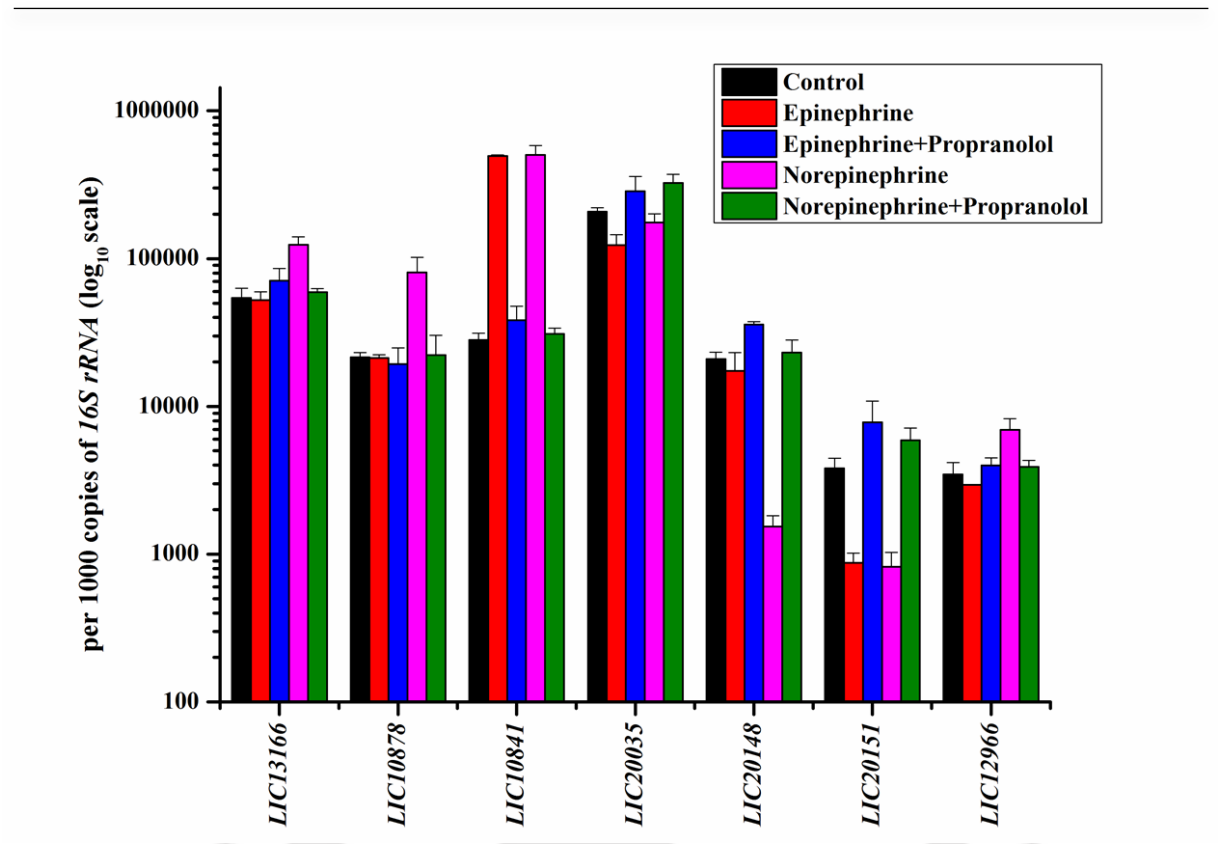

Figure 3.8. Effect of catecholamines and its inhibitor on the selective gene transcripts of *L. interrogans* serovar Copenhageni. The differential transcription of 7 orthologous genes of *L. interrogans* serovar Copenhageni has been represented here. The differential transcripts of 7 genes in the presence of catecholamines were restored to the basal level on the addition of its inhibitor propranolol (PO). The patterns of differential transcription of 7 orthologs of *L. interrogans* serovar Copenhageni to *L. interrogans* serovar Lai are very close. Student's paired *t*-test was used to determine the significance of the gene transcription and $P<0.05$ was considered statistically significant. The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

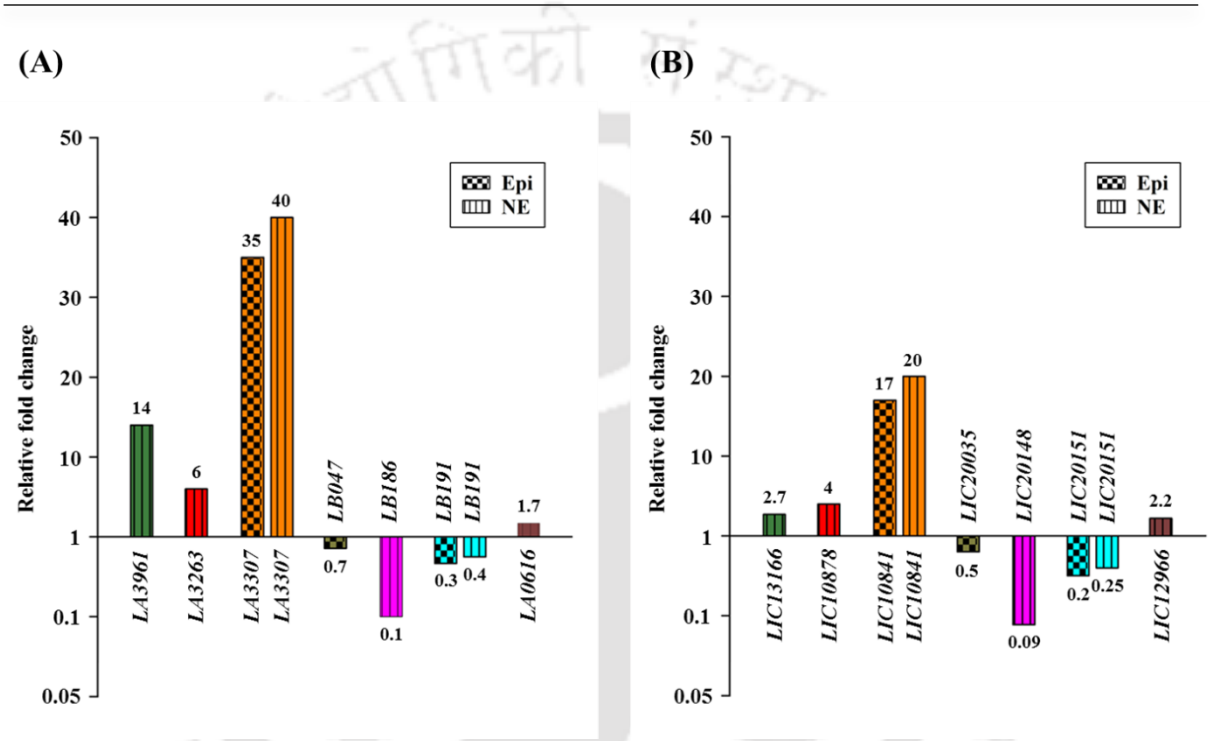

Figure 3.9. The fold change of the genes of *L. interrogans* responding significantly to catecholamines. (A) The fold change in gene transcription of *L. interrogans* serovar Lai responding significantly to catecholamines. Each gene is represented by a unique color bar along with fold change value at the apex of each bar. **(B)** The fold change in gene transcription of *L. interrogans* serovar Copenhageni responding significantly to catecholamines.

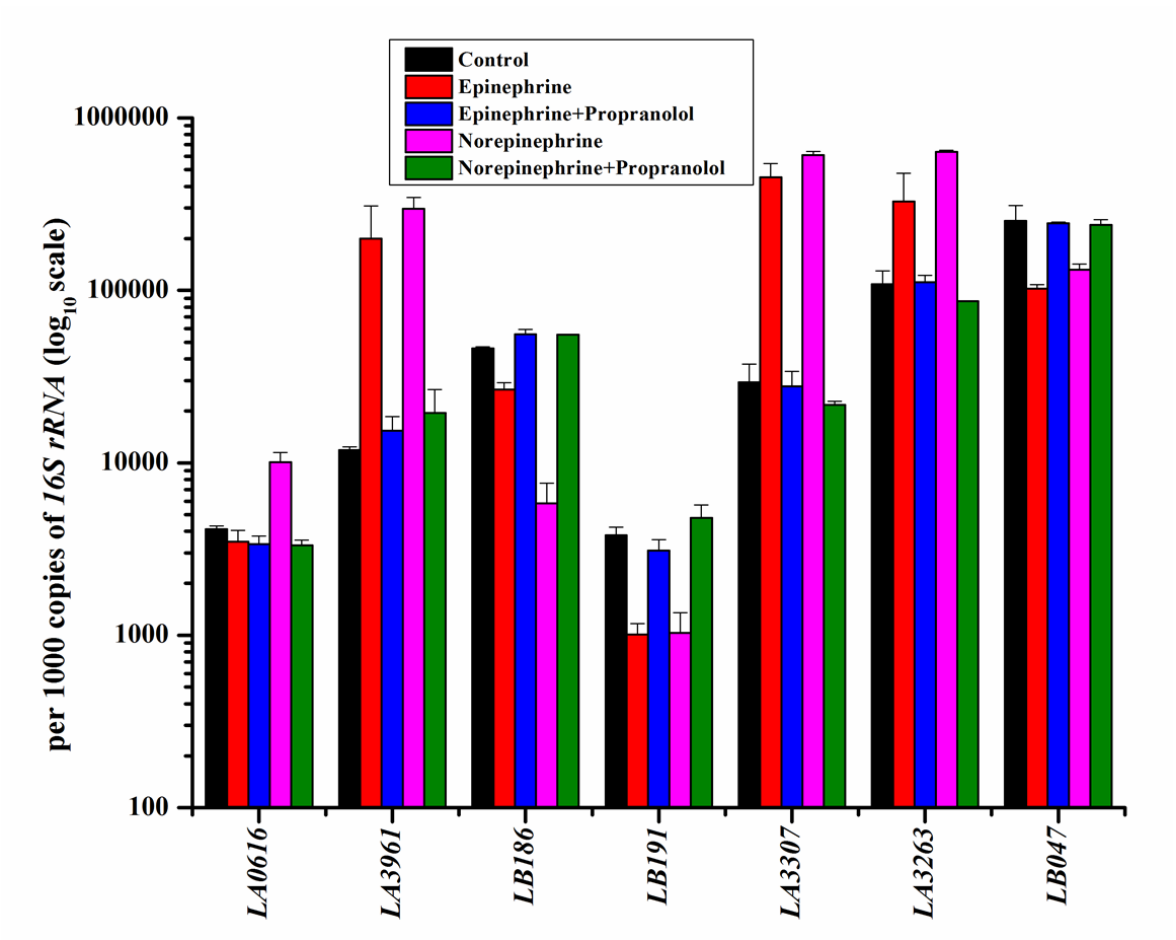

Figure 3.10. Effect of catecholamines on the selective gene transcripts of spirochetes at physiological concentration of host. Transcript analyses of the 7 out of 44 by qRT-PCR of the cDNA synthesized from *L. interrogans* serovar Lai grown in the presence of catecholamines (50 µM) and its inhibitor propranolol (PO). This graph represents the same set of 7 genes which were found to be differentially transcribed in the presence of catecholamines at a concentration of 500 µM as shown in Figure 3.4. The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

Among the 7 genes differentially transcribed due to the presence of catecholamines, the *rfe* gene (*LA3307/LIC10841*) in our experimental condition was found to be many folds (35/40

folds) upregulated in the presence of Epi/NE. The molecular function of *rfe* gene is predicted to be involved in lipopolysaccharide (LPS) synthesis (Meier-Dieter et al., 1990). The LPS in Gram negative bacteria characteristically consists of lipid A, core oligosaccharide, and O-antigen (Reeves et al., 1996). The O antigen, which is a type of Enterobacterial Common Antigen (ECA) is synthesized through a lipid-linked intermediate pathway by all the members of the family Enterobacteriaceae as shown in Fig. 3.11.

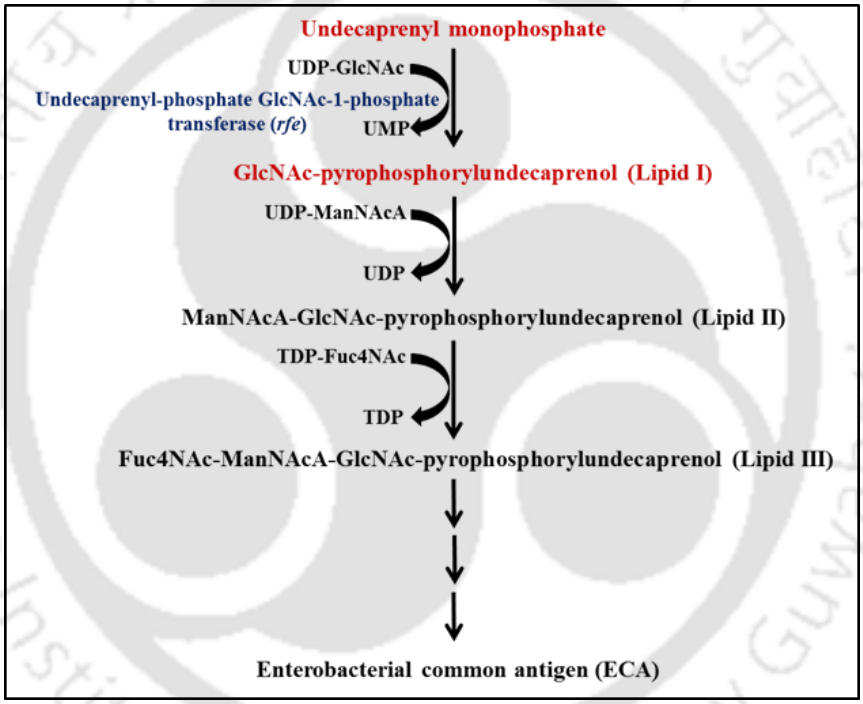

Figure 3.11. Synthesis of lipid-linked intermediates involved in Enterobacterial common antigen biosynthesis. The ECA is synthesized through a series of steps of which the first step is catalyzed by the rfe enzyme to synthesize Lipid I in the Enterobacteriaceae family (Meier-Dieter et al., 1990).

The ECA is an outer membrane glycolipid by all the members of the Enterobacteriaceae family. The carbohydrate portion consists of N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-

mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (Meier-Dieter et al., 1990). Briefly, GlcNAc-1-Phosphate from Uridine diphosphate-GlcNAc is transferred to undecaprenyl monophosphate forming GlcNAc-pyrophosphorylundecaprenol which is the Lipid I (Barr and Rick, 1987). Next, ManNAcA from Uridine diphosphate-ManNAcA is transferred to lipid I to yield ManNAcA-GlcNAc-pyrophosphorylundecaprenol (Lipid II) (Barr and Rick, 1987). Finally, Fuc4NAc from Thymidine diphosphate-Fuc4NAc is transferred to lipid II to yield Fuc4NAc-ManNAcA-GlcNAc-pyrophosphorylundecaprenol (Lipid III) which in turn is used for the synthesis of ECA heteropolysaccharide chains (Barr et al., 1989). The first step which involves the synthesis of lipid I is catalyzed by Undecaprenyl-phosphate GlcNAc-1-phosphate transferase (rfe) in Enterobacteriaceae (Meier-Dieter et al., 1990).

The *rfb* locus encodes the enzymes responsible for LPS biosynthesis in *L. interrogans*. The O-antigenic side chains are synthesized and assembled by a set of enzymes encoded by the *rfb* gene cluster, in addition to the *rfe* gene in *L. interrogans* (Mitchison et al., 1997; Ren et al., 2003). Previous reports have compared the *rfb* loci of strains Lai and Fiocruz L1-130 (both belonging to the same serogroup, Icterohaemorrhagiae), which revealed only minor gene diversity (He et al., 2007). This suggests that the compositions of the *rfb* locus genes from strains of the same serogroup are more similar to each other than those belonging to different serogroups (He et al., 2007). The LPS has been thought to be critical for leptospiral survival and one plausible reason for this conclusion stems from the fact that the *rfb* locus was relatively free of insertions in a random transposon mutagenesis study (Murray et al., 2009a). Taking the previous reports into consideration, we were interested to study the effect of catecholamines on the expression of the *rfb* gene cluster. It was previously reported that

there are a total of 6 genes namely *rfbA* (*LA1662*/*LIC12123*), *rfbB* (*LA1661*/*LIC12124*), *rfbD* (*LA1660*/*LIC12125*), *rfbC* (*LA1659*/*LIC12126*), *rfbF* (*LA1664*/*LIC12121*) and *rfbG* (*LA1632*/*LIC12151*) present in the *rfb* gene cluster in *L. interrogans* (Ren et al., 2003). On performing a quantitative analysis of gene transcripts by qRT-PCR, all the 6 genes of the *rfb* cluster showed significant ($P < 0.05$) differential upregulation in the presence of Epi/NE and the effect of which was restored to the basal level (control) in the presence of its antagonist, propranolol (Fig. 3.12).

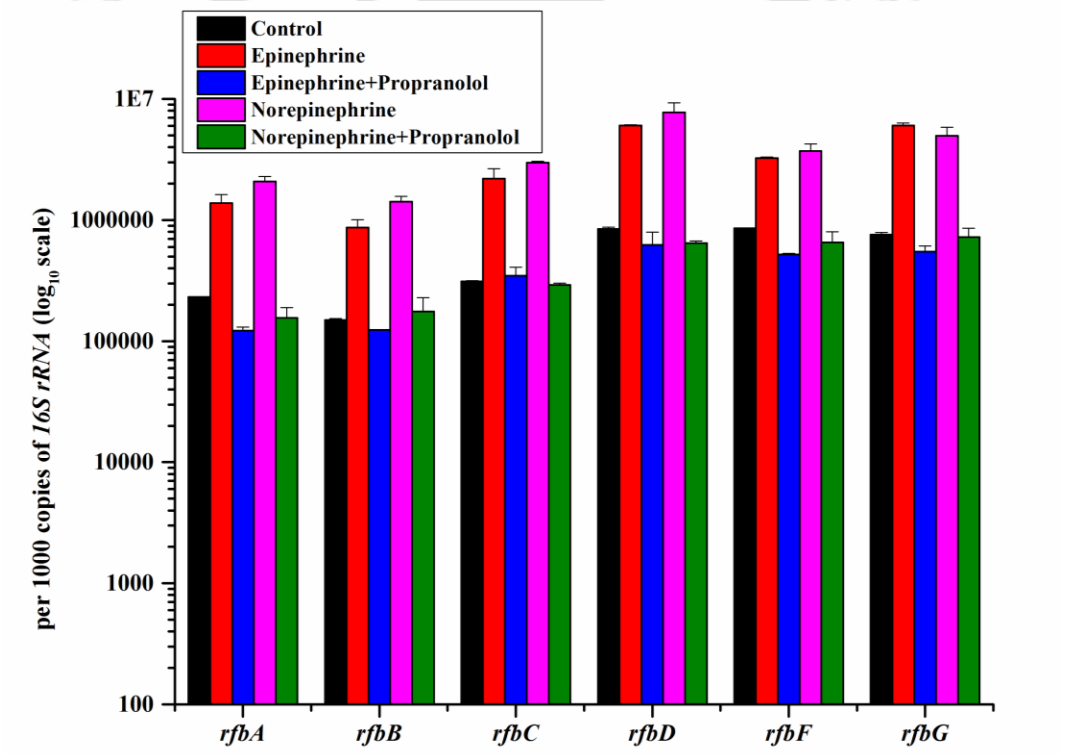

Figure 3.12. Differential transcription of *rfb* gene clusters of *L. interrogans* in the presence of catecholamines and its antagonist, propranolol. Transcript analyses of the 6 genes comprising the *rfb* locus by qRT-PCR of the cDNA synthesized from *Leptospira* grown in the presence of catecholamines and its inhibitor, propranolol (500 µM). The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

3.4.4 Effect of oxidative stress on spirochetes selective transcripts

To test if the 7 genes of *Leptospira* getting differentially expressed in the presence of catecholamines also responded to an oxidative stress condition, *L. interrogans* serovar Copenhageni was exposed to varying concentrations of hydrogen peroxide (1 mM and 10 mM) for 1 h at 37ºC after growing it till log phase at 29ºC. Interestingly, all the 7 genes namely *LIC12966 (lipL41), LIC13166 (ompL36/fcpA), LIC20148* (heme oxygenase), *LIC20151* (TonB dependent receptor), *LIC10841 (rfe), LIC10878* and *LIC20035* were getting significantly differentially modulated in the presence of hydrogen peroxide (Fig. 3.13). It was also seen that exposure to either 1 mM or 10 mM hydrogen peroxide yielded similar pattern of gene transcription as shown in previous reports (Eshghi et al., 2012a). One probable reason can be that hydrogen peroxide serves as an environmental cue in the range used in our study and influences the gene transcription in a similar fashion. It has been reported that 10 mM hydrogen peroxide is the maximal end of oxidative stress that *L. interrogans* can effectively withstand (Eshghi et al., 2012a).

The genes *LB047*/*LIC20035*, *LA3307*/*LIC10841* and *LA3263*/*LIC10878* were found to be differentially regulated in the presence of oxidative stress in a similar fashion to host factor catecholamines. The genes involved in the iron-uptake (*LB186*/*LIC20148* and *LB191*/*LIC20151*) were upregulated in the presence of oxidative stress. In contrast, in the presence of host factor catecholamines, the transcription of *LB186*/*LIC20148* and *LB191*/*LIC20151* was repressed. On the same note, *LA0616*/*LIC12966* (*lipL41*) and *LA3961*/*LIC13166* (*ompL36/fcpA*) gene transcripts showed downregulation in transcription in the presence of oxidative stress but were getting upregulated in the presence of host factor

catecholamines. The possible reason for such discrepancy may be due to the variable effect of host factors on the transcription factors involved in the gene expression of *L. interrogans*.

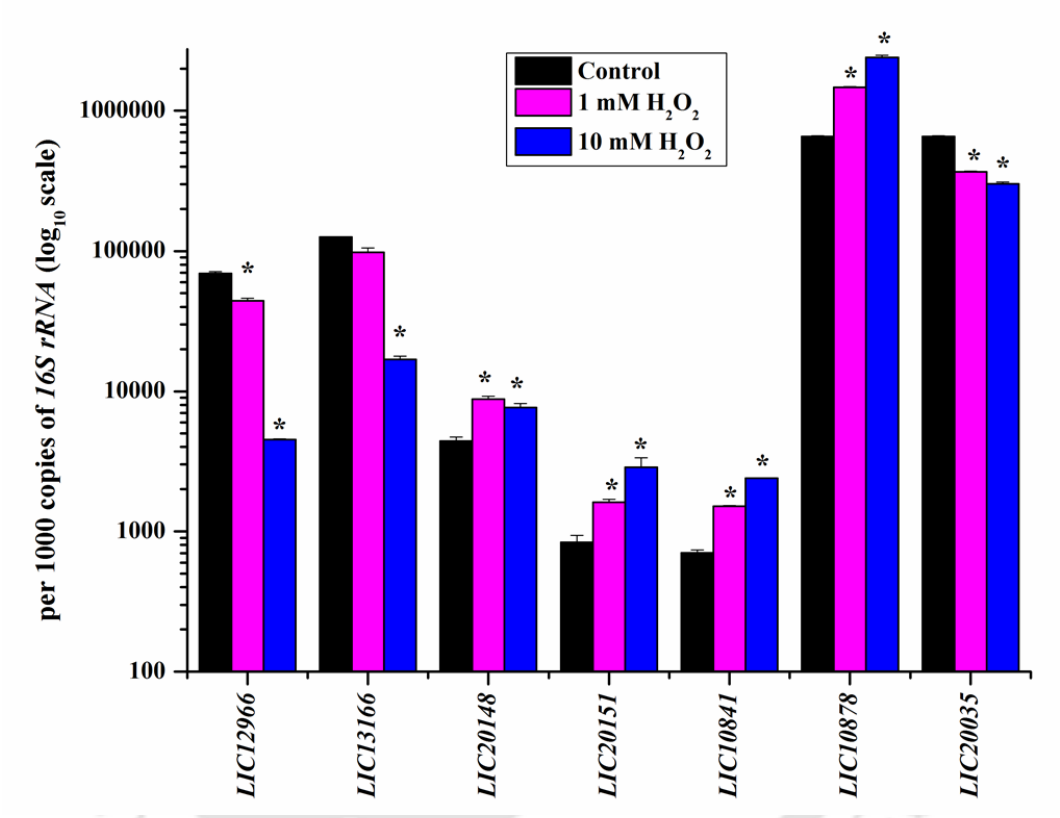

Figure 3.13. Effect of oxidative stress on *L. interrogans* serovar Copenhageni selective transcripts at 37°C. Transcript analyses of the 7 genes encoding membrane proteins by qRT-PCR of the cDNA synthesized from *Leptospira* grown in the presence and absence of hydrogen peroxide. Student's paired t-test was used to determine the significance of the gene transcription and $P<0.05$ was considered statistically significant which is indicated by an asterisk. The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

3.4.5 Effect of temperature on spirochetes selective transcripts

As reported in the literature, one of the main host factors is the temperature encountered inside the host (Fraser and Brown, 2017; Nally et al., 2001b). It was interesting to see if there is any differential transcription of these 7 genes at elevated temperature of 37ºC. *L. interrogans* serovar Copenhageni grown at elevated temperature of 37ºC, showed significant downregulation of gene transcripts *LA0616*/*LIC12966, LA3961*/*LIC13166, LB186*/*LIC20148,* and *LB047*/*LIC20035* by qRT-PCR (Fig. 3.14).

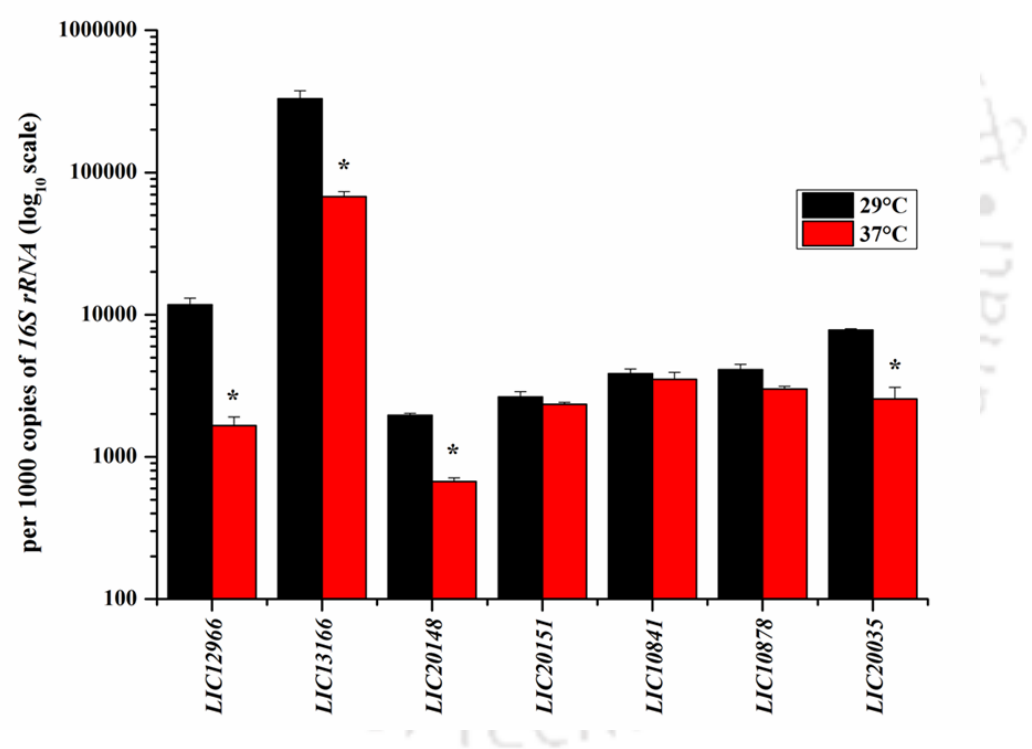

Figure 3.14. Effect of temperature on *L. interrogans* serovar Copenhageni selective transcripts at 29°C and 37°C. Comparison of transcripts of the 7 genes encoding membrane proteins by qRT-PCR of the cDNA synthesized from *Leptospira* grown at 29°C and 37°C. Student's paired t-test was used to determine the significance of the gene transcription and P<0.05 was considered statistically significant which is indicated by an asterisk. The gene transcription data has been log transformed to make it symmetrical for a more statistical relevance. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

Previous studies have shown that temperature can modulate gene transcription of selected genes in *L. interrogans* (Carvalho et al., 2009; Nally et al., 2001c; Stamm et al., 1991a). Two of the genes, *LB186*/*LIC20148* and *LB047*/*LIC20035* showed differential downregulation when individually grown at elevated temperature and in the presence of catecholamines. On the other hand, *LA0616*/*LIC12966* and *LA3961*/*LIC13166* gene transcripts were showing differential downregulation in the presence of elevated temperature and oxidative stress. Intriguingly, *LB047*/*LIC20035* was the only gene which showed a differential downregulation when individually grown in the presence of catecholamines, hydrogen peroxide (oxidative stress) and elevated temperature.

3.4.6 Analysis of the gene *LA4185*/*LIC13341* transcription at physiological osmolarity and temperature

As mentioned in section 3.4.3, *LA4185*/*LIC13341* transcripts could not be detected from the cDNA of *L. interrogans*, although it could be amplified from the genomic DNA of *L. interrogans*. To check for the transcription of *LA4185*/*LIC13341* in the other pathogenic serovars of *Leptospira*, a PCR using the genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Lai strain Lai, *L. interrogans* serovar Canicola strain Hond Utrecht IV, *L. interrogans* serovar Pomona, and *L. interrogans* serovar Bataviae was set up. An amplified fragment of 1,065 bp indicated the presence of *LA4185*/*LIC13341* in all the *L. interrogans* serovars examined (Fig. 3.15A). In contrast, no amplification of *LIC13341* was observed in the non-pathogenic *L. biflexa* serovar Patoc strain Patoc 1. However, *flaB*, a constitutive gene of 849 bp amplified in all the tested pathogenic and non-pathogenic serovars of *Leptospira*. A reverse transcription-polymerase chain reaction (RT-PCR) was performed for amplifying *LIC13341* from the cDNA

synthesized from the various serovars of *L. interrogans*. The amplification of *fla*B gene could be observed but no transcription of *LA4185*/*LIC13341* could be validated in the pathogenic *Leptospira* serovars (Fig. 3.15B).

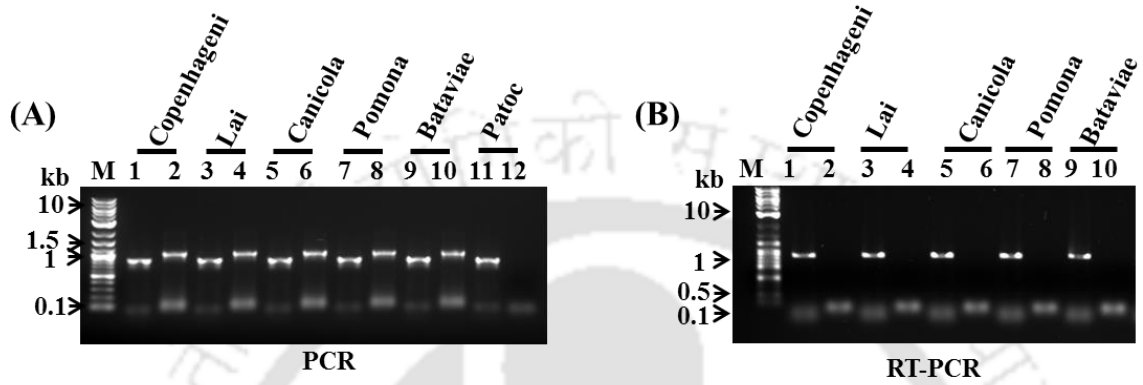

Figure 3.15. Molecular analysis of *LA4185/LIC13341*. Using PCR and RT-PCR, the presence of *LA4185*/*LIC13341* and its transcription was evaluated in pathogenic and non-pathogenic serovars of *Leptospira,* respectively. **(A)** PCR of *LA4185*/*LIC13341* using genomic DNA as the template of pathogenic and non-pathogenic serovars of *Leptospira*. An amplicon size of 1,065 bp confirmed the existence of *LA4185*/*LIC13341* in pathogenic *Leptospira* serovars Copenhageni, Lai, Canicola, Pomona and Bataviae (Lane 2, 4, 6, 8 & 10). The constitutive gene, *flaB* (849 bp) was used as a positive control for determining the quality of DNA (Lane 1, 3, 5, 7, 9 & 11). No amplification of *LA4185*/*LIC13341* was observed in the non-pathogenic serovar (Patoc) of *Leptospira* (Lane 12). **(B)** RT-PCR was performed using the cDNA synthesized from the mid-log phase culture of pathogenic *Leptospira* serovars to investigate transcripts of *LA4185*/*LIC13341* grown in EMJH medium at 29°C. No transcription of *LA4185*/*LIC13341* were detected in the pathogenic serovars of *Leptospira* grown in EMJH medium at 29°C (Lanes with the even number) whereas *fla*B gene transcripts could be detected (Lanes with the odd number).

It is now well documented that environmental factors such as temperature and osmolarity can influence the transcription of many genes of *L. interrogans* (Atzingen et al., 2008; Oliveira et al., 2010). To investigate if host factors such as osmolarity and temperature could induce the transcription of *LA4185*/*LIC13341*, *L. interrogans* was grown in the presence of physiological osmolarity and temperature. Induction of *LA4185*/*LIC13341* transcription

under physiological osmolarity was assessed by growing *Leptospira* cultures at 29°C in EMJH medium supplemented with 10% rabbit serum and re-suspending mid-log phase culture in fresh EMJH medium (control) or in EMJH medium containing 120 mM NaCl for an overnight period. The addition of 120 mM NaCl (ionic osmolarity) to the medium mimics physiological conditions (~300 mOsm) encountered by leptospires upon entry into the host (Matsunaga et al., 2007a; Oliveira et al., 2011). We also evaluated gene transcripts of *LA4185*/*LIC13341* from *in vitro* mid-log phase culture subjected to temperature upshifts from 29ºC to 37ºC of culture during an overnight period to simulate conditions experienced by leptospires in the early stages of infection and during febrile stage as described previously (Oliveira et al., 2011). Transcript analysis of *LA4185*/*LIC13341* of *L. interrogans* grown *in vitro* at physiological osmolarity or temperature (37ºC) did not lead to detection of *LA4185*/*LIC13341* transcripts. To validate the quality of the cDNA synthesized in our study, some genes previously shown to respond to physiological osmolarity or temperature were selected. We were able to reproduce the differential gene transcription of previously reported genes *LIC10464* (*ligB*) (Matsunaga et al., 2007a), *LIC11335* (*groEL*) (Nally et al., 2001c) and *LIC10314* (*lsa63*) (Vieira et al., 2010c) under physiological osmolarity or temperature (37°C) using qRT-PCR (Fig. 3.16A and Fig. 3.16B). Thus, we were unable to switch on the transcription of *LA4185*/*LIC13341* in the presence of physiological osmolarity or temperature used in our present study.

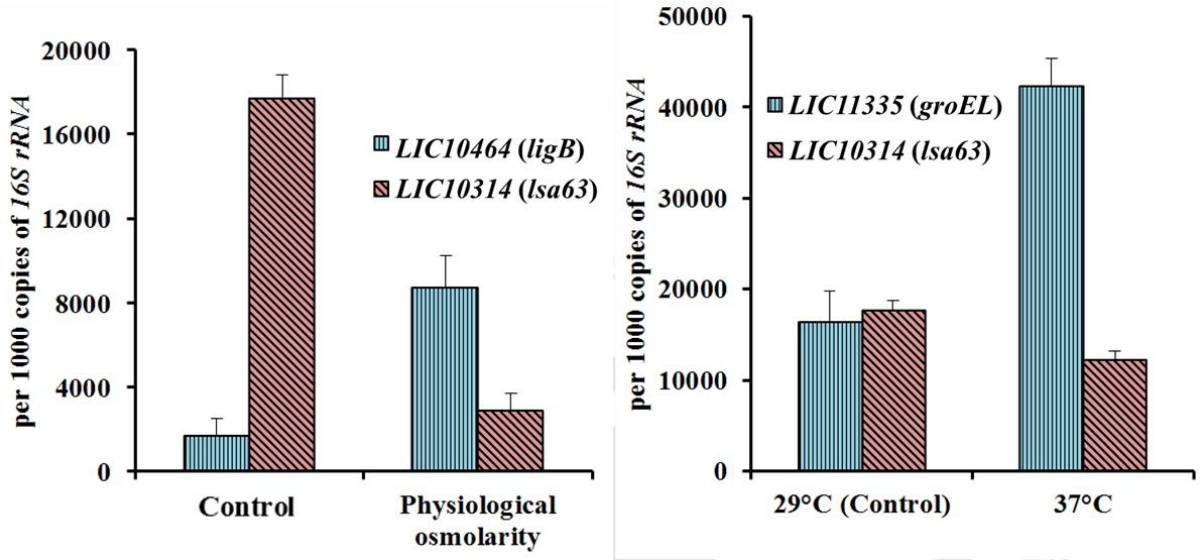

Figure 3.16. Validation of gene transcription from the cDNA of *L. interrogans* grown at physiological osmolarity or temperature using previously reported genes responding to osmolarity or temperature. **(A)** The qRT-PCR was used to quantify *LIC10464* (*ligB*) and *LIC10314* (*lsa63*) transcripts which are known to be differentially expressed at physiological osmolarity (120 mM NaCl) at 29°C. Transcription of target genes were quantified and normalized with *16S rRNA* using $2^{-\Delta\Delta}$Ct method. At physiological osmolarity, there was an upregulation in the transcription of *LIC10464* (*ligB*) whereas, a downregulation of the transcription was observed for *LIC10314* (*lsa63*). **(B)** Evaluation of gene transcription of *LIC11335 (groEL)* and *LIC10314* (*lsa63*) under physiological temperature (37 ˚C) culture condition of *Leptospira.* The shift in temperature from 29°C to 37°C under *in vitro* culture condition leads to upregulation of *groEL* and downregulation of *lsa63* transcription. The gene transcription is depicted as number of copies of the gene transcribed per 1000 copies of *16S rRNA*. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

3.4.7 Analysis of the gene *LA4185/LIC13341* transcription using low passage *L. interrogans*

It had been previously reported that in *L. interrogans*, there can be a loss in the virulence of the bacteria on continuous *in vitro* passaging. To check if this may be the reason for not detecting the transcription of *LA4185/LIC13341*, we procured low passaged strains of

Leptospira. Interestingly, as is evident from Fig. 3.17, we were able to demonstrate the transcription of *LA4185/LIC13341* in all the low passage pathogenic serovars of *L. interrogans* tested.

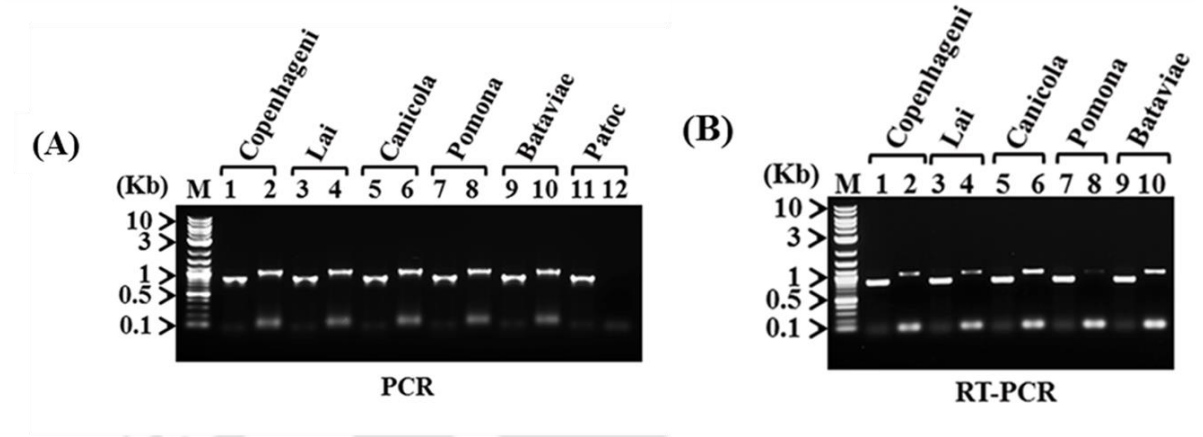

Figure 3.17. Molecular analysis of *LA4185/LIC13341* in low passage *L. interrogans.* (A) PCR was performed for *LA4185/LIC13341* gene using genomic DNA as the template of low passage pathogenic and non-pathogenic serovars of *Leptospira*. An amplicon size of 1,065 bp confirmed the existence of *LA4185*/*LIC13341* in pathogenic *Leptospira* serovars Copenhageni, Lai, Canicola, Pomona and Bataviae (Lane 2, 4, 6, 8 & 10). The constitutive gene, *flaB* (849 bp) was used as a positive control for determining the quality of DNA (Lane 1, 3, 5, 7, 9 & 11). No amplification of *LA4185*/*LIC13341* was observed in the non-pathogenic serovar (Patoc) of *Leptospira* (Lane 12). **(B)** RT-PCR was performed using the total RNA isolated from the mid-log phase culture of low passage pathogenic *Leptospira* serovars to investigate transcripts of *LA4185/LIC13341* grown in EMJH medium at 29°C (Lanes with the even number) whereas *fla*B gene transcripts could be detected (Lanes with the odd number).

3.4.8 Analysis of the *LA1939*/*LIC11966* transcription at physiological osmolarity

As mentioned in section 2.3.2, LA1939/LIC11966 was predicted to be a hypothetical lipoprotein with its location predicted to be in outer membrane of *L. interrogans*. As per previous reports, the transcription of *LA1939*/*LIC11966* is modulated by mimicking *in vivo*

conditions, like iron limitation with the presence of serum (Eshghi et al., 2009) and physiological osmolarity (Matsunaga et al., 2007a). A common conclusion from these reports is that the transcription of *LA1939*/*LIC11966* was downregulated under both these conditions. Bioinformatics indicate that *LA1939*/*LIC11966* is exclusively present in the pathogenic species of *Leptospira*. As this hypothetical protein was responding to osmolarity (which is another host factor), we were interested to understand the function of this hypothetical protein. For this, as mentioned in section 3.4.6, *L. interrogans* was grown in the presence of physiological osmolarity and the transcription of *LA1939*/*LIC11966* was analyzed along with some other known genes like *LIC10464* (*ligB*) (Matsunaga et al., 2007a) and *LIC10314* (*lsa63*) (Vieira et al., 2010d) which respond to osmolarity. *LA1939*/*LIC11966* was getting downregulated at physiological osmolarity in *L. interrogans* in concordance with the previous report (Matsunaga et al., 2007a). We were able to recapitulate the transcription pattern of both *LIC10464* and *LIC10314* from the cDNA synthesized from *L. interrogans* grown under physiological osmolarity (Fig. 3.18).

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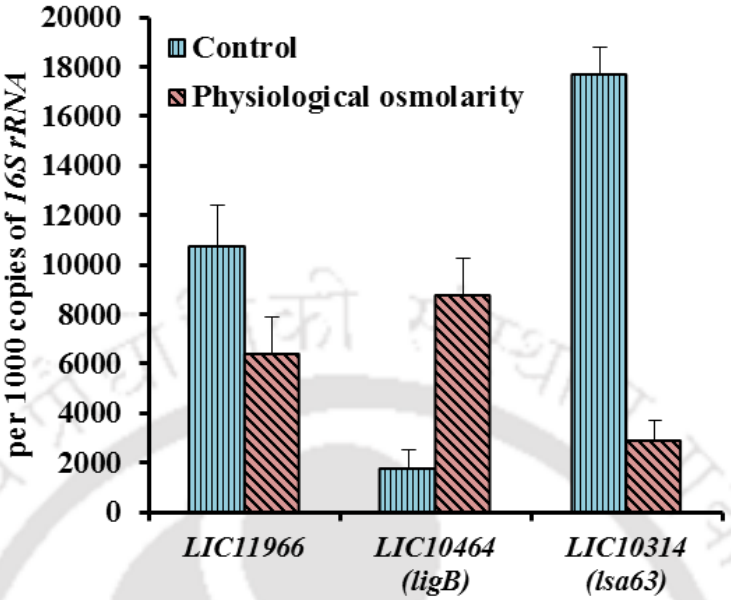

Figure 3.18. Effect of physiological osmolarity on *L. interrogans* serovar Copenhageni selective transcripts. Transcript analysis of *LIC11966* by qRT-PCR of the cDNA synthesized from *Leptospira* grown at physiological osmolarity. *LIC10464* and *LIC10314* were also found to respond to physiological osmolarity as per previous reports. Bars denote the mean standard deviation from 2 independent qRT-PCR analyses.

3.5 DISCUSSION

A wide range of pathogenic bacteria like *Salmonella* (Toscano et al., 2007), *Helicobacter* (Doherty et al., 2009), and *Staphylococcus* (Freestone et al., 2008a) have been found to be responsive to the host stress hormones. However, very little is known about the spirochetes response to stress hormones in a mammalian host. Therefore, in the present study, the effects of stress hormones on the transcription of selective membrane proteins of *Leptospira* were investigated. The typical concentration of stress hormone used in various studies of microbes-hormone chemical communication was in the range of 50-500 µM which is equivalent to the physiological concentration detected in the host (Freestone et al., 2008a;

Lyte, 2004). In this study, catecholamines or its antagonist, alone or in combination did not alter the rate of growth of *Leptospira* under *in vitro* condition at 29ºC. These were in agreement with the growth pattern of another spirochete *Borrelia borgdorferi* in the presence of the catecholamines and its antagonist propranolol (Scheckelhoff et al., 2007). However, in numerous other bacteria like Enterobacteria and many gram-negative bacteria, growth rates are augmented in the presence of catecholamines (Sandrini et al., 2014; Sharaff and Freestone, 2011). Apparently, the response of bacteria to catecholamines on its growth rate is variable.

It is evident from the present work that the *Leptospira* in the presence of stress hormones can modulate its gene transcripts selectively and the effect of which can be inhibited in the presence of its antagonist. The mechanistic of such gene modulation in spirochetes due to catecholamine is under infancy till date, however, Sperandio and his co-workers have reported that the catecholamines can induce expression of virulence factors of *E. coli* 0157:H7 by mimicking the action of the autoinducer-3 (AI-3) quorum sensing (QS) system in bacteria (Sperandio et al., 2003). AI-3 and Epi/NE are thought to be recognized by the same two-component histidine kinase (HK) receptor (QseBC) of *E. coli* 0157: H7, it will be a fascinating topic to identify this interaction between endocrinology and quorom sensing pathways in bacteria (Walters et al., 2006). We speculate that a similar form of interaction with mammalian catecholamine and HK receptor of *L. interrogans* might be playing a role in the differential transcription of *Leptospira* genes. Using PSORT program, it is estimated that *Leptospira* possesses 29 HK two-component receptors, out of which 19 are predicted to be located in the inner membrane, 9 in the cytoplasm and one in the periplasm of *Leptospira* (Nascimento et al., 2004c). These studies indicate that bacterial pathogens use molecular

sensors to ease their adaptation in the mammalian host. Nevertheless, understanding the exact mechanism of differential transcription of genes in response to catecholamine in *Leptospira* is warranted for future studies.

Catecholamines have been reported to bind to the ferric iron binding proteins like transferrin, lactoferrin etc. of the host (Freestone et al., 2007) and facilitate in the release of iron for microbial uptake. Therefore, 10 genes (*LA1005, LA1796, LA4253, LA0634, LB194, LB187, LB191, LB183, LA2579, LB186*) of *Leptospira* involved in iron-uptake (Lo et al., 2010) were included in this study. Our results indicate that only 2 genes of *Leptospira* involved in iron-uptake (*LB186* and *LB191*) were repressed in the presence of catecholamines. The possible reason for such discrepancy in gene transcription may be due to the difference in the availability of iron source under *in vitro* and *in vivo* condition (Xue et al., 2010). In other words, the available iron in the culturing EMJH medium exists as free Fe^{2+} (Murray et al., 2008; Murray et al., 2009b) whereas in the host it is in bound form.

Besides iron-uptake regulators, two genes namely *LA3961/LIC13166* (*ompL36/fcpA*) and *LA0616/LIC12966* (*lipL41*) were upregulated in the presence of NE. Leptospires outer membrane lipoprotein OmpL36 which has recently been renamed to FcpA, is a flagellar component of both pathogenic and non-pathogenic strains of *Leptospira* (Pinne and Haake, 2009; Wunder et al., 2016). While *Leptospira* LipL41, the third most predominant outer membrane lipoprotein (Cullen et al., 2005) is absent in the saprophytic strains (Bulach et al., 2006). The trend of upregulation of *ompL36* transcripts in the presence of NE was possibly a token towards the maintenance of the hook shaped morphology, coiled flagellar phenotype, and translational motility in the host during stress condition leading to dissemination of spirochetes to various host tissues (Wunder et al., 2016). Our findings that OMPs (LipL41

and OmpL36) are differentially regulated due to host hormonal stress is in agreement with an erstwhile microarray study where LipL41 and OmpL36 were highly repressed in the presence of macrophages (Xue et al., 2010).

The molecular function of one the differential expressed *rfe* gene (*LA3307/LIC10841*) has been predicted to be involved in the lipopolysaccharide (LPS) synthesis. This *rfe* gene in our experimental condition was found to be many folds (35/40 folds) upregulated in the presence of Epi/NE. Catecholamines have been also shown to upregulate *rfb* gene clusters which encode enzymes responsible for LPS biosynthesis, thus highlighting the fact that the LPS synthesis is affected when *L. interrogans* transitions from the environment to the host tissues. Hence, it will be an interesting topic of future study to understand the exact effect of catecholamines on the LPS synthesis pathway. In addition, two hypothetical proteins (HP) namely LB047/LIC20035 and LA3263/LIC10878 were found to be differentially regulated in the presence of Epi or NE, respectively. This gene regulation was endorsed by the recent report of differentially regulated genes of *Leptospira* grown inside dialysis membrane chamber or in the presence of macrophages (Caimano et al., 2014; Xue et al., 2010).

Oxidative stress along with temperature represents two of the main external factors that influence the virulence and viability of pathogenic *Leptospira*. Bacteria are unavoidably exposed to reactive oxygen species (ROS) during the course of aerobic metabolism. ROS include superoxide anion radicals and hydrogen peroxide produced by the sequential reduction of oxygen catalyzed by membrane-associated respiratory chain enzymes and hydroxyl radicals subsequently produced from hydrogen peroxide through the Fenton reaction (Winterbourn, 2008). *Leptospira* is exposed to ROS during the infection process (Eshghi et al., 2012b). ROS in the form of oxygen radicals can be generated by immune cells

including macrophages and neutrophils (Nathan and Shiloh, 2000). These generated oxygen radicals can be used to produce hydrogen peroxide, hypochlorous acid etc. which are all responsible in mediating bacterial killing (Eshghi et al., 2012b). Defense against these toxic compounds are therefore crucial for bacterial survival inside and outside the host. Among the multiple enzymes dedicated to ROS detoxification, catalase and other peroxidases catalyze the degradation of H_2O_2 to H_2O. The genes encoding peroxidases in many gram-positive bacteria, like *Deinococcus radiodurans* (Chen et al., 2008a) etc. are under the transcriptional control of H_2O_2 sensors, OxyR, and peroxide stress regulator (PerR) (Dubbs and Mongkolsuk, 2012). In *Leptospira*, several genes like *katE, clpB,* etc have been shown to be involved in the resistance to hydrogen peroxide (Eshghi et al., 2012a; Lourdault et al., 2011). It was noteworthy that all the 7 genes showing differential expression in the presence of catecholamines were also found to be differentially regulated in the presence of oxidative stress. However, the trend of the regulation varied from gene to gene basis. A set of *Leptospira* genes namely *LB047*/*LIC20035*, *LA3263*/*LIC10878,* and *LA3307*/*LIC10841* were showing downregulation individually in the presence of catecholamines (Fig. 3.4) and oxidative stress (Fig. 3.13) suggesting an uniform effect of these host factors on the gene regulators. However, the other 4 genes (*LA0616*/*LIC12966*, *LA3961*/*LIC13166*, *LB186*/*LIC20148,* and *LB191*/*LIC20151*) were showing a reverse trend of gene expression in the presence of oxidative stress when compared to catecholamine gene expression analysis. Previous reports evidenced that 2 genes (*LA1402*/*LIC12339* and *LA2020*/*LIC11888*) which were strongly induced by osmolarity were downregulated by elevated temperatures, thus indicating that in case of some genes, there can be a variation in the gene transcription pattern in the presence of different host factors (Matsunaga et al., 2007a). This ultimately

indicates that there can be the involvement of different transcription factors in the regulation of these genes. The exact mechanistic of how *Leptospira* responds to these host factors is still unclear.

Temperature is another key environmental factor known to affect leptospiral gene expression (Lo et al., 2006). *Leptospira* responds to temperature upshifts by increased synthesis of heat shock proteins such as GroEL, DnaK, and Hsp15 (Lo et al., 2006) and LpxD involved in the biosynthesis of Lipid A which is a constituent of the LPS (Eshghi et al., 2015a). The present study indicates that out of the 7 genes studied for the gene expression in the presence of elevated temperature; only 4 of them viz. *LA0616*/*LIC12966*, *LA3961*/*LIC13166*, *LB186*/*LIC20148*, and *LB047*/*LIC20035* were showing a differential expression. The transcripts of *LA0616*/*LIC12966* (*lipL41*) were differentially downregulated in the presence of both host factors, elevated temperature and oxidative stress which is in concordance to a previous report that indicates that *lipL41* transcription is downregulated in the presence of macrophage-derived cells (Xue et al., 2010). The fact that the predominant OMPs like LA0616/LIC12966 (LipL41) and LA3961/LIC13166 (OmpL36/FcpA) have been shown to be differentially modulated due to host factors in this study, suggests that the interaction with the host during infection is an important signal for regulating the transcription of these genes (Monahan et al., 2008; Nally et al., 2007). Our findings also highlight the fact that downregulation of these major OMPs may be critical for the immune evasion of pathogenic *Leptospira*, in a way similar to another spirochete, *B. borgdorferi* (Crother et al., 2003; Liang et al., 2002). Intriguingly, *LB047*/*LIC20035* transcripts were consistently showing a downregulation in the presence of the host factors (catecholamines, oxidative stress and elevated temperature) tested in the current study. However, it must be mentioned that the *in*

vitro culture conditions are very different from the condition found inside the host or in the environment, e.g. EMJH is a rich medium with low osmolarity (67 mOsm) and low cultivation temperature (29 °C) when compared to the physiological levels of 300 mOsm and 37 °C respectively (Haake and Matsunaga, 2010). Hence these transcription analyses results should be further validated by mimicking the *in vitro* conditions as closely as that of the *in vivo* conditions as possible (Xue et al., 2010).

The *LA4185*/*LIC13341* transcript was not detected in any of the tested pathogenic *Leptospira* under normal *in vitro* culture condition. We were interested to switch-on the transcription of *LA4185*/*LIC13341* under *in vitro* culture condition and thus set out to investigate the possible cause for such loss in gene transcription. Various experiments have previously described that spirochetes tend to change its gene expression pattern as per the environmental factor like temperature (Barnett et al., 1999; Cullen et al., 2002; Haake, 2000; Lo et al., 2006; Nally et al., 2001c) and chemical signals in the form of osmotic stress (Choy et al., 2007; Matsunaga et al., 2005; Pappas and Picardeau, 2015). Studies report that expression of genes like *sph2*, *ligA* (Matsunaga et al., 2007b) and *lsa21* (Atzingen et al., 2008) could be induced by physiological osmolarity and *lipL53* gene expression could be induced by elevated temperature of 37°C (Oliveira et al., 2010). To our surprise, *LA4185*/*LIC13341* gene transcription did not switch-on either by the sudden shift in temperature to 37°C or by mimicking host osmotic stress condition under *in vitro* culture condition. However, *LIC11335* (*groEL*) and *LIC10314* (*lsa63*) reported elsewhere (Nally et al., 2001c; Vieira et al., 2010c) to be differentially regulated at high temperature was recapitulated at our experimental conditions using qRT-PCR. Similarly, under high osmolarity, the trend of upregulation of *LIC10464* (*ligB*) and downregulation of *LIC10314* (*lsa63*) was observed as

reported previously (Matsunaga et al., 2007b; Vieira et al., 2010c). It is also well known that prolonged *in vitro* passaging promotes leptospiral virulence attenuation (Haake et al., 1991), and it has been suggested that expression of many virulence factors may be downregulated or lost upon continuous passaging (Matsunaga et al., 2003). On procuring low passage strains of *Leptospira*, we were able to detect the gene transcripts of *LA4185*/*LIC13341*, thus indicating the fact that the virulence of *Leptospira* had been attenuated due to continuous passaging *in vitro*.

Among the differentially regulated genes, *LIC20035/LB047* was further characterized as it codes for a conserved hypothetical protein in *Leptospira* and it was differentially downregulated in the presence of the host factors like catecholamines, oxidative stress and elevated temperature. Along with LIC20035, one putative lipoprotein, LA4185/LIC13341, whose function was unknown, was selected for further characterization as it was interesting to investigate the unknown host factor(s) or transcription factor(s) which induce(s) *LA4185*/*LIC13341* gene transcription. The gene *LA1939*/*LIC11966* annotated as hypothetical lipoprotein was found to be downregulated at physiological osmolarity which is a host factor. Hence, LA1939/LIC11966 was chosen for further characterization. Another gene *LA0957*/*LIC12693* encoding an outer membrane efflux protein was previously found to be downregulated in mammalian host-adapted *Leptospira* and hence was selected for further characterization.

Chapter 4

Cloning, expression, and characterization of selected membrane proteins for the serological diagnosis of leptospirosis

CHAPTER 4

Cloning, expression, and characterization of selected membrane proteins for the serological diagnosis of leptospirosis

4.1 Abstract

In the previous chapters, we developed a novel way of finding/screening genes responding to host factors. These genes may encode antigens of diagnostic importance for leptospirosis. In this chapter, the comprehensive analysis of four hypothetical OMPs namely LB047/LIC20035, LA4185/LIC13341, LA1939/LIC11966 and LA0957/LIC12693 was performed. The catecholamine modulated gene LIC20035 exhibited immunogenic and adhesin property to host extracellular matrices. Protease-accessibility assay and phase-partition of integral membrane proteins of *Leptospira* describe LIC20035 to be outer membrane surface-exposed protein. The recombinant-LIC20035 protein can be serologically detected using human/bovine sera positive for leptospirosis. Moreover, the recombinant-LIC20035 can bind to diverse host extracellular matrices and with a higher affinity towards collagen and chondroitin sulfate. Another hypothetical lipoprotein, LIC13341, whose function was unknown, was selected for further characterization as it was interesting to investigate the unknown host factor(s) or transcription factor(s) which induce(s) *LIC13341* gene transcription. Another reason for the loss of *LIC13341* transcription could be because of the attenuation of virulent genes during *in vitro* passaging. Interestingly, low passage *L. interrogans* demonstrated the transcription of *LIC13341* in all the pathogenic serovars of *L. interrogans* tested. The secondary structure of the bacteria-expressed affinity-purified recombinant LIC13341 protein (r-LIC13341) was predicted to contain 8.02% of α-helices

and 22.14% of β-strands by circular dichroism spectroscopy. Mouse polyclonal antibodies against r-LIC13341 can detect LIC13341 in *Leptospira* lysates suggesting that the antigen LIC13341 is immunogenic. Phase separation and protease assays show that LIC13341 to be a surface-exposed outer membrane protein of *Leptospira*. The r-LIC13341 can bind to a wide spectrum of host extracellular matrix (ECM) components. The specific adherence of *Leptospira* to laminin and hyaluronic acid of the ECM was competitively inhibited in the presence of r-LIC13341. ELISA and the immunoblot assay performed using the serum samples from confirmed human or bovine leptospirosis cases could detect r-LIC13341, suggesting LIC13341 is expressed in diverse hosts during *Leptospira* infection. The gene *LIC11966* encodes a hypothetical lipoprotein which was found to be downregulated at physiological osmolarity which is a host factor, was found to be a surface exposed outer membrane lipoprotein. It was also found to bind to a diverse group of ECM and most notably to fibrinogen. This protein can also be serologically detected in diverse infected hosts. Another gene *LIC12693* encoding an outer membrane efflux protein was previously found to be downregulated in mammalian host-adapted *Leptospira* was predominantly located in the detergent-insoluble phase, possibly indicating it to be a transmembrane protein. LIC12693 was also found to bind to laminin and fibronectin. In summary, *L. interrogans* LIC20035, LIC13341, LIC11966 and LIC12693 are outer membrane adhesins and are antigens of diagnostic importance across a wide host spectrum.

4.2 Introduction

Leptospirosis is an important re-emerging zoonotic disease caused by spirochetes of genus *Leptospira*. These spirochetes affect a broad spectrum of mammalian hosts including humans (Andersen-Ranberg et al., 2016). It is estimated that more than a million cases of

leptospirosis are reported annually worldwide, and over 70% of cases of them are from tropical countries (Costa et al., 2015).

Outer membrane proteins of *Leptospira* are core components by which pathogenic *Leptospira* interact with the host and play an essential role as adhesins (51), receptors for various host molecules (44), and key mediators for adaptation to change in the environment (49). In the last few years, numerous studies have been done in regard to the effect of host factors on the differential expression of outer membrane proteins (OMPs) of infectious agents. In *Escherichia coli*, catecholamines can induce genes associated with adhesion to host cells (Chen et al., 2006; Hendrickson et al., 1999; Lyte et al., 1997; Vlisidou et al., 2004), affect chemotaxis, colonization to Hela cells (Bansal et al., 2007) and can even enhance toxin production in them (Lyte et al., 1996; Voigt et al., 2006). In the same way, in response to catecholamine, *Actinobacillus pleuropneumoniae* (Li et al., 2012) and *Borrelia burgdorferi* (Scheckelhoff et al., 2007) actively regulate virulence genes. Such studies have proved that the pathogens take advantage of the host response on causing infection and illustrate the way in which pathogens can intercept host hormonal signals to its advantage. Substantial evidence from the aforementioned studies conveys that bacteria have a catecholamine response system(s) that possess(s) pharmacological similarity to the mammalian adrenoceptors and dopamine receptors.

The molecular mechanisms of *Leptospira* pathogenesis are not well elucidated. However, it is conceived that the adhesion of *Leptospira* to host tissue components is a crucial step for establishing infection and pathogenesis. The extracellular matrix (ECM) is produced by host cells and is known to facilitate bacterial cell attachment and in some cases to pathogen dissemination. In the last two decades, many known leptospiral adhesins of *Leptospira*, have

been reported as the virulence factors that may be critical in the pathogenesis of leptospirosis (Vieira et al., 2014). In addition, virulence factors of pathogens expressed in the host during infection are expected to elicit specific humoral immune responses and, thus, may serve as candidate markers for recombinant protein-based serodiagnostic tests. Among other spirochetes, *Borrelia* spp. (Guo et al., 1998; Parveen et al., 2003; Parveen and Leong, 2000) and *T. pallidum* (Cameron et al., 2004) have also been shown to express various outer membrane proteins (OMPs), that confers attachment to various extracellular matrices.

The ECM is a complex association of macromolecules constituted by polysaccharides chains of glycosaminoglycans (GAGs) and fibrous proteins such as laminin, collagen, elastin, and fibronectin. The most important GAGs for the ECM composition are hyaluronic acid, chondroitin sulfate, and heparan sulfate. Hyaluronic acid (HA), a major ECM component, is a non-sulfated glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and N-acetyl-D-glucosamine (Weissmann and Meyer, 1954). Interestingly, HA is the only GAG that is not attached to a core protein. On the other hand, laminins are heterotrimers that form the major constituents of the basement membrane of epithelium and endothelium. Laminin-binding proteins have been reported for several pathogens, e.g. EhaB of *E. coli* (Wells et al., 2009), Tp0136 of *Treponema pallidum* (Brinkman et al., 2008), OmpL37 (Pinne et al., 2010), Lsa24, Lsa27 and Lsa63 of *Leptospira* (Vieira et al., 2014).

In this chapter, we have characterized LIC20035, LIC13341, LIC11966 and LIC12693 of *L. interrogans*. LIC20035 which is modulated in the presence of catecholamines is a hypothetical protein with no assigned function. The gene *LIC13341* encodes for a hypothetical protein which was not transcribed from the cDNA of *L. interrogans*. The possible reason speculated were some unknown host factor(s) or transcription factor(s) which

induce(s) *LIC13341* gene transcription or the possibility of gene attenuation due to continuous passaging *in vitro*. To investigate the gene attenuation aspect, low passage *L. interrogans* were used to check for *LIC13341* transcription. Interestingly, *LIC13341* transcription was observed for all the pathogenic *Leptospira* tested. Both LIC20035 and LIC13341 of *Leptospira* were evaluated for their cellular location, ability to bind to host ECM components and for their potential diagnostic application for leptospirosis. We have also characterized a putative lipoprotein of *L. interrogans*, LIC11966 which was found to be downregulated in the presence of physiological osmolarity. The *LIC12693* encoding an outer membrane efflux protein which was previously found to be downregulated in mammalian host-adapted *Leptospira* was also characterized in the present study.

4.3 Materials and Methods

4.3.1 Bacterial strains, media and growth condition

A panel of three pathogenic *Leptospira* reference strains i.e., *L. interrogans* serovar Copenhageni str. Fiocruz L1-130, *L. interrogans* serovar Lai str. Lai, *L. interrogans* serovar Canicola str. Hond Utrecht IV and one non-pathogenic *Leptospira* strain *L. biflexa* serovar Patoc str. Patoc 1 was obtained from Indian Council of Medical Research, Regional Medical Research Centre (ICMR, RMRC), Port Blair, Andaman and Nicobar Islands, India. Other reference strains, *L. interrogans* serovar Pomona str. Pomona and *L. interrogans* serovar Bataviae str. Swart was obtained from Indian Council of Agricultural Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru, India. A total of human (n=50), bovine (50) and canine (n=18) serum samples tested positive for leptospirosis by microscopic agglutination test (MAT) along with the MAT-negative serum samples of human (n=20), bovine (n=20) and canine (n=11) were obtained from

ICAR-NIVEDI. Spirochetes were grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium (Difco) maintained at 29°C with 10% heat-inactivated rabbit serum (56°C for 30 min) unless stated. A 7-day-old culture of *Leptospira* (500 µL) was inoculated in 5 mL of EMJH medium with or without 5-Fluorouracil (HiMedia) and was subcultured successively at 7-day intervals for genomic DNA or total RNA isolation. The growth conditions of *Leptospira* in the presence of catecholamines and its antagonist, propranolol has been described in section 3.3.2.1. Bacterial cells of *E. coli* strains DH5α and BL21 (DE3) were grown at 37ºC in Luria-Bertani (LB) liquid medium or LB-agar with or without ampicillin or kanamycin (SRL) in 100 µg/mL concentration for cloning, transformation, and expression studies.

4.3.2 Nucleic acid isolation and qRT-PCR

Total RNA of spirochetes were isolated as described in section 3.3.2.3. Plasmids (pTZ57R/T or pET28a) were isolated from 5 mL of *E. coli* culture using plasmid purification kit (HiMedia). Standard procedures were followed for generation of recombinant plasmid. The QIAquick gel extraction kit (Qiagen, New Delhi, India) was used for the isolation of DNA fragments from agarose gel. All DNA fragments were resolved onto 0.8% agarose gel. All enzymes used for DNA manipulations were obtained from New England Biolabs or Fermentas (Mumbai, India).

4.3.3 Protein overexpression and purification

The predicted coding DNA sequences (CDS) of *LIC20035* (1275 bp), *LIC13341* (1065 bp), *LIC11966* (414 bp), *LIC12693* (1590 bp) including *LIC10191* encoding *loa22* (519 bp) were amplified without the signal peptides using the genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. InstaTA cloning kit (Thermo Fisher Scientific) was used

to facilitate gene cloning. Thereafter, *LIC20035*, *LIC13341*, *LIC11966*, and *LIC10191* were individually cloned in pET28a vector and the recombinant plasmid was used for over-expressing in *E. coli* BL21 (DE3) competent cells. *LIC12693* was cloned in pET23a vector as we were unable to express LIC12693 which was cloned in pET28a vector. The oligomers used in the cloning of *LIC20035*, *LIC13341*, *LIC11966*, *LIC12693* and *LIC10191* genes in pET vector (Novagen San Diego, CA, USA) are mentioned in Table 4.1. The restriction endonuclease sites are in bold, italicized, and underlined.

Table 4.1. List of oligonucleotides used for cloning in this chapter

Gene ID	Gene name/ function	Gene size (bp)	5' to 3' Forward/Reverse Sequence
LIC20035	Hypothetical protein	1323	CTA***GCTAGC***TGTAACTCCACTCCTAATGTGGAG
			CCG***CTCGAG***TTATTTACAACCTTGCATTTCTCC
LIC10191	*Loa22*, OmpA lipoprotein	588	CTA***GCTAGC***GCTGAAAAAAAAGAGGAATCCG
			CCG***CTCGAG***TTATTGTTGTGGTGCGGAAG
LIC12693	Hypothetical protein	1674	CTA***GCTAGC***CATCATCATCATCATCACGAGGAC ATACTTCCGGAAGAAA
			CCG***CTCGAG***TTATTTTTCGGAAGTTTCCGTAG
LIC13341	Putative lipoprotein	1230	CTA***GCTAGC***AAAACACCTCCTGATTCCAAA
			CCG***CTCGAG***TCATTCTTGCTTGGAAACCA
LIC11966	Putative lipoprotein	480	CTAG***CTAGC***TGCAAACAAGATCCAGTAGAT
			CCG***CTCGAG***TTATTGAGAAGCGTATTCTTTC
LIC11889	Flagellin (*flab*)	852	ATGATTATCAATCACAACCTGAGTG
			TTAGATCTGCTGCAGAAGCTTG

Recombinant LIC20035 and LIC10191 (Loa22) proteins were purified using hybrid method from E. coli lysate. Briefly, *E. coli* BL21 (DE3) cells were induced using 0.5 mM IPTG in 1 liter of LB medium at 37ºC for 4 h. The cell pellet obtained was lysed with cold denaturing lysis buffer (8 M Urea, 20 mM Na_3PO_4, pH-7.8; 500 mM NaCl) and thereafter, sonicated for 15 min with 6 s on and off cycles. The resulting homogenate was centrifuged at 12,000 g for 30 min to remove insoluble debris. Induced recombinant proteins were purified by affinity column chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resins (Invitrogen) using the protocol published elsewhere (Kumar et al., 2010). Briefly, N-terminal 6X His-tagged recombinant proteins bound to Ni-NTA resins were washed with denaturing wash buffer (8 M Urea, 20 mM Na_3PO_4, pH 6.0; 500 mM NaCl) and subsequently with native wash buffer (500 mM NaCl, 20 mM Imidazole and 50 mM NaH_2PO_4, pH 8.0). The recombinant proteins were then eluted out using elution buffer (500 mM NaCl, 250 mM Imidazole and 50 mM NaH_2PO_4, pH-8.0). Thereafter, the purified proteins were dialyzed (Pierce protein dialyzing cassette) and concentrated (Corning Centricon Spin-x-UF) to 0.4 mg/mL in dialysis buffer (50 mM Tris-Cl buffer pH 8 and 100 mM NaCl) before storing it at -80ºC. The recombinant LIC12693 could not be purified in the required amount after exhaustive attempts.

The r-LIC13341 and r-LIC11966 were purified using the native method from *E. coli* lysate. The purified recombinant protein r-LIC13341 was dialyzed (Pierce protein dialyzing cassette) and concentrated (Corning Centricon Spin-x-UF) to 0.4 mg/mL in phosphate-buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) before storing it at -80°C. The purified r-LIC11966 was also dialyzed and concentrated to 5 mg/mL in a buffer containing 50 mM sodium phosphate, pH 7.4 and 100 mM NaCl.

4.3.4 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy measurements were performed for r-LIC20035 and r-LIC13341 at room temperature using a Jasco J-815 spectropolarimeter (Japan Spectroscopic, Tokyo) at a scanning speed of 100 nm/min. Far-UV CD spectra were measured using a 5 mm and 1 mm path-length cell at 0.5 nm intervals for r-LIC20035 and r-LIC13341 respectively. The difference in the path lengths of the cuvettes used for the experiments was because of the unavailability of the 5 mm path length cuvette during the r-LIC13341 CD analysis. The spectra were presented as an average of 3 scans recorded from 190 to 260 nm and smoothed using Savitzky–Golay filter (Savitzky and Golay, 1964). The molar ellipticity (Φ) is expressed in degree.cm^2.dmol^{-1}. Spectra data were submitted to the K2D2 web server (Perez-Iratxeta and Andrade-Navarro, 2008) that calculated the secondary structure content from the ellipticity experimental data. The theoretical secondary structure was calculated using PSIPRED v3.3 (Jones, 1999).

4.3.5 Generation of polyclonal antibodies against purified recombinant proteins of *Leptospira*

The purified recombinant proteins of LIC20035, LIC13341, LIC11966 and LIC12693 were used as antigens to immunize 4-6 weeks old female BALB/c mice. About 10 µg per mouse of recombinant protein emulsified in Freund's complete adjuvant (FCA, Cat No. sc-3727 from Santa Cruz Biotechnology) was used for primary immunization (5 mice per group) by subcutaneous injection. A negative control group was injected with equal volume phosphate buffer saline (PBS) along with the adjuvant. Immunized mice were further given two booster injections of recombinant protein emulsified in Freund's incomplete adjuvant (FIA, Cat No. 3726 from Santa Cruz Biotechnology) at 14 and 24 days of primary immunization. At 10

days of the second booster, blood was collected from each mouse by retro-orbital bleeding and then was sacrificed using atlantooccipital dislocation method as described before (Kumar et al., 2010). Sera obtained were pooled for antibody titer analysis by ELISA before experimental use. Immunization experiments in mice were performed in Department of Veterinary Microbiology, College of Veterinary Science, Assam Agriculture University Guwahati, India, after approval by Institutional Animal Ethics Committee. Antibodies against r-Loa22 were generated in rabbit by outsourcing the purified protein to Abgenex, Bhubaneswar, India.

4.3.6 Enzyme-linked immunosorbent assay (ELISA) for titer determination

Disposable 96-well polystyrene plate was coated with 50 µL of r-LIC20035, r-LIC12693, r-LIC11966 or r-LIC13341 (400 ng/well) and incubated overnight at 4ºC. It was blocked with 100 µL of 3% bovine serum albumin (BSA) at 37ºC for 2 h. After three times washing of wells with 200 µL of phosphate buffer saline containing 0.05% Tween 20 (PBS-T), plate was incubated with 50 µL of mouse anti-LIC20035/ LIC13341/ LIC12693/ LIC11966 of various dilutions (1:1000; 1:5,000; and 1:10,000) at 37ºC for 2 h. Pooled pre-immune serum of mice was used as a negative control. After three washing, wells were probed with 50 µL of goat anti-mouse IgG HRP-conjugated (1:5000) for 1 hour at 37. The plate was washed as described above and the binding was detected by adding TMB (Tetramethyl Benzidine) peroxidase substrate (Thermo Fisher Scientific) for 10 min at 37ºC. Final optical density (OD) was taken at 450 nm wavelength using ELISA plate reader (Infinite 200 Pro, Tecan) after terminating the reaction with 1 M H_2SO_4. The endpoint titer was determined visually, with the highest serum dilution giving a positive color development.

4.3.7 Immunoblot assay

Whole-cell lysates of *L. interrogans* serovar Lai grown in the presence and absence of catecholamines were resolved onto 12% SDS-PAGE and transferred to a nitrocellulose membrane (HiMedia). Membranes were blocked with 5% non-fat dried milk diluted in Tris-buffer saline (TBS, pH 8) containing 0.05% Tween 20 (TBS-T) and probed with anti-LIC20035 (1:500) mouse polyclonal serum for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000; Sigma) in TBS for 1 h. The protein reactivity was revealed by adding chemiluminescence substrate (Thermo scientific, Catalog no. 32209) over nitrocellulose membrane and imaged under Chemidoc (Biorad XRS+). Band densitometry of the LB047/LIC20035 expression obtained from immunoblot was quantified using Image Lab software (Biorad) as described before (Taylor et al., 2013) and the density values were normalized with LipL31 expression in the immunoblot. The relative densitometry of LB047 expression was calculated in terms of percentage from two independent immunoblot experiments.

The whole-cell lysates of *L. interrogans* serovars (1×10^9 cells) and/or the r-LIC13341/r-LIC12693/r-LIC11966 were run onto 12% SDS-polyacrylamide gel which was subsequently transferred to a nitrocellulose membrane. It was then probed depending on the experiments with either mouse anti-LIC13341/anti-LIC11966/anti-LIC12693 polyclonal serum (1:1000 each) or human/bovine pooled serum (1:200) tested positive or negative for leptospirosis, for 2 h at room temperature. After washing, the nitrocellulose membranes were incubated with HRP conjugated secondary antibodies of goat anti-mouse IgG (1:5000; Sigma)/goat anti-human IgG (1:5000; Genei)/rabbit anti-bovine IgG (1:5000; Sigma) in TBS-T for 1 h. The

detection was performed by adding chemiluminescence substrate over the nitrocellulose membrane and imaged under Chemidoc.

4.3.8 Phase separation of an integral membrane protein using Triton X-114

Phase separation of the integral membrane proteins of *Leptospira* to localize LIC20035 was performed using Triton X-114 solution as described elsewhere (Bordier, 1981). Briefly, 1×10^9 spirochetes grown in EMJH medium were centrifuged (5,000 g) and washed 3 times with phosphate buffer saline (PBS). The pellet obtained was sonicated four times with 20 s burst after suspension in 800 µL of PBS. The suspension was added with 200 µL of 10% Triton X-114 (Sigma, Cat No. X114) and was rocked overnight at 4°C. The insoluble debris was removed by centrifugation at 13,000 g for 15 min at 4°C and placed in 37°C water bath for 10 min. The suspension was centrifuged for 10 min at 13,000 g at room temperature to separate the detergent (D) and the aqueous phase (A). The detergent phase (50 µL) was mixed with 1 mL of original buffer at 0°C rewarmed at 37°C and spun in a microcentrifuge as before. Later, the final aqueous (A) and detergent phase (D) obtained after three times wash were precipitated with 10 volumes of chilled acetone. The aqueous (A) and detergent phase (D) was then resolved on 10% SDS-PAGE before transferring to nitrocellulose membrane (Himedia) for immunoblot. The antibodies against LipL32 raised in rabbits were obtained as a kind gift from Dr. David Haake (Los Angeles, California, USA).

Phase separation of the integral membrane proteins of *Leptospira* to localize LIC13341, LIC11966, and LIC12693 were performed using Triton X-114 solution as described elsewhere (Haake et al., 2000b). Briefly, a 50 mL mid-log-phase culture of *L. interrogans* Copenhageni (5×10^9 cells) was harvested and washed in PBS containing 5 mM $MgCl_2$. The membrane proteins were extracted at 4°C in the presence of 1% Triton X-114 (Sigma), 150

mM NaCl, 10 mM Tris (pH 8) and 1 mM EDTA. The insoluble debris was removed by centrifugation at 12,000×*g* for 15 min and then 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by warming the supernatant at 37°C and subjecting it to centrifugation for 10 min at 1,000 g. Remaining procedures remained the same as mentioned above for LIC20035 phase separation experiment.

4.3.9 Protease-accessibility assay for validating the cellular localization of outer membrane proteins (LB047, LIC13341, and LIC11966)

Proteinase K assay was performed for cellular localization of LB047, LIC13341, and LIC11966 using the procedures described elsewhere (Domingos et al., 2012). Briefly, suspensions of 15 mL of 6 days old live *L. interrogans* serovar Lai/ *L. interrogans* serovar Copenhageni (2.5×10^8 spirochetes/mL) were harvested and resuspended in 6 mL of PBS containing 25 µg of Proteinase K (SRL). Tubes containing 1 mL of resuspended spirochetes were then incubated for 0, 1, 3, and 5 h before the addition of 10 µl of 100 mM phenylmethylsulfonyl fluoride (PMSF) to terminate the Proteinase K activity. The suspensions were subsequently pelleted by centrifugation at 5000 g for 15 min, washed twice with PBS and resuspended in 1 mL of PBS for performing ELISA using antibodies against OmpL54, LB047/LIC13341/LIC11966, and LipL31. OmpL54 and LipL31 are outer and cytoplasmic membrane proteins of *L. interrogans* that were used in this experiment as positive and negative controls respectively (Haake and Matsunaga, 2002; Pinne and Haake, 2009). The antibodies against OmpL54 and LipL31 raised in rabbits were obtained as a kind gift from Dr. David Haake (Los Angeles, California, USA). For the cellular localization of LB047/LIC13341/LIC11966 by ELISA, 100 µl of the Proteinase K treated leptospires were coated onto the microtiter plates and incubated for 16 h at room temperature. The primary

antibody against OmpL54, LIC20035/LIC13341/LIC11966, and LipL31 was used at a dilution of 1:50, 1:500/1:1000/1:1000 and 1:1000, respectively. Goat anti-rabbit/anti-mouse IgG HRP-conjugated secondary antibodies were used at a dilution of 1:5000. The binding was detected as described in section 4.3.6.

4.3.10 Quantifying the binding of r-LIC20035, r-LIC13341, r-LIC11966, and r-LIC12693 to host extracellular matrix (ECM) components using ELISA

Host ligands included in the study were fibronectin (Sigma, Cat No. F4759), laminin (Sigma, Cat No. L2020), collagen from calf skin (Sigma; Catalog No. C9791), collagen type I from rat tail (Merck; Catalog No. 08-115), elastin (Sigma, Cat No. E1625), chondroitin sulphate A & B (Sigma, Catalog No. C9819/C3788), hyaluronic acid (Sigma, Catalog No. H7630), heparan sulphate (Sigma; Catalog No. H7640) and fibrinogen (Sigma; Catalog No. F3879). As negative controls for the ligand, fetuin from fetal calf serum and BSA (New England Biolabs) were used. Recombinant-Loa22 was used as a negative control antigen, as it is previously shown to interact moderately with the tested ECM components (Barbosa et al., 2006). The binding of recombinant proteins to these host ECM components were analyzed by indirect ELISA. Briefly, ELISA plates were coated in duplicates with 1 µg of the solubilized ECM components, BSA (negative control ligand) and fetuin (highly glycosylated attachment negative control ligand) in 50 µL of PBS for 2 h at 37ºC. Further procedures were followed as described in section 4.3.6.

4.3.11 Dose-response curves and equilibrium dissociation constant determination

ELISA plates were coated overnight with 1 µg of different ECM components (i.e. fibronectin, laminin, collagen from calf skin, collagen type I from rat tail, elastin, chondroitin sulphate A & B, hyaluronic acid, heparan sulphate, and fibrinogen). Each plate was blocked

and increasing concentrations of purified r-LIC20035 (0-8 µM), was added (50 µL per well, in PBS) followed by incubation for 2 h at 37ºC. For r-LIC13341, the protein concentrations were used from 0-24 µM. The assessment of bound protein was performed with polyclonal antiserum raised in mice against LIC20035 and LIC13341 followed by HRP-conjugated anti-mouse IgG. The ELISA data, when the reactions reached a saturation point, were used to calculate the equilibrium dissociation constant (K_D), according to a method described elsewhere (Lin et al., 2009) by following the equation $K_D=(A_{max}[protein])/A)-[protein]$, where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium) and [protein] is the protein concentration.

4.3.12 Inhibition of live leptospires adherence to laminin and hyaluronic acid by r-LIC13341

ELISA plates were coated with laminin or hyaluronic acid (1 µg/well) for overnight at 4ºC. The microtiter plates were washed and blocked with 3% BSA for 2 h at 37ºC. Thereafter, the wells of microtiter plates were incubated with increasing concentrations of r-LIC13341 (0-10 µg) as described previously (Domingos et al., 2012). After 3 washings, live *L. interrogans* Copenhageni (1×10^8 spirochetes per well in 100 µL) were allowed to adhere to the antigen-coated microtiter plate for 90 min at 37ºC. After washing of the microtiter plates, the bound leptospires were quantified indirectly by probing with rabbit anti-Loa22 antibody (1:1,000) and its secondary HRP-conjugated antibodies (1:5,000). Final OD was taken at 450 nm wavelength using ELISA plate reader after terminating the reaction with 1 M H_2SO_4.

4.3.13 Effect of laminin oxidation on the protein-laminin interaction

To evaluate the role of carbohydrate moiety of laminin on binding with r-LIC13341, microtiter plate wells were coated with 1 µg of laminin in 50 mM sodium acetate buffer, pH

5.0, and incubated for 16 h at 4°C. Wells were washed 3 times with 50 mM sodium acetate buffer, pH 5.0, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 - 100 mM) in the same buffer for 15 min at 4°C in the dark as described previously (Barbosa et al., 2006). After 3 washes with 50 mM sodium acetate buffer, wells were blocked with 100 µL of 1% BSA for 1 h at 37ºC. Binding of r-LIC13341 (1 µg in PBS per well) to periodate-treated laminin was assessed as described in section 4.3.9.

4.3.14 Effect of r-LIC13341 denaturation on its interaction with hyaluronic acid

The untreated and heat denatured (95°C for 10 min) recombinant LIC13341 (0.25 - 4 µM) was allowed to bind to the hyaluronic acid (1 µg per well) coated microtiter plate. The binding was measured by indirect ELISA as described in section 4.3.10.

4.3.15 ELISA for recognition of recombinant-LIC20035, LIC11966, and LIC13341 using sera tested positive for leptospirosis

Serum samples of human (n=50), bovine (n=50) and canine (n=18) which had tested positive for leptospirosis through microscopic agglutination test (MAT), were used for recognition of r-LIC20035, r-LIC11966, and r-LIC13341 by ELISA. Another antigen r-LIC10191 (Loa22), a known surface-exposed lipoprotein of leptospirosis diagnosis (Chalayon et al., 2011), was taken in an equivalent amount to testify the ELISA results and to scale the absorbance obtained for both the recombinant proteins. The control human (n=15)/bovine (n=10)/canine (n=11) serum tested MAT-negative were also used for recognition of recombinant proteins by ELISA. Microtiter plates were coated in duplicates with an equal amount (400 ng/well) of recombinant proteins (r-LIC20035, r-LIC13341, r-LIC11966, and r-Loa22) overnight at 4ºC and thereafter unbound surface was blocked with 3% BSA at 37ºC for 2 hours. After 3 washing of the plate with 200 µL of phosphate buffer saline containing 0.05% Tween 20

(PBS-T), wells were probed with human/bovine/canine leptospirosis serum (1:100) at 37 °C for 2 hours. Microtiter wells were probed with secondary HRP-conjugated anti-human IgG (Genei, Catalog No. 62114028001A) /anti-bovine IgG (Sigma, Cat No. A5295)/ anti-dog IgG (Sigma, Cat No. A6792) for 1 hour (1:5,000) at 37ºC and the binding was measured as described in end-point titer determination. The cut-off value for antibody reactivity was calculated as described previously (Chalayon et al., 2011). Briefly, the average of the absorbance values and their standard deviation (SD) were calculated from the MAT negative serum groups and the cut-off value of the average + 2 SD was used for analysis. All the absorbance values obtained from the ELISA assay which equaled or exceeded the cut-off value was considered as positive for infection and thus sensitivity (%) of the assay was calculated. The specificity (%) of the assay was calculated on the number of samples of the MAT negative group below the calculated cut-off.

4.3.16 Statistical analysis

All results are expressed as the mean ± standard error. Student's paired t-test was used to determine the significance of differences between means and $P < 0.05$ was considered statistically significant. Two independent experiments were performed, each one in duplicate or triplicate.

4.4 RESULTS

4.4.1 Molecular characterization of hypothetical protein LIC20035/LB047

Among the seven genes that were significantly differentially expressed in the presence of catecholamine(s), *LIC20035/LB047* encoding hypothetical outer membrane protein in *Leptospira* was selected for further characterization as this gene was differentially expressed in the presence of all the host factors tested i.e. catecholamines, oxidative stress and osmolarity. Protein BLAST was performed to identify orthologs of LIC20035 in the pathogenic, intermediate and saprophytic strains of *Leptospira*. As is evident from Table 4.2, LIC20035 is conserved (88-99% amino acid sequence identity) across pathogenic leptospires in comparison to intermediates (62-64%) and saprophytic strains (39-40%).

The *LIC20035* was cloned without its predicted signal peptide using genomic DNA of *L. interrogans* serovar Copenhageni (Fig. 4.1). The molecular characterization of *LIC20035* was performed by analyzing the existence of this gene by PCR in *L. interrogans* serovars like Copenhageni, Lai, and Canicola of *Leptospira* and saprophytic (*L. biflexa* serovar Patoc) *Leptospira*. Using PCR, amplification of 1275 bp confirmed the existence of *LIC20035* in various pathogenic spirochete serovars viz. Copenhageni, Lai, and Canicola of *Leptospira*. Interestingly no amplicon was detected in non-pathogenic serovar Patoc (Fig. 4.2A), although there is an existence of the ortholog of LIC20035 with 39% amino acid identity. However, amplification of the *fla*B gene of 852 bp could be seen in all pathogenic and non-pathogenic serovars of *Leptospira* (Fig. 4.2A).

Table 4.2. Comparative analyses of the protein sequence identity of LIC20035 in the different species of *Leptospira*.

Leptospira species	Pathogenic (++), Intermediate (+) or Saprophyte (-)	Query coverage (%)	Identity (%)	NCBI Accession number
kirschneri	++	99	99	WP_004765841.1
noguchii	++	99	96	WP_061247126.1
santarosai	++	99	90	WP_004475508.1
weilii	++	99	90	WP_002999236.1
alexanderi	++	99	90	WP_078124341.1
alstonii	++	99	90	WP_020775287.1
kmetyi	++	99	90	WP_010572225.1
mayottensis	++	99	89	WP_002764046.1
borgpetersenii	++	99	88	WP_011671265.1
wolffii	+	99	64	WP_016545620.1
fainei	+	99	64	WP_016551189.1
inadai	+	99	63	WP_010410057.1
broomii	+	99	63	WP_010568341.1
licerasiae	+	98	62	WP_008589084.1
wolbachii	-	99	40	WP_015682912.1
biflexa	-	100	39	WP_012476493.1

The over-expressed r-LIC20035 was purified using Ni-affinity column chromatography (Fig 4.2B). Subsequently, the purified recombinant protein was used to generate antibodies in BALB/c mice. The polyclonal antibodies generated against r-LIC20035 showed an end-point titer of 1:5000 (Fig. 4.2C). The immunoblot of the *L. interrogans* Lai grown in the presence of catecholamines and its antagonist demonstrated that the generated polyclonal anti-LIC20035 were able to recognize LB047 at the expected size of 50 kDa. Moreover, there was a repression of the LB047 in the presence of Epi in comparison to the basal expression in

control (Fig. 4.2D). The expression of LB047 in the spirochetes grown in the presence of Epi and its antagonist combined was considerable of the same magnitude to that of control. The consistent expression of LipL31 in the host and under IVCL has been previously described and thus LipL31 used in our experiment demonstrated equal loading of all the experimental samples (Fig. 4.2D). In the same context, the relative densitometry of LB047 expression obtained from immunoblot illustrated LB047 to be repressed in the presence of Epi and its expression was restored to the basal level in the presence of antagonist, PO (Fig. 4.2E). Further, before using LIC20035 to understand its function, the secondary structure of the purified r-LIC20035 was evaluated by circular dichroism (CD). The CD spectroscopy data showed maxima at 215 nm and minima at 200 nm wavelengths (Fig 4.2F). The spectroscopic data analysis using program K2D2 revealed 2% α-helix and 52% β-sheet. This was in close agreement to the theoretical secondary structure of the r-LIC20035 which predicted 0% α-helix and 66% β-sheet using program PSIPRED.

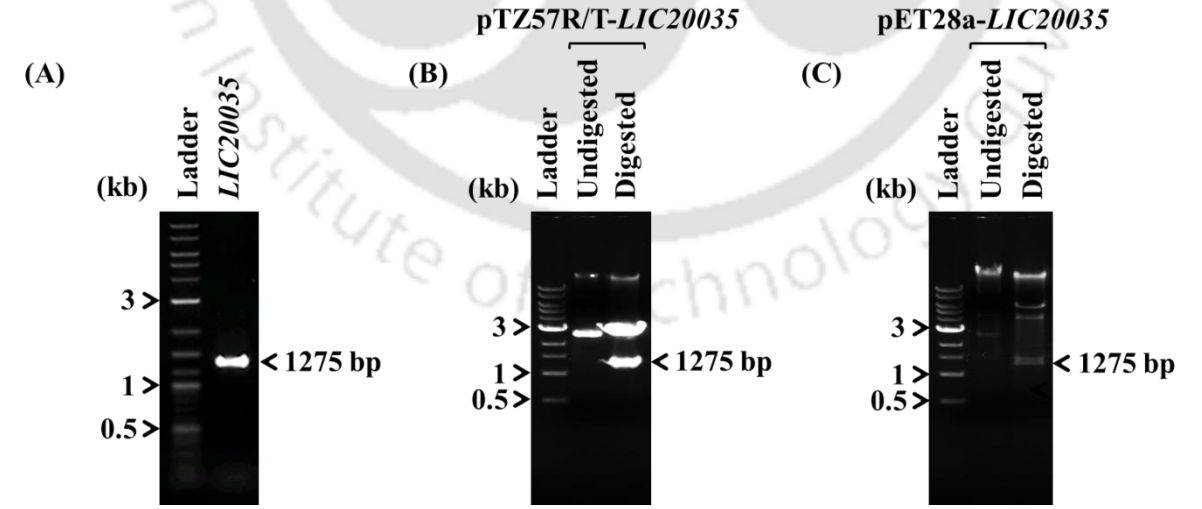

Figure 4.1. Cloning of *LIC20035* in pET28a vector. (A) PCR amplification of *LIC20035* gene (1,275 bp) using genomic DNA of pathogenic *L. interrogans* Copenhageni after deleting the signal peptide. **(B)** Cloning of amplified *LIC20035* in PTZ57R/T vector. The double digestion

with *Nhe*I and *Xho*I shows a fall-out of 1,275 bp. **(C)** Cloning of *LIC20035* in pET28a vector. A fall-out of 1,275 bp using *Nhe*I and *Xho*I confirms the cloning.

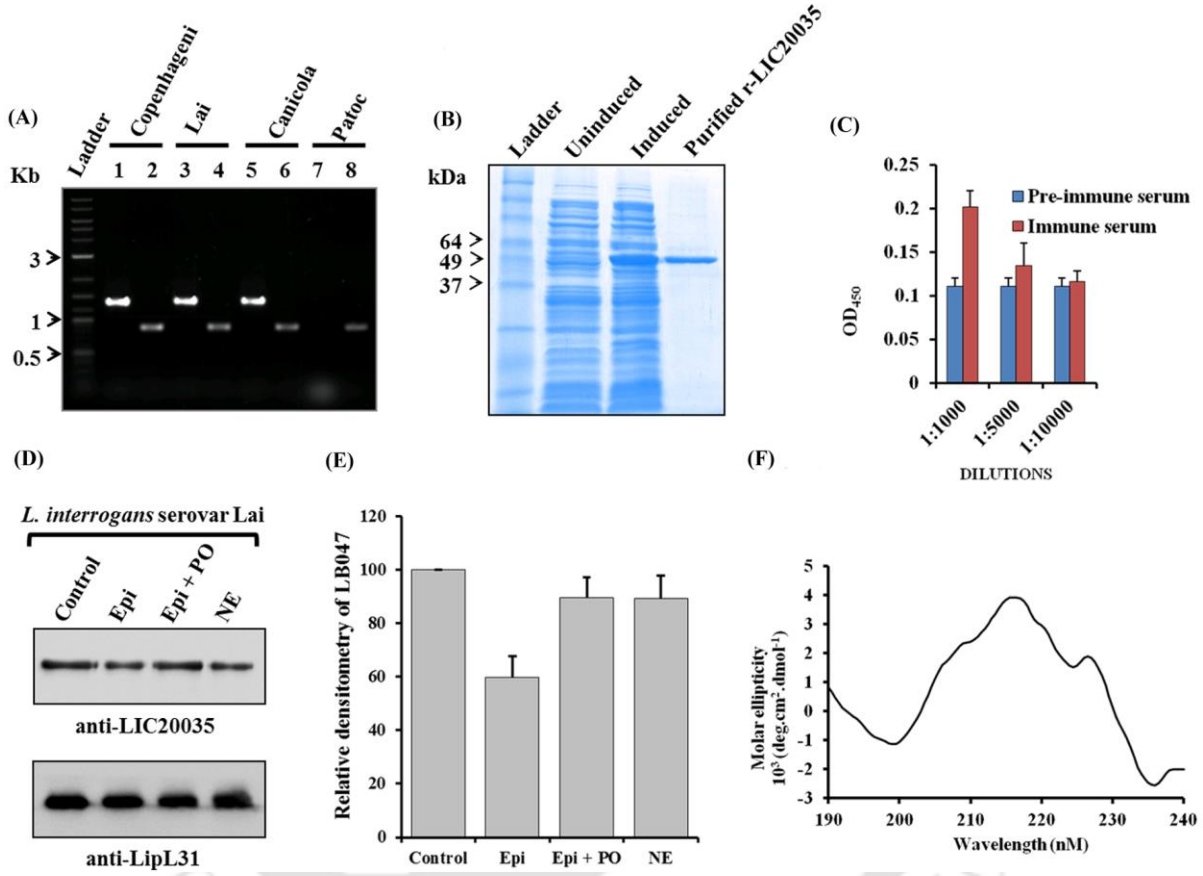

Figure 4.2. Characterization of hypothetical protein LIC20035/LB047. (A) PCR for *LIC20035* gene using genomic DNA of pathogenic (Copenhageni, Lai, Canicola) and non-pathogenic serovars (Patoc) of *Leptospira*. An amplicon size of 1,275 bp by agarose gel electrophoresis confirmed the existence of *LIC20035* gene in pathogenic serovars (Lane 1, 3 and 5). In contrast, no amplification of *LIC20035* was observed in the non-pathogenic serovar of *Leptospira* (Lane 7). Primers of constitutive *flaB* gene were used as a positive control for determining the quality of DNA (Lane 2, 4, 6 and 8). **(B)** Induction and purification of recombinant-LIC20035. Induction of r-LIC20035 expression was done using 0.5 mM IPTG and its purification was performed using Ni-NTA column chromatography under hybrid conditions. The uninduced and induced lysates of BL21 *E. coli* along with the purified recombinant-LIC20035 are shown on 12% SDS-PAGE stained with Coomassie. **(C)** Generated polyclonal antibody titer raised against r-LIC20035. The pooled mice immune sera obtained after 10 days of second booster dose was used to calculate the titer of polyclonal antibodies generated against r-LIC20035 using ELISA. Serum obtained before the immunization of r-LIC20035

antigen was used as a control for evaluation of antibody titer and data is represented as mean± standard error mean (SEM) of two independent experiments. **(D)** Immunoblot demonstrates the differential expression of LB047 in *L. interrogans* serovar Lai grown in the presence of catecholamines and its inhibitor. There was a repression of LB047 in the presence of Epi alone in comparison to control and NE, the effect of which was restored to the basal levels when grown along with its inhibitor propranolol (PO). Anti-LipL31 was used as a control to demonstrate equal loading of *Leptospira* lysates. **(E)** Relative densitometry of the immunoblot shown in Fig. 4.2D was calculated and normalized with the band intensity of LipL31 using Image Lab software from two independent experiment. The repression of LB047 was evident in the presence of Epi and the effect of which was restored to the basal level using its inhibitor PO. **(F)** Far-ultraviolet circular dichroism (CD) of r-LIC20035. The CD spectra are depicted in the range of 190-240 nm wavelengths showing a predominant signal of *β*-strand. CD spectra are shown as an average of 3 scans with a scanning speed of 100 nm/minute.

4.4.2 LIC20035 is a predominant outer membrane surface-exposed protein

Cellular localization of LIC20035 was assessed by Triton X-114 detergent solubilization and phase partitioning. Solubilizing *Leptospira* in 1% Triton X-114 yields a detergent-insoluble fraction known as protoplasmic cylinder (PC) fraction and a detergent soluble fraction (Haake et al., 1991). The Triton X-114 soluble fraction is resolved into two phases by heating above the cloud point (37ºC) of this detergent, resulting in separation of the detergent-hydrophobic phase (D) from the detergent-poor aqueous phase (A). In fact, previous cellular localization studies on *Leptospira* show that the outer membrane lipoproteins separate in Triton X-114 detergent phase, while periplasmic proteins separate into the aqueous phase of the soluble fraction. The immunoblot using polyclonal anti-LIC20035 detected the LIC20035 to be predominantly present in the detergent phase of the Triton X-114; however, a small amount of the protein could also be traced in the aqueous phase (Fig. 4.3A). Additionally, to validate the Triton X-114 phase separation experiment, anti-LipL32 was used as a positive control to detect LipL32 which was reported to be present only in detergent phase (Haake et al., 2000a). A conspicuous band of LipL32 observed only in the detergent phase of the

Leptospira lysates by immunoblot underpins experimental procedures for separating the membrane-bound proteins (Fig. 4.3A).

To validate LB047 (ortholog of LIC20035 in *L. interrogans* serovar Lai) is a surface-exposed membrane protein; a protease-accessibility assay was performed for *L. interrogans* serovar Lai. The integrity of the spirochete cell wall during Proteinase K treatment was confirmed by performing ELISA using antiserum against LipL31, a previously described cytoplasmic membrane protein. Similarly, an antibody against a known surface-exposed protein OmpL54 was used as a positive control. After 1 to 5 h treatment with Proteinase K, LB047 showed a decrease in recognition with its antiserum, similar to the positive control, OmpL54 (Fig. 4.3B). Around 50% reduction in LB047 recognition was observed after 5 h of protease assay. In contrast, no significant reduction in reactivity with anti-LipL31 describes the integrity of spirochete membrane was not compromised during the Proteinase K assay (Fig. 4.3B). Taken together, the cellular localization using Triton X-114 and Proteinase K assay suggest LB047/LIC20035 to be a surface-exposed membrane protein.

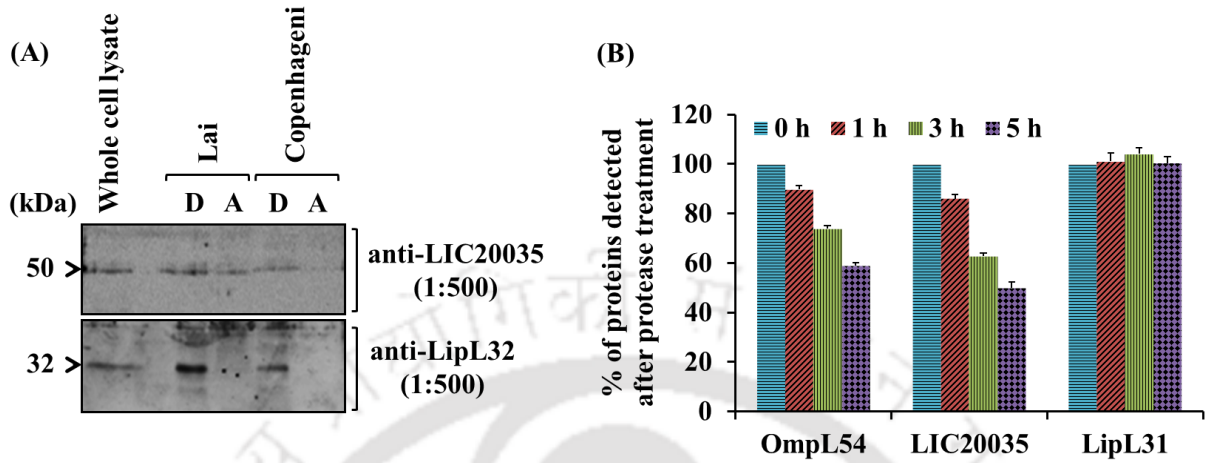

Figure 4.3. Cellular localization of LIC20035/LB047. (A) Triton-X-114 phase partitioning of spirochetes proteins. Spirochetes lysates were subjected to Triton X-114 phase partition of aqueous (A) and detergent phase (D). The aqueous and detergent phase fraction was resolved onto 12% SDS PAGE and was immunoblotted with anti-LIC20035 serum (top panel). A major portion of LIC20035 was in the detergent phase and partly in the aqueous phase in both serovars Lai and Copenhageni. Whole-cell lysates of serovar Copenhageni in lane 1 was used as a molecular marker for LIC20035. A leptospiral outer membrane lipoprotein LipL32, which is known to localize predominantly in the detergent phase of Triton X-114, was used to validate the experiment. Anti-LipL32 (1:500) detected LipL32 only in the detergent phase (D) fraction of serovars Lai and Copenhageni (bottom panel). Whole-cell lysates of serovar Copenhageni in lane 1 was used as a molecular marker for LipL32. **(B)** Proteinase K accessibility assay of *L. interrogans* Lai for surface exposed LB047. Spirochetes were incubated with 25 µg of Proteinase K at various time interval up to 5 h. The spirochete suspensions were washed with PBS and coated onto microtiter plate. Using ELISA, a drastic decrease in the signal for LB047 reactivity with its antiserum was observed after 1 to 5 h Proteinase K treatment similar to OmpL54, a known outer membrane protein. LipL31 was used as a control to keep a check on the cellular integrity of spirochetes during treatment with Proteinase K. Error bars represent the standard deviations of the three replicates. Statistical analysis was performed by Student's *t*-test by comparing the signals obtained for 0 h and another time point of treatment with Proteinase K ($P<0.05$).

4.4.3 Cloning, over-expression, and purification of Loa22 (LIC10191)

To scale the recognition capacity of the infected serum samples, Loa22, a known diagnostic marker was overexpressed and purified from the prokaryotic expression system. Briefly, the

CDS of *LIC10191* was amplified without the signal peptide and cloned in pET28a vector as (Fig. 4.4A and Fig. 4.4B). The overexpression was achieved by the addition of 0.5 mM IPTG. The recombinant Loa22 protein was purified using hybrid method (Fig. 4.4C). The purified r-Loa22 protein was used for all the ELISA experiments as control. Antibodies against r-Loa22 were generated in rabbit by outsourcing the purified protein to Abgenex, Bhubaneswar, India. The polyclonal antibodies were able to specifically recognize Loa22 in the lysates of pathogenic *Leptospira* serovars (Copenhageni, Lai, and Canicola) and the recombinant protein using anti-Loa22 antibody (Fig. 4.4D).

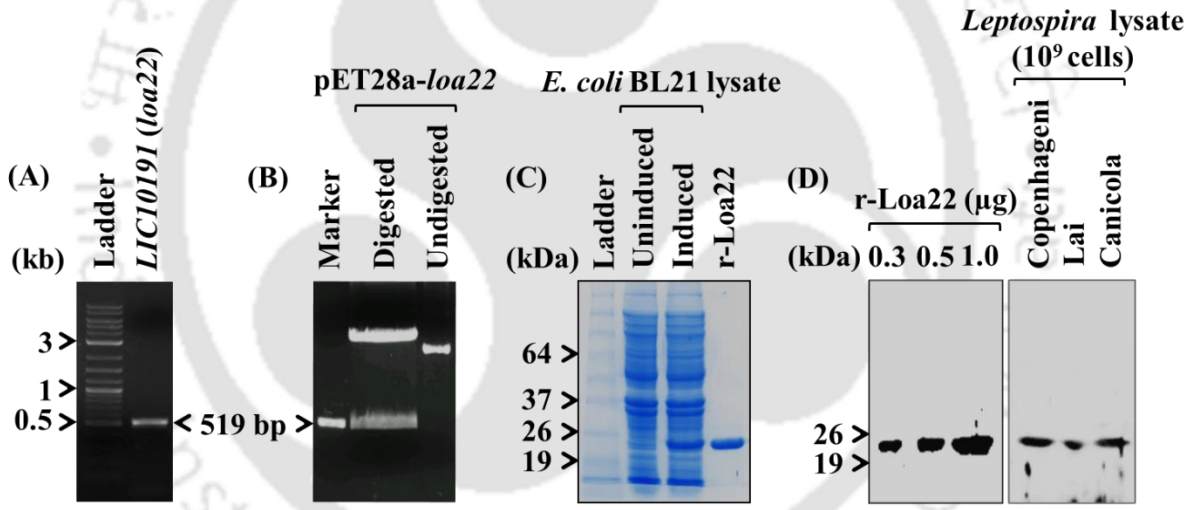

Figure 4.4. Cloning, overexpression and purification of r-Loa22. (A) PCR amplification of *loa22* gene using genomic DNA of *L. interrogans* serovar Copenhageni as a template. **(B)** Cloning of *loa22* gene in pET28a bacterial expression vector. The double digestion using *Nhe*I and *Xho*I shows a fallout of 519 bp. **(C)** Over-expression of r-Loa22 in *E. coli* BL21 (DE3) cells and purification of recombinant protein. **(D)** Immunoblot to detect recombinant and native Loa22 antigen. Various amount of r-Loa22 (0.3-1 µg) and *Leptospira* lysates of various pathogenic serovars (Copenhageni, Lai, and Canicola) were probed with rabbit anti-Loa22 antibody. Single specific band of native Loa22 was recognized in the lysates using anti-Loa22 antibody.

4.4.4 LIC20035 is detected by antibodies of human and bovine leptospirosis serum

As LIC20035 is localized in the outer membrane of spirochetes, it was interesting to evaluate its serological recognition using immunoglobulins of *Leptospira*-infected hosts. The recombinant proteins (Loa22 and LIC20035) were coated on microtiter plates and were probed with human or bovine serum tested positive for leptospirosis (n=50) through microscopic agglutination test (MAT +ve). As a control, serum samples of human (n=15) and bovine (n=10) tested negative for leptospirosis (MAT -ve) were included in the serological assay. The average OD for serological detection of LIC20035 antigen in humans (Fig. 4.5A) was found to be 0.5618. The calculated sensitivity and specificity of serological detection of LIC20035 by human serum was 98% and 100% respectively. In contrast, Loa22 showed sensitivity and specificity of 100% for human serum (Fig. 4.5B). The average OD for the serological detection of LIC20035 antigen in bovine was 0.4449 (Fig. 4.5C). The calculated sensitivity and specificity of serological detection of LIC20035 by bovine serum was 100%. The antigen Loa22 also showed sensitivity and specificity of 100% for bovine serum (Fig. 4.5D). Interestingly, the difference between the absorbance obtained for Loa22 (0.6404) and LIC20035 (0.4449) for bovine infected serum samples was very high (Fig. 4.5C and Fig. 4.5D).

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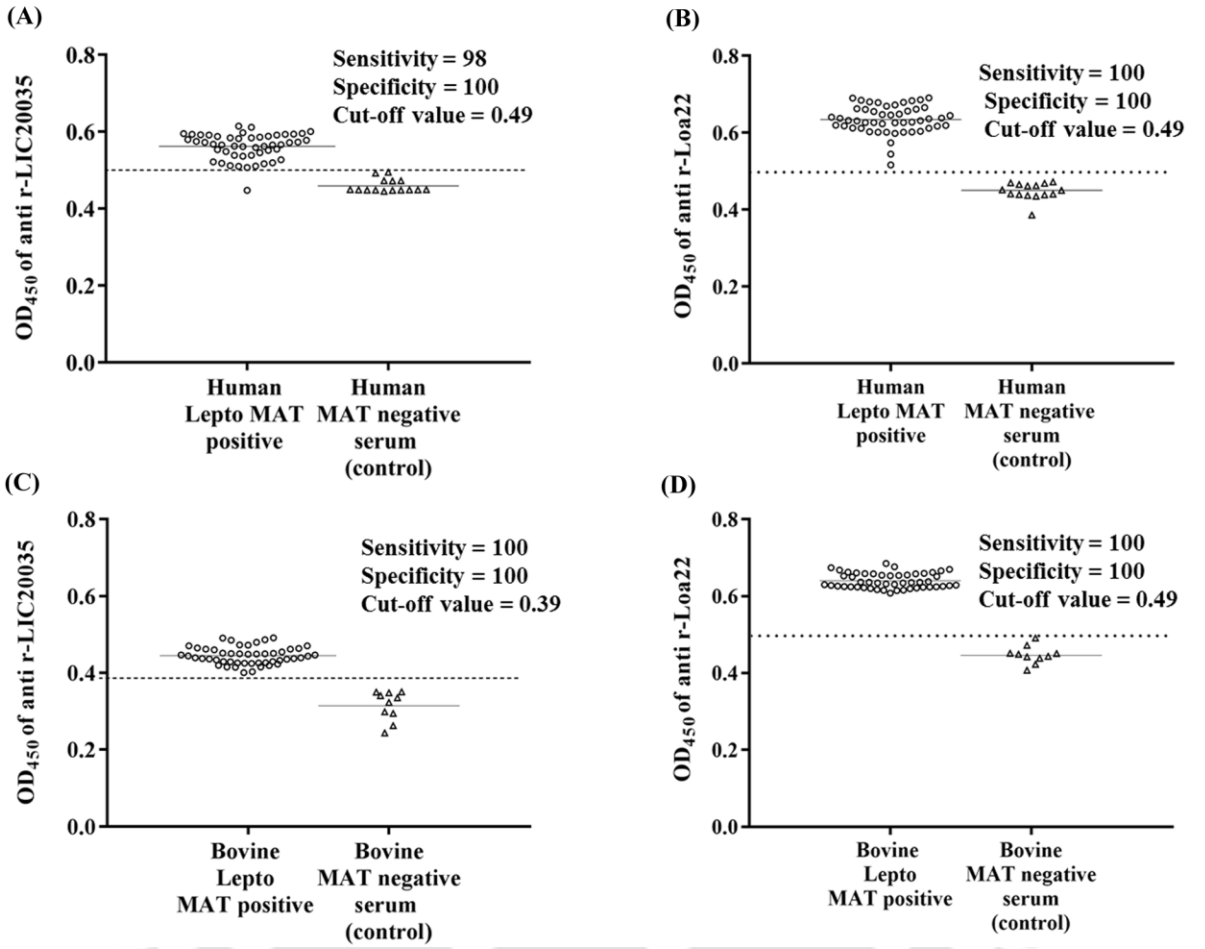

Figure 4.5. The recombinant-LIC20035 is recognized by leptospirosis positive sera. Enzyme-linked immunosorbent assay (ELISA) was performed to detect r-LIC20035 or r-LIC10191 using the sera of humans (1:100) and bovines (1:100) tested MAT-positive (n=50) and control serum tested MAT-negative for leptospirosis. The cut-off value of the assay was derived from the mean (M) of the control group plus 2SD (standard deviation) for each recombinant antigens (dotted black lines). The mean (M) of each group represents as black horizontal lines. **(A)** ELISA to detect r-LIC20035 (400 ng/well) using human sera tested positive for leptospirosis. The sensitivity and specificity of the assay were 98 and 100, respectively. **(B)** ELISA to detect r-LIC10191 (400 ng/well) using human sera tested positive for leptospirosis. Recombinant-LIC10191 (r-Loa22) of an equivalent amount (400 ng/well) was used to scale the recognition capacity of human sera. **(C)** ELISA to detect r-LIC20035 (400 ng/well) with bovine sera tested positive for leptospirosis. The sensitivity and specificity of the serological assay were 100. **(D)** ELISA to detect r-Loa22 with bovine sera tested positive for leptospirosis. Recombinant-Loa22 of the equivalent amount was used to scale the recognition capacity of bovine sera.

4.4.5 LIC20035 binds to extracellular matrix components of the host

The numerous study about the attachment of *L. interrogans* to extracellular matrices of the host gave a clue to the existence of several adhesion molecules (Barbosa et al., 2006). Thus, ELISA was performed to study the interaction of r-LIC20035 with the ECM components. The r-LIC20035 along with r-Loa22 was allowed to bind with the immobilized ECM components (i.e. laminin, fibronectin, collagen (calf skin and rat tail), hyaluronic acid, chondroitin sulfate A and B, and elastin) on a 96-well plate with BSA and the highly glycosylated serum protein, fetuin, as negative controls. The r-LIC20035 showed significant binding ($P<0.001$) with all the ECM components in contrast to Loa22 (Fig. 4.6A).

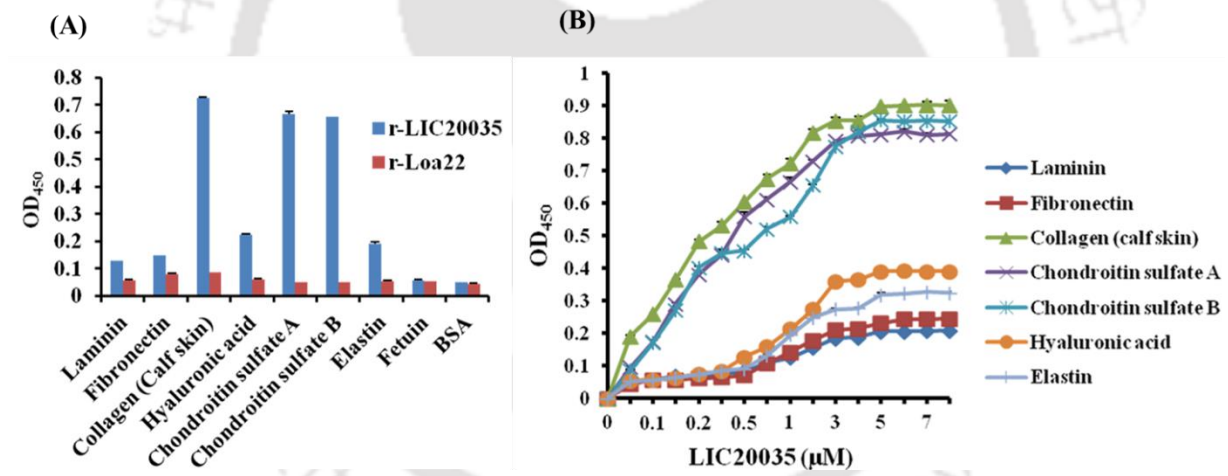

Figure 4.6. The recombinant-LIC20035 binds to host extracellular matrix components. **(A)** ELISA depicts r-LIC20035 interacts with extracellular matrix (ECM) components, laminin, fibronectin, collagen (calf skin), hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, and elastin. Bovine serum albumin (BSA) and the highly glycosylated serum protein, fetuin, were used as controls for non-specific binding. Loa22 was included as a negative control for non-specific binding with the ECM components. Recombinant-LIC20035 exhibited significant binding with all ECM components as compared to fetuin or BSA ($P<0.001$) and with a higher affinity towards chondroitin sulfate and collagen. In contrast, Loa22 showed moderate binding with all the ECM components used in this study. Results are indicative of two independent experiments performed. **(B)** Dose-dependent binding of the LIC20035 to ECM components. One microgram of specific ECM coated microtiter plate was incubated with increasing

concentrations (0-8 µM) of r-LIC20035. Binding assay of LIC20035 was measured using the anti-LIC20035 serum at an appropriate dilution. The mean absorbance values of r-LIC20035 binding to ECM of two experiments are shown at 450 nm.

The highest affinity of r-LIC20035 was seen with collagen type I and chondroitin sulfate. Additionally, quantitative analyses of the binding of r-LIC20035 with each of these ECM components are shown in Fig. 4.6B. A dose-dependent saturable binding was observed with increasing concentration of the recombinant protein (0-8 µM) over a fixed amount of the ECM components (1 µg). The r-LIC20035 protein interacts with the host ligands in a dose-dependent and saturable fashion with the calculated K_D of 200 nM, 250 nM and 200 nM for collagen type I and chondroitin sulfate A, and chondroitin sulfate B, respectively.

4.4.6 *In silico* analysis of LIC13341 and identification of its orthologs

The hypothetical protein, LIC13341 was characterized in the present study as the gene transcript of *LIC13341* was not transcribing from the cDNA of *L. interrogans*. It became interesting to investigate the unknown host factor(s) or transcription factor(s) which might induce(s) the *LIC13341* gene transcription. Another reason for the loss of *LIC13341* transcription could be because of the attenuation of virulent genes during *in vitro* passaging. After procuring low passage *L. interrogans*, we were able to demonstrate the transcription of *LIC13341* in all the low passage pathogenic serovars of *L. interrogans* tested. Bioinformatics analysis of LIC13341 using SignalP 3.0 program (Dyrløv Bendtsen et al., 2004) predicted a signal peptide at the N-terminal with the cleavage site between 55^{th} and 56^{th} amino acid residues. In addition, CELLO (Yu et al., 2006; Yu et al., 2004) and PSORT programs (Nakai and Horton, 1999) predicted LIC13341 to be an outer membrane or periplasmic protein of *Leptospira*, respectively. The amino acid sequence of LIC13341 analyzed manually, had

signal peptide which may be recognized by the spirochetal lipoprotein signal peptidase (Lsp) (Cullen et al., 2004). The CDS of *LIC13341* fulfilled the requirements established for predicting spirochetal proteins to be lipoprotein in nature (Cullen et al., 2004; Haake et al., 2000b; Setubal et al., 2006). BLAST search analysis was performed using available LIC13341 sequences from the NCBI database and 27 representative leptospiral sequences were used to construct the phylogenetic tree. The resulting phylogram showed a high level of sequence conservation among pathogenic spp. of *Leptospira* for LIC13341 (71-96% amino acid sequence identity) in comparison to intermediates (64-66%) and saprophytic spp. of *Leptospira* (42 - 46%) (Fig. 4.7 and Table 4.3).

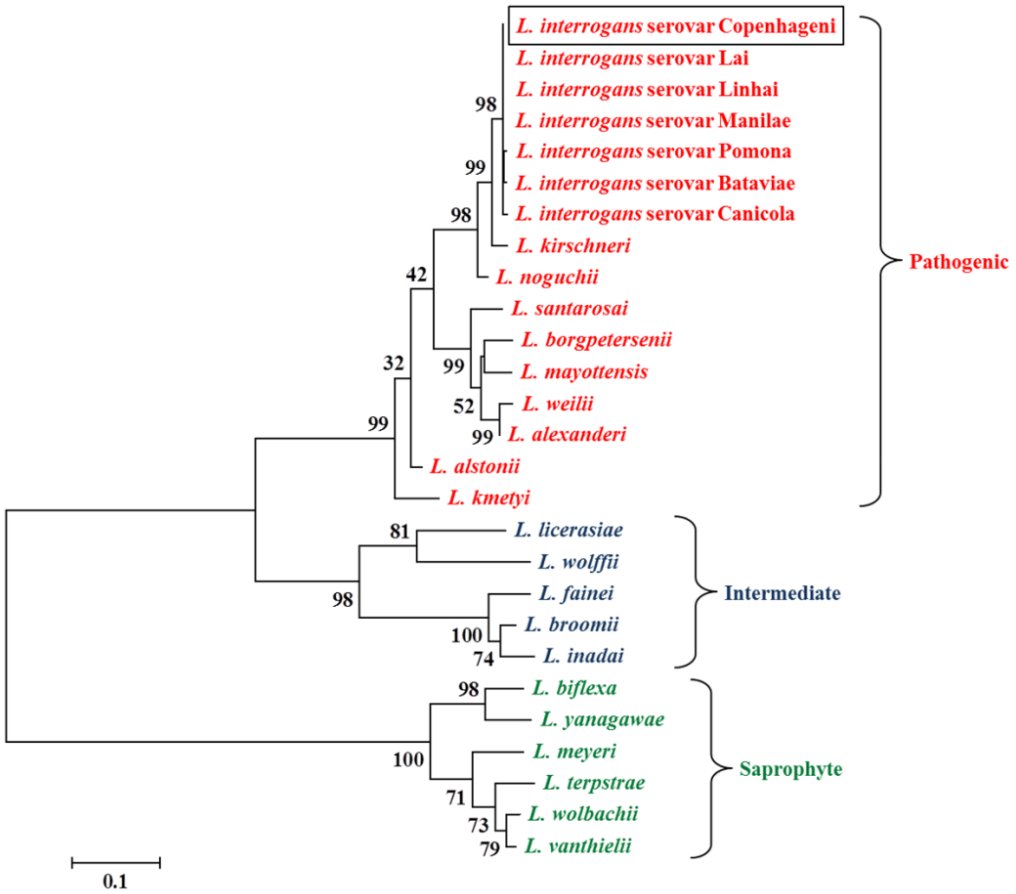

Figure 4.7. Phylogenetic analysis of *Leptospira* spp. using LIC13341 protein sequence. A total of 26 orthologs of LIC13341 were retrieved through BLAST search of NCBI database. The phylogenetic tree was constructed with the highest log likelihood (-4351.42) using the MEGA7

program where pathogenic, intermediate and saprophytic *Leptospira* are displayed in red, blue and green font colors, respectively. The percentage of the tree in which the associated taxa clustered together is shown next to the branches. A bootstrap value greater than 50 at each cluster shows the reliability of data. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The resulting phylogram show the high level of sequence conservation for LIC13341 (black box) among pathogenic *Leptospira* serovars.

Table 4.3. Comparative analyses of different *Leptospira* spp. based on LIC13341 protein sequence identity.

Leptospira species	Type of species	Query coverage (%)	Identity (%)	NCBI Accession number
kirschneri	++	100	92	WP_082281515.1
noguchii	++	100	90	WP_061250804.1
santarosai	++	89	96	WP_020765729.1
borgpetersenii	++	100	79	WP_032854494.1
mayottensis	++	100	78	WP_002746098.1
weilii	++	100	71	WP_061223798.1
alexanderi	++	100	79	WP_010577242.1
alstonii	++	100	82	WP_020775322.1
kmetyi	++	91	83	EQA53100.1
licerasiae	+	91	66	WP_008594170.1
wolffii	+	89	64	WP_016546685.1
fainei	+	89	65	WP_016550275.1
broomii	+	89	66	WP_010571642.1
inadai	+	89	65	WP_010416930.1
biflexa	-	97	42	ABZ96280.1
yanagawae	-	87	44	WP_015676698.1
meyeri	-	90	45	WP_004785067.1
terpstrae	-	90	45	WP_002972612.1
wolbachii	-	88	45	WP_015681989.1
vanthielii	-	88	46	WP_002977118.1

++, pathogenic; +, intermediate; -, saprophyte

4.4.7 Molecular characterization of the hypothetical protein LIC13341

To validate the bioinformatics analysis of LIC13341, genomic DNA or total RNA was isolated from *Leptospira* serovars for molecular detection of *LIC13341* by PCR and RT-PCR. An amplified fragment of 1,065 bp indicated the presence of *LIC13341* in the pathogenic *L. interrogans* serovar Copenhageni (Fig. 4.8A). This amplified product was cloned in the pET28a via TA cloning (Fig. 4.8B and Fig. 4.8C).

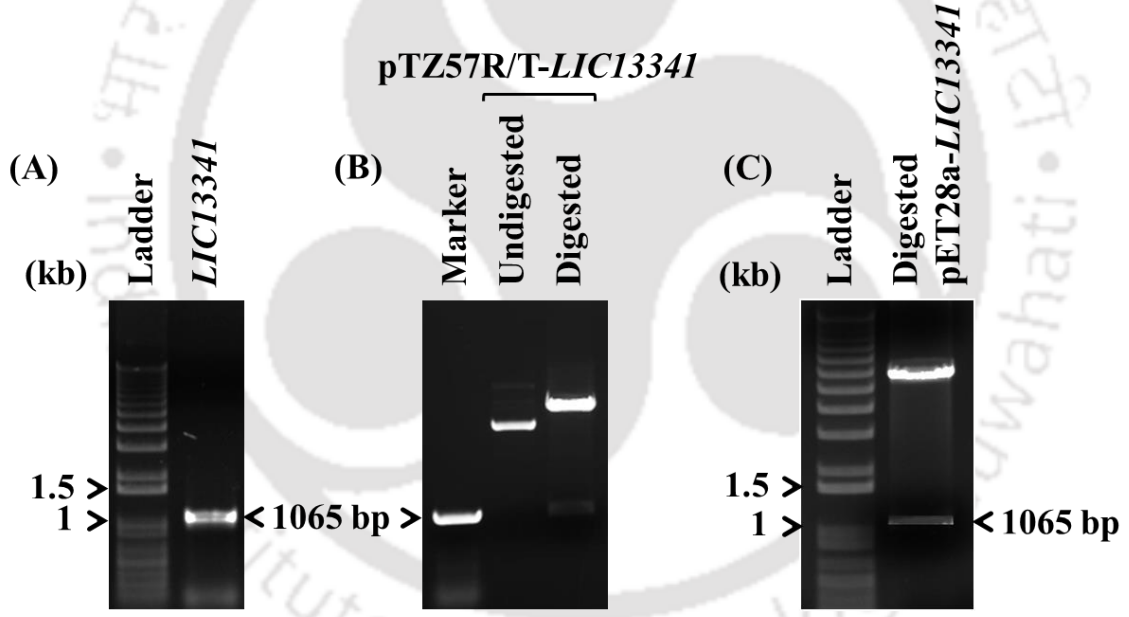

Figure 4.8. Cloning of *LIC13341* in pET28a vector. (A) PCR amplification of *LIC13341* gene (1,065 bp) using genomic DNA of pathogenic *L. interrogans* Copenhageni after deleting the signal peptide. **(B)** Cloning of amplified *LIC13341* in PTZ57R/T vector. The double digestion with *Nhe*I and *Xho*I shows a fall-out of 1.065 bp. **(C)** Cloning of *LIC13341* in pET28a vector. A fall-out of 1,065 bp using *Nhe*I and *Xho*I confirms the cloning.

The gene encoding hypothetical lipoprotein LIC13341 in *L. interrogans* serovar Copenhageni genome was expressed in *E. coli* BL21 cells after cloning into a pET28a expression vector (Fig. 4.9A). Induction of protein expression was done using 0.5 mM IPTG where overexpression of r-LIC13341 (~42 kDa) was evidently observed (Fig. 4.9A). Using the purified r-LIC13341, polyclonal antibodies were raised in BALB/c mice. Immunoblotting of the r-LIC13341 and the lysates of pathogenic *Leptospira* serovars (Copenhageni, Lai, and Canicola) using mouse anti-LIC13341 antibody recognized both native and the r-LIC13341 (Fig. 4.9B). These results suggest that leptospiral native proteins shared a common epitope with the recombinant protein. Interestingly, mouse anti-LIC13341 antibody detected an additional band of LIC13341 with lower molecular size exclusively in the tested serovars of pathogenic *Leptospira* (Fig. 4.9B). However, there was no detection of the LBF0133 (ortholog of LIC13341 in *L. biflexa*) by anti-LIC13341 antibody in the lysate of *L. biflexa* serovar Patoc (Fig. 4.9B). Interestingly, anti-LIC13341 antibody recognized an additional lower band with higher mobility in the immunoblot. This variation in the mobility of LIC13341 may be due to lipidation.

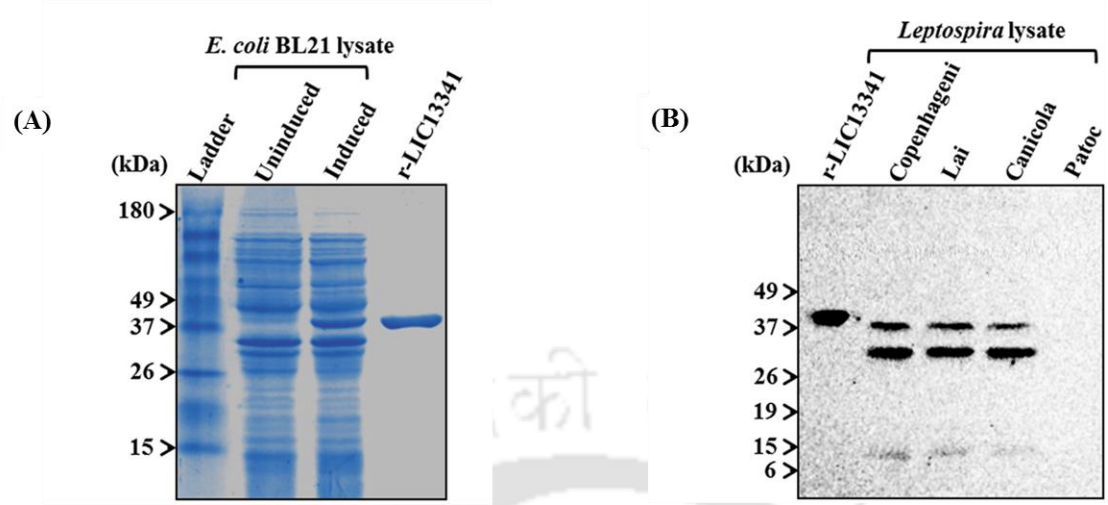

Figure 4.9. Characterization of the hypothetical protein LIC13341. The CDS of *LIC13341* obtained from the NCBI genome database were validated in pathogenic and non-pathogenic *Leptospira* serovars at DNA and protein level. **(A)** Induction and purification of recombinant-LIC13341. The CDS of *LIC13341* of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was cloned in pET28a expression vector and overexpressed in *E. coli* BL21 cells. Induction of r-LIC13341 expression was done using 0.5 mM IPTG, and its purification was done using Ni-NTA column chromatography under native conditions. The uninduced and induced lysates of *E. coli* BL21 cells along with the purified recombinant LIC13341 are shown on a 12% SDS-polyacrylamide gel stained with Coomassie. **(B)** Immunoblot analysis of native and recombinant-LIC13341 antigen with the anti-LIC13341 antibody. Generated anti-LIC13341 antibody can detect the purified r-LIC13341 and the native LIC13341 in pathogenic *Leptospira* serovars. An additional band with higher mobility in *Leptospira* lysates recognized by the anti-LIC13341 antibody may be due to LIC13341 lipidation.

4.4.8 LIC13341 is an outer membrane surface-exposed lipoprotein

Cellular localization of LIC13341 was analyzed by phase separation of *L. interrogans* serovar Copenhageni proteome using Triton X-114. Herein, the immunoblot analysis performed using polyclonal mouse anti-LIC13341 antibody detected the LIC13341 to be predominantly in the detergent phase of the Triton X-114; however, a small amount of the protein could also be found in the aqueous phase (Fig. 4.10A). Previous cellular localization studies of *Leptospira* (Haake et al., 2000b; Haake et al., 1991) using Triton X-114 have

shown that the outer membrane lipoproteins separate in detergent (hydrophobic) phase, while periplasmic proteins separate into the aqueous phase. Additionally, immunoblot analysis using anti-LipL32 antibody detected LipL32 predominantly in the detergent phase, as reported previously (Abreu et al., 2017; Barbosa et al., 2010), validating the Triton X-114 phase separation experiment (Fig. 4.10A).

In addition, to determine whether LIC13341 is a surface exposed outer membrane protein; a protease-accessibility assay was performed for *L. interrogans* serovar Copenhageni. By performing ELISA with r-LIC13341 antiserum on live *Leptospira* treated with proteinase K during the period of 0-5 h, there was a reduction in recognition of LIC13341 (Fig. 4.10B). The integrity of the spirochete cell wall during proteinase K treatment was confirmed by ELISA using antiserum against LipL31, a previously described cytoplasmic membrane protein (Haake and Matsunaga, 2002). In addition, under similar condition, an antibody against a known surface-exposed protein OmpL54 (Pinne and Haake, 2009) showed a reduction in recognition of OmpL54 (Fig. 4.10B). Around 70% reduction in LIC13341 recognition in live *Leptospira* was observed after 5 h of protease assay, implying that LIC13341 is a surface-exposed membrane protein. In contrast, no significant reduction in reactivity of live spirochete with anti-LipL31 antibody was noticed indicating that the integrity of spirochete membrane was not compromised during the proteinase K assay (Fig. 4.10B).

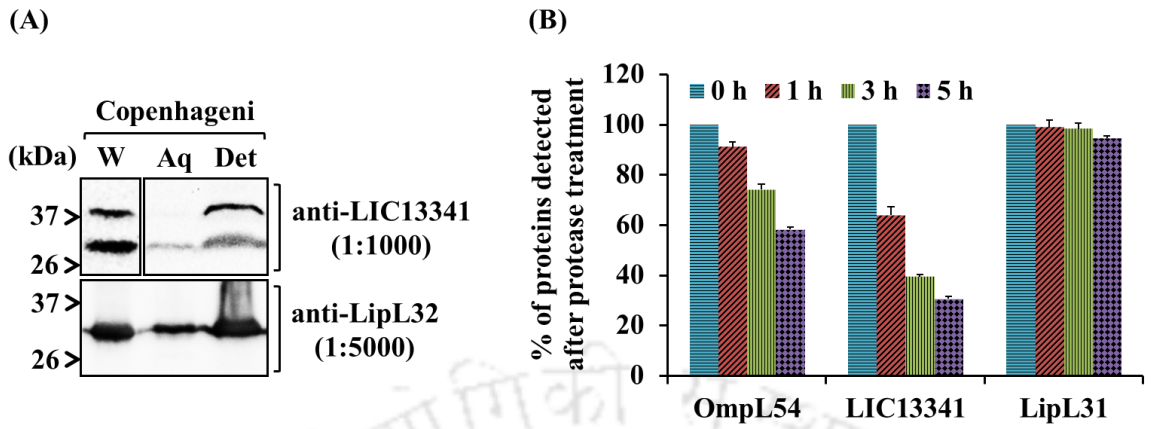

Figure 4.10. Cellular localization of the hypothetical protein LIC13341. (A) *Leptospira* LIC13341 is a membrane-bound protein. Spirochetes whole cell lysates (W) were subjected to Triton X-114 phase partition into aqueous (Aq) and detergent phase (Det). The aqueous and detergent phase fraction was resolved onto 12% SDS-polyacrylamide gel and subsequently immunoblotted with anti-LIC13341 serum. A major portion of LIC13341 was present in the detergent phase and partly in the aqueous phase. The anti-lipl32 antibody, used as positive control detected LipL32 predominantly in the detergent phase in comparison to the aqueous phase. **(B)** Protease accessibility assay of *L. interrogans* serovar Copenhageni describes LIC13341 to be a surface-exposed protein. Spirochetes were incubated with 25 µg of proteinase K at various time intervals up to 5 h at 37°C. The protease treated spirochete suspensions were washed with PBS and used to coat a microtiter plate. Using ELISA, a drastic decrease in the signal for LIC13341 reactivity with its antiserum was observed after 1 to 5 h of proteinase K treatment, similar to the case with OmpL54, a known outer membrane protein. LipL31 was used as a control to check the cellular integrity of the spirochetes during treatment with proteinase K. Error bars represents the standard deviations (SDs) from the three replicates. Statistical analysis was performed by Student's *t*-test by comparing the signals obtained for 0 h and another time point of treatment with proteinase K ($P<0.05$).

4.4.9 Recombinant LIC13341 binds to the host extracellular matrix macromolecules

The function of LIC13341 is not known to date; therefore, the bacteria-expressed affinity-purified r-LIC13341 was validated for its secondary structure integrity before performing any biochemical analysis. The structural integrity of r-LIC13341 was evaluated by CD spectroscopy (Fig. 4.11A). Analysis of the experimental CD spectra data of r-LIC13341 by K2D2 program (Perez-Iratxeta and Andrade-Navarro, 2008) predicted 8.02% of α-helix and

22.14% of β-strand indicating its suitability for further biochemical studies. The secondary structure prediction of r-LIC13341 using the program PSIPRED (Buchan et al., 2013) was closer to the experimental CD data.

Several comprehensive studies on the adherence of *L. interrogans* to extracellular matrices of hosts suggested the existence of numerous adhesion molecules on the outer membrane of *Leptospira* which are surface exposed (Ghosh et al., 2018; Robbins et al., 2015). In such studies, Loa22, another outer membrane protein of *Leptospira* due to its moderate attachment to host ECM (Barbosa et al., 2006; Ghosh et al., 2018) is an ideal antigen for comparative analysis of antigen-ECM interaction. Herein, to investigate r-LIC13341 interaction with ECM ligands by ELISA, r-Loa22 along with its specific antibody was included in the study. To evaluate LIC13341 interaction with the ECM components using ELISA, a soluble form of r-LIC13341 was allowed to bind to the immobilized host ECM components on microtiter plates. The antigen r-LIC13341 exhibited significant binding to laminin, fibronectin, collagen, hyaluronic acid, chondroitin sulfate A, elastin, and heparan sulfate compared to control protein fetuin or BSA ($P<0.001$) as a ligand (Fig. 4.11B). Among these extracellular matrices, laminin and hyaluronic acid showed the highest affinity for binding to r-LIC13341 on the microtiter plate. However, as anticipated, no specific binding of the antigen r-Loa22 to the target ECM macromolecules was detected in comparison to BSA or fetuin. Nevertheless, the binding of r-LIC13341 with laminin and hyaluronic acid was also assessed on a quantitative basis (Fig. 4.11C). A dose-dependent and saturable binding were observed when increasing concentrations of the r-LIC13341 (0 - 24 µM) were allowed to bind to a fixed amount of laminin and hyaluronic acid (1 µg). The saturation level of r-LIC13341 binding to laminin and hyaluronic acid was reached at a concentration of 22 µM and 20 µM,

respectively (Fig. 4.11C). The calculated K_D of the r-LIC13341 binding reactions was 420±57 nM and 299±35 nM to laminin and hyaluronic acid, respectively. In addition, the effect of the gradient increase in the amount of r-LIC13341 on the inhibition of live leptospires adherence to laminin and hyaluronic acid was examined by ELISA. The results indicate that the addition of r-LIC13341 in an increasing amount (0-10 µg) reduced the leptospiral adherence to both laminin and hyaluronic acid in a dose-dependent manner (Fig. 4.11D).

To examine the role of carbohydrate moieties of laminin in binding to r-LIC13341, laminin was oxidized by increasing concentrations of sodium metaperiodate (5 - 100 mM) at 4˚C for 15 min. The inhibition of laminin binding to r-LIC13341 due to gradual increase in sodium metaperiodate concentration is shown in Fig. 4.12A. This mild treatment ensures cleavage of vicinal carbohydrate hydroxyl groups while the polypeptide chain structure remains intact (Woodward et al., 1985). The oxidation effect was dose-dependent and the r-LIC13341 attachment to metaperiodate-treated laminin got reduced to less than 50% at a 100 mM concentration of periodate (Fig. 4.12A). These results indicate that the carbohydrate residues of laminin are critical for the interaction of r-LIC13341 with this major ECM glycoprotein. To further confirm the results of specific ECM binding, r-LIC13341 was heat-denatured before the binding reaction to hyaluronic acid. A sharp reduction in the binding of r-LIC13341 to hyaluronic acid under denaturing condition demonstrates the influence of antigen structure on the binding with hyaluronic acid (Fig. 4.12B). Moreover, the decline in binding under denaturing condition suggests conformational epitopes in LIC13341 are important for these interactions.

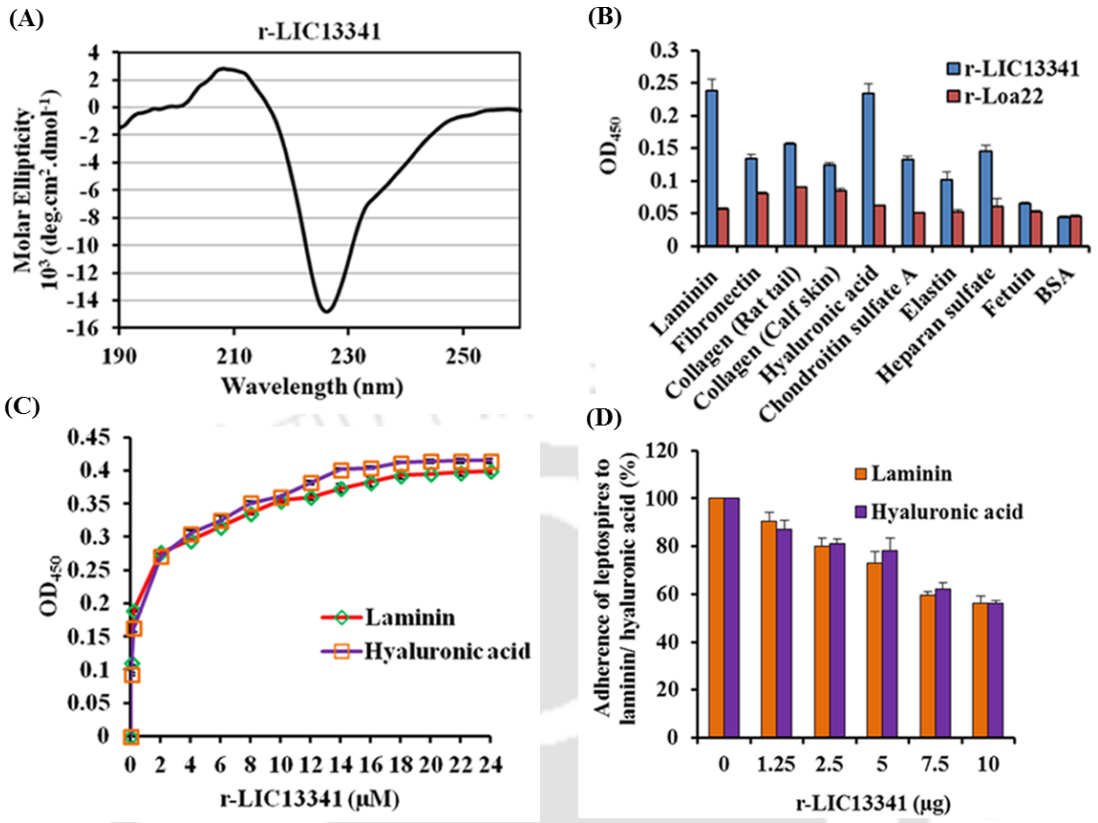

Figure 4.11. The recombinant-LIC13341 binds to host extracellular matrix components. **(A)** Far-ultraviolet circular dichroism (CD) spectra of r-LIC13341. The spectra are depicted in the range of 190-260 nm wavelengths showing the presence of both α-helix and β-strand with a predominant signal of β-strand. CD spectra are shown as an average of 3 scans with a scanning speed of 100 nm min^{-1}. **(B)** Enzyme-linked immunosorbent assay (ELISA) shows r-LIC13341 interaction with extracellular matrix components. Bovine serum albumin (BSA) and the highly glycosylated serum protein, fetuin, were used as controls for non-specific binding. Recombinant Loa22 was included as a negative control for non-specific binding with the ECM components. Recombinant-LIC13341 exhibited significant binding to all ECM components compared to fetuin or BSA ($P<0.001$) and with a higher affinity towards laminin and hyaluronic acid. In contrast, Loa22 showed moderate binding with the ECM components used in this study. Results are indicative of two independent experiments. **(C)** Dose-dependent binding of the r-LIC13341 to laminin and hyaluronic acid. Laminin or hyaluronic acid coated microtiter plates were incubated with increasing concentrations of r-LIC13341 (0-24 µM). Binding of LIC13341 was measured using the anti-LIC13341 serum at an appropriate dilution. The mean absorbance values of r-LIC13341 binding to laminin and hyaluronic acid at 450 nm are shown. **(D)** Inhibition of *Leptospira* adherence to ECM in the presence of r-LIC13341 using ELISA. Laminin or hyaluronic acid (1 µg per well) was coated onto the microtiter plates followed by incubation with increasing concentrations of r-LIC13341 (0-10 µg) for 90 min at 37°C. Live *L. interrogans* serovar Copenhageni were added to microtiter plates and incubated for 90 min at 37°C. After washing, the quantification of bound leptospires was performed by anti-Loa22 serum. The

absorbance was measured at 450 nm wavelength using ELISA plate reader. The binding of *L. interrogans* to laminin or hyaluronic acid in the absence of r-LIC13341 was considered as 100% binding. A gradual reduction in *Leptospira* attachment to ECM was observed with increasing concentration of r-LIC13341. Error bars represent the standard deviations of the three replicates.

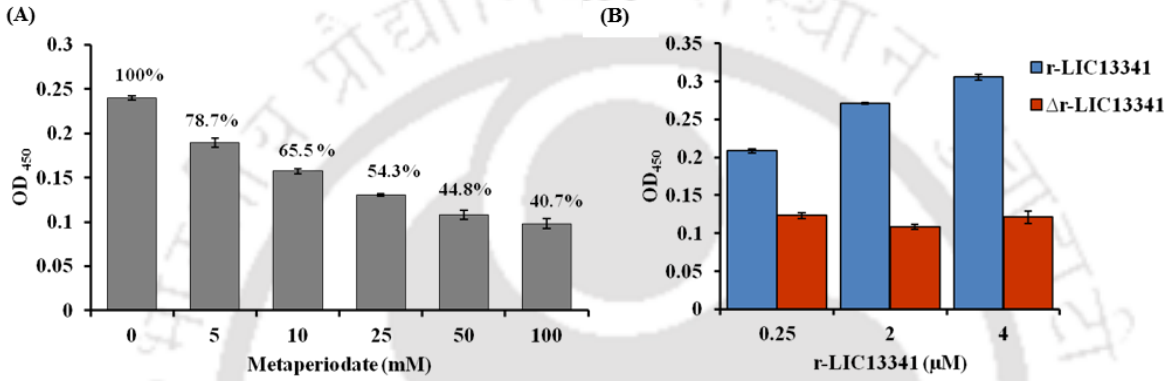

Figure 4.12. Binding of recombinant LIC13341 to laminin and hyaluronic acid is physio-chemically dependent. (A) Laminin oxidation reduces r-LIC13341-laminin interaction. Immobilized laminin on microtiter plates was oxidized with various concentrations of sodium metaperiodate (5 to 100 mM) for 15 min at 4˚C. The mean absorbance values of r-LIC13341 binding to oxidized or untreated laminin are compared. The reduction in the binding percentage of r-LIC13341 to oxidized laminin as a function of periodate concentration is indicated above each bar. **(B)** Heat-denatured r-LIC13341 fails to bind hyaluronic acid. Hyaluronic acid coated (1 µg per well) microtiter plates were allowed to bind with increasing concentration of r-LIC13341 (0.25-4 µM) or its heat denatured form (Δr-LIC13341). Binding was detected using the anti-LIC13341 serum at an appropriate dilution. The mean absorbance values at 450 nm (± the standard errors of two independent experiments) are shown.

4.4.10 Recognition of recombinant LIC13341 by serum antibodies of human and bovine leptospirosis cases

As LIC13341 is localized in the outer membranes of spirochetes, it was interesting to evaluate its recognition using immunoglobulins of *Leptospira*-infected hosts. ELISA was conducted to evaluate the recognition of r-LIC13341 with the human or bovine serum samples that tested positive for leptospirosis (n=50) by the microscopic agglutination test

(MAT). As a control, serum samples of human (n=15) and bovine (n=10) that tested negative for leptospirosis by the MAT were included in the serological assay. MAT positive serum samples, in contrast to MAT negative serum samples, could agglutinate the reactive *Leptospira* serogroups at titer ≥100. The calculated sensitivity and specificity of the assay to recognize r-LIC13341 with human leptospirosis serum was 100% (Fig. 4.13A and Table 4.4). In contrast, the bovine serum showed the lower percent of sensitivity (78) than human serum (100) to recognize r-LIC13341 (Fig. 4.13B and Table 4.4). Nevertheless, the specificity for detecting r-LIC13341 by bovine serum was 100% (Fig. 4.13B). Moreover, the immunoblot analysis performed using pooled serum of human/bovine positive for leptospirosis recognized both r-LIC13341 and r-Loa22 compared to leptospirosis MAT-negative serum samples (Fig. 4.13C and Fig. 4.13D).

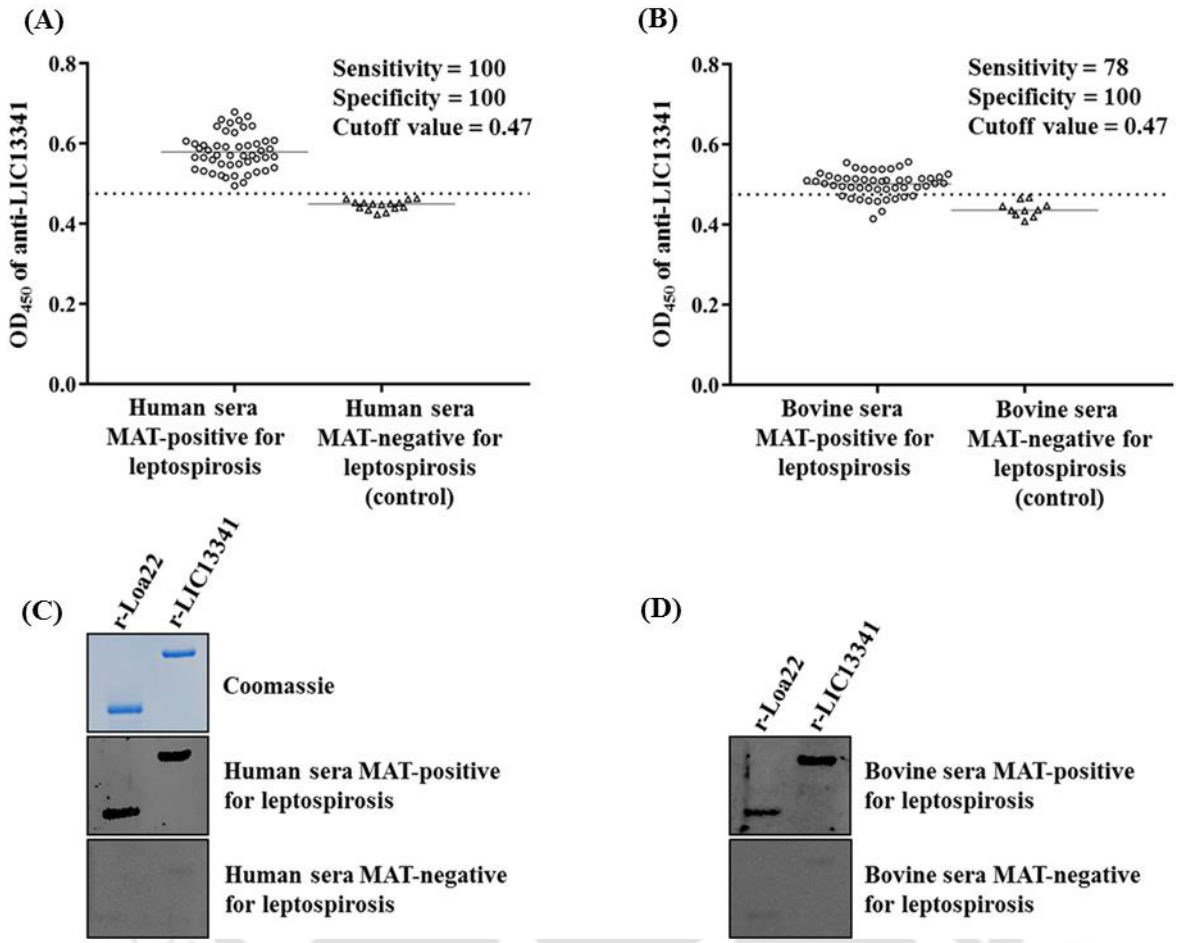

Figure 4.13. Recombinant-LIC13341 is recognized by leptospirosis-positive sera. ELISA was performed to detect r-LIC13341 using the sera of humans and bovines testing MAT positive (n=50) and control serum testing MAT negative for leptospirosis. The absorbance obtained from each serum samples were plotted as empty circles or triangles for MAT positive and negative serum samples, respectively. The cut-off value of the assay was derived from the mean of the control group plus 2SDs (standard deviations) for the antigen used (dotted black lines). The mean of each group is represented by black horizontal lines. **(A)** ELISA to detect r-LIC13341 (400 ng per well) using human sera testing positive for leptospirosis. The sensitivity and specificity of the assay were both 100%. **(B)** ELISA to detect r-LIC13341 (400 ng per well) with bovines sera testing positive for leptospirosis. The sensitivity and specificity of the assay were 78% and 100%, respectively. **(C)** Immunoblot using pooled leptospirosis-positive serum of human recognizes r-LIC13341 and r-Loa22 antigens. Recombinant antigens (r-LIC13341 and r-Loa22; 1 µg per lane) were separated on 12% SDS-PAGE and stained with Coomassie dye (top panel). Immunoblot using pooled human serum testing positive for leptospirosis recognized each antigen (r-LIC13341 and r-Loa22) with equal intensity (middle panel). In contrast, there was no remarkable recognition of these antigens using pooled human serum testing negative for leptospirosis (lower panel). **(D)** Immunoblot analysis using pooled leptospirosis-positive serum samples of bovine recognizes r-LIC13341 and r-Loa22 antigens. Each antigen (r-

LIC13341 and r-Loa22) was recognized with equal intensity in the immunoblot (upper panel). In contrast, there was no remarkable recognition of these antigens using pooled bovine serum testing negative for leptospirosis (lower panel).

Table 4.4. Measured ELISA data for immunodetection of recombinant-LIC13341 using serum of human/bovine positive for leptospirosis

Host	Human		Bovine	
Serum (samples)	**MAT (+ve) (n=50)**	**MAT (-ve) (n=15)**	**MAT (+ve) (n=50)**	**MAT (-ve) (n=10)**
Mean OD ± SD	0.5791 ± 0.045	0.4462 ± 0.012	0.5009 ± 0.030	0.4383 ± 0.019
Cut-off (Mean OD + 2SD)	-	0.4702	-	0.4757
Sensitivity (%)	100	-	78	-
Specificity (%)	-	100	-	100

MAT: Microscopic agglutination test

SD: Standard deviations

OD: Optical density

4.4.11 Molecular characterization of the hypothetical lipoprotein LIC11966

The gene *LIC11966* which encodes a hypothetical lipoprotein was characterized in the present study as it was found to downregulate at physiological osmolarity. To validate the existence of *LIC11966* in the pathogenic serovars of *L. interrogans*, the genomic DNA or the total RNA was isolated from the pathogenic *Leptospira* serovars and the saprophyte *L. biflexa*. The primers were designed to amplify *LIC11966* without it signal peptide. An amplified fragment of 414 bp confirmed the existence of *LIC11966* in all the pathogenic serovars of *L. interrogans* tested in the present study (Fig. 4.14A). No amplification was observed in *L. biflexa* serovar Patoc (Fig. 4.14A). A reverse transcription PCR validated the

presence of *LIC11966* transcripts in all the pathogenic serovars of *L. interrogans* tested (Fig. 4.14B). The constitutive *flaB* gene amplified from all the *Leptospira* serovars in the PCR and RT-PCR reaction (Fig. 4.14A and Fig. 4.14B).

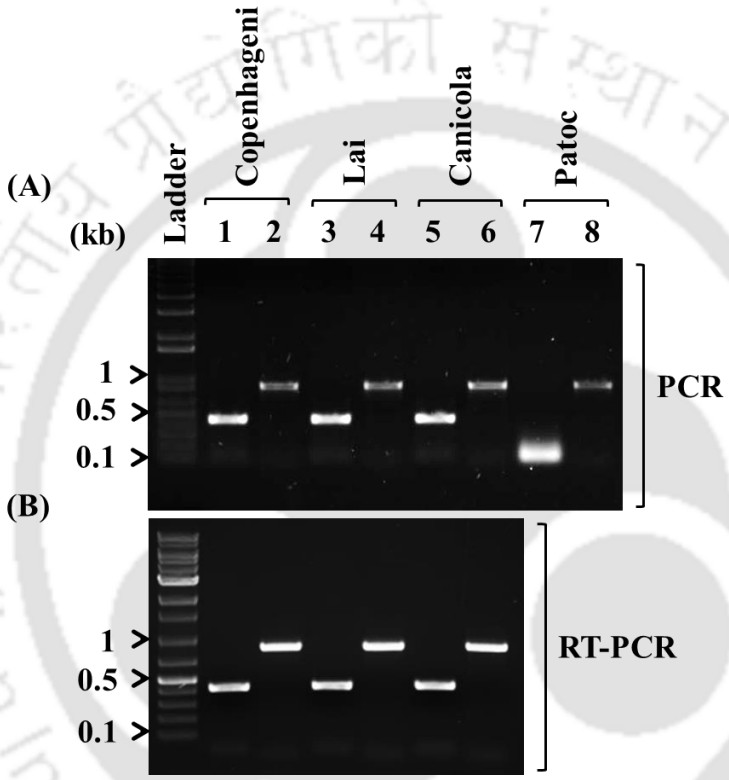

Figure 4.14. Characterization of the hypothetical lipoprotein LIC11966. (A) PCR was performed for amplification of *LIC11966* (414 bp) from genomic DNA of indicated serovars of pathogenic *L. interrogans* (Copenhageni, Lai and Canicola). The amplicon size of 414 bp were detected (Lane 1, 3 and 5) which confirmed the existence of the *LIC11966* gene and its orthologs in *L. interrogans* serovars tested. Template DNA from non-pathogenic *L. biflexa* serovar Patoc was used in PCR as a negative control where amplicon of *LIC11966* was not detected (Lane 7). **(B)** Transcription of *LIC11966* was confirmed in the pathogenic *L. interrogans* serovars by RT-PCR (lanes labelled with odd numbers). Specific primers of a constitutive *flaB* gene (852 bp) of *Leptospira* was used in PCR and RT-PCR (lanes labelled with even numbers) to check the quality of template used in the PCR reaction.

4.4.12 *In silico* analysis of LIC11966 and identification of its orthologs

BLAST search analysis was performed using available LIC11966 sequences from the NCBI database and 15 representative leptospiral sequences were used to construct the phylogenetic tree. The resulting phylogram showed a high level of sequence conservation among pathogenic spp. of *Leptospira* for LIC11966 (100–57% amino acid sequence identity) (Fig. 4.15 and Table 4.5). It is also noteworthy that this protein is absent in intermediates and saprophytes. This indicates that this gene is exclusively present in the pathogenic serovars of *Leptospira*. This also pinpoints to the fact that this gene might be one of the virulence genes of *L. interrogans*.

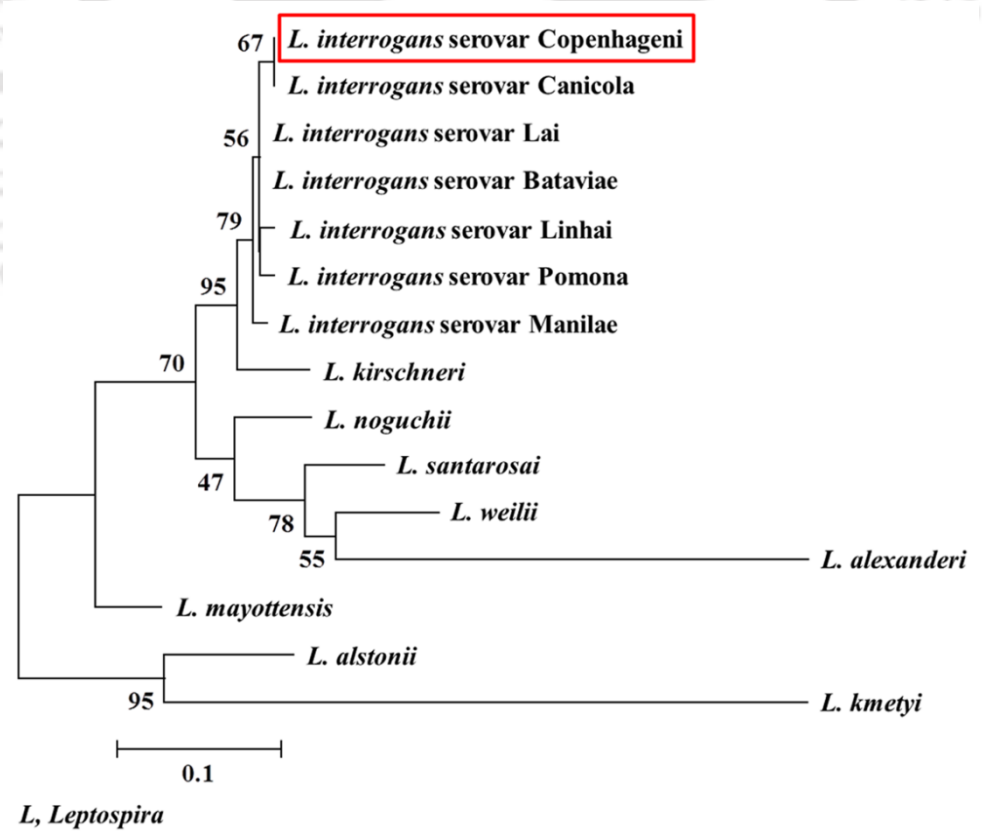

Figure 4.15. Phylogenetic analysis of *Leptospira* spp. using LIC11966 protein sequence. A total of 14 orthologs of LIC11966 were retrieved through BLAST search of NCBI database. The phylogenetic tree was constructed with the highest log likelihood (-987.90) using the MEGA7

program. The percentage of the tree in which the associated taxa clustered together is shown next to the branches. A bootstrap value greater than 50 at each cluster shows the reliability of data. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The resulting phylogram show the high level of sequence conservation for LIC11966 (red box) among pathogenic *Leptospira* serovars.

Table 4.5. Comparative analyses of the protein sequence identity of LIC11966 in the different species of *Leptospira*.

Leptospira species	**Query Coverage (%)**	**Identity (%)**	**NCBI Accession number**
L. interrogans serovar Canicola	100	100	OCC30350.1
L. interrogans serovar Lai	100	99	NP_712120.1
L. interrogans serovar Linhai	100	99	AJR14687.1
L. interrogans serovar Manilae	100	99	EYU63405.1
L. interrogans serovar Bataviae	100	99	OAM75663.1
L. interrogans serovar Pomona	100	99	EMI70432.1
L. kirschneri	100	92	WP_004755938.1
L. noguchii	100	85	WP_002151500.1
L. mayottensis	100	79	WP_117340238.1
L. santarosai	100	78	WP_004476249.1
L. weilii	98	80	WP_002622075.1
L. alstonii	98	71	WP_020772825.1
L. kmetyi	98	57	WP_040912836.1
L. alexanderi	76	64	WP_078128751.1

4.4.13 Molecular characterization of LIC11966 of *L. interrogans* serovar Copenhageni

The full-length *LIC11966* of *L. interrogans* Copenhageni without the signal peptide was amplified from genomic DNA (Fig. 4.16A). This PCR amplified DNA fragment was cloned in pTZ57R/T (TA) vector (Fig. 4.16B). The *Nhe*I and *Xho*I double digested insert was

subsequently cloned into pET28a vector (Fig. 4.16C) and overexpressed in *E. coli* BL21 (DE3) cells (Fig. 4.16D). The protein was purified by the native method and concentrated to 5 mg/mL (Fig. 4.16D).

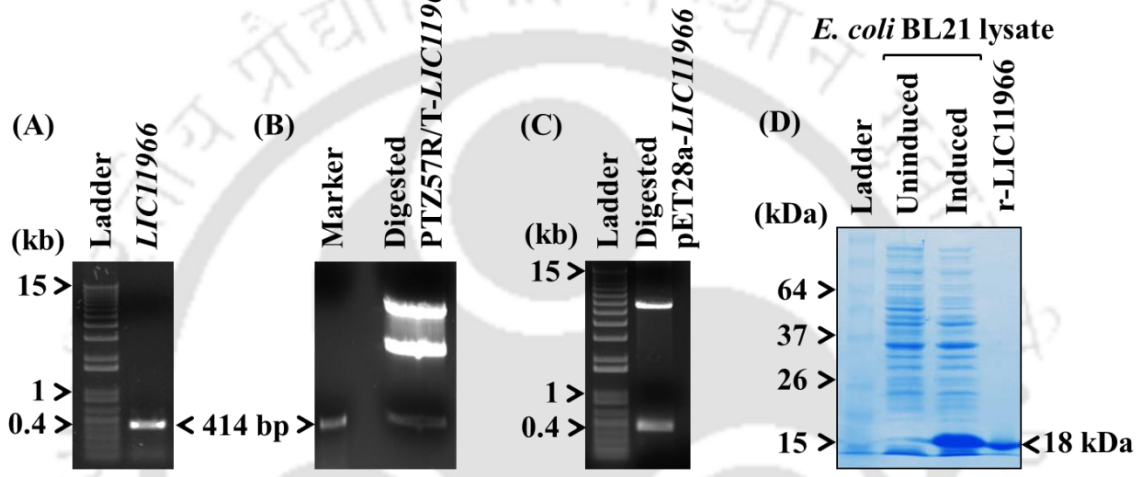

Figure 4.16. Cloning of *LIC11966* in pET28a vector. (A) PCR amplification of *LIC11966* gene (414 bp) using genomic DNA of pathogenic *L. interrogans* Copenhageni after deleting the signal peptide. **(B)** Cloning of amplified *LIC11966* in PTZ57R/T vector. The double digestion with *Nhe*I and *Xho*I shows a fall-out of 414 bp. **(C)** Cloning of *LIC11966* in pET28a vector. A fall-out of 414 bp using *Nhe*I and *Xho*I confirms the cloning. **(D)** Over-expression of r-LIC11966 in *E. coli* BL21 (DE3) cells and purification of recombinant protein using the native method.

Using the purified r-LIC11966, polyclonal antibodies were generated in BALB/c mice. The titer was found to be 1000 (Fig. 4.17A). Immunoblotting of the r-LIC11966 and the lysates of pathogenic *Leptospira* serovars (Copenhageni, Lai, and Canicola) using mouse anti-LIC11966 recognized both native and the r-LIC11966 (Fig. 4.17B). A non-pathogenic *L. biflexa* serovar Patoc was used as negative control in the immunoblot.

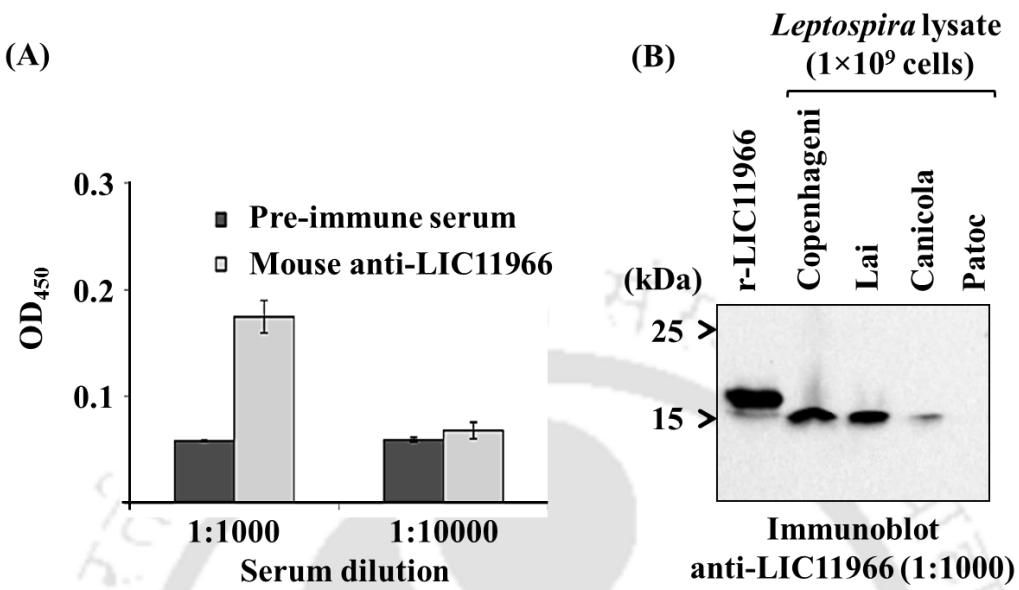

Figure 4.17. Evaluation of LIC11966 expression using specific anti-LIC11966 antibodies. **(A)** The generated polyclonal anti-LIC11966 titer. The serum obtained before the immunization of r-LIC11966 was used as a control for evaluation of antibody titer. The data is represented as mean ± standard error of two independent experiments. **(B)** Immunoblot analysis of recombinant and native-LIC11966 antigen with anti-LIC11966 antibody. Pooled polyclonal antisera (1:1000) from mice immunized with r-LIC11966 can detect the recombinant and the native-LIC11966 antigen in the whole cell lysate prepared from 1×10^9 cells of pathogenic *Leptospira* (*L. interrogans* serovars; Copenhageni, Lai or Canicola). A non-pathogenic *L. biflexa* serovar Patoc was used as negative control in the immunoblot.

4.4.14 LIC11966 is an outer membrane surface-exposed protein

The subcellular localization of LIC11966 was analyzed by phase separation of *L. interrogans* serovar Copenhageni proteome using Triton X-114 detergent. The immunoblot performed using polyclonal anti-LIC11966 detected LIC11966 to be predominantly present in the detergent phase of the Triton X-114; however, a small amount of the protein could also be seen in the aqueous phase (Fig. 4.18A). To validate the Triton X-114 phase separation experiment, immunoblot using anti-LipL32 was performed. LipL32 was found to be

predominantly located in the detergent phase in comparison to aqueous phase as reported previously (Fig. 4.18A) (Abreu et al., 2017; Barbosa et al., 2010).

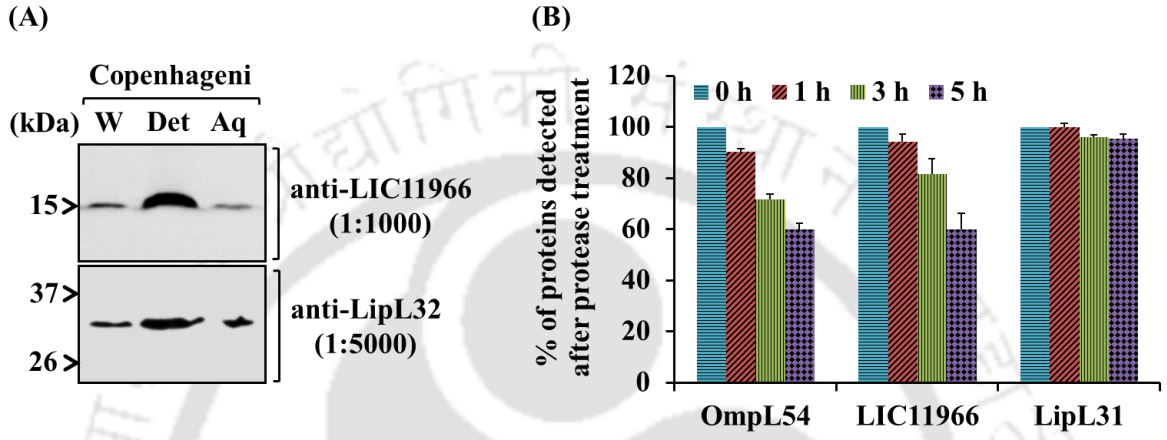

Figure 4.18. Subcellular localization of hypothetical protein LIC11966. (A) *Leptospira* LIC11966 is a membrane-bound protein. Spirochetes whole cell lysates (W) were subjected to Triton X-114 phase partition to separate into aqueous (Aq) and detergent phase (Det). A major portion of LIC11966 was present in the detergent phase and partly in the aqueous phase. Anti-LipL32, used as positive control detected LipL32 predominantly in the detergent phase in comparison to the aqueous phase. **(B)** Protease accessibility assay of *L. interrogans* Copenhageni indicates that LIC11966 is a surface-exposed protein. Spirochetes were incubated with 25 µg of proteinase K at various time intervals up to 5 h at 37°C. The protease treated spirochete suspensions were washed with PBS and used to coat a micro test plate. Using ELISA, a drastic decrease in the signal for LIC11966 reactivity with its antiserum was observed after 1 to 5 h of proteinase K treatment, similar to the case with OmpL54, a known outer membrane protein. LipL31 was used as a control to check the cellular integrity of the spirochetes during treatment with proteinase K. Error bars represents the standard deviations (SDs) from the three replicates. Statistical analysis was performed by Student's *t*-test by comparing the signals obtained for 0 h and another time point of treatment with proteinase K ($P<0.05$).

To determine whether LIC11966 is a surface exposed outer membrane protein; a protease-accessibility assay was performed for *L. interrogans* serovar Copenhageni. By performing ELISA with r-LIC11966 antiserum on live *Leptospira* treated with proteinase K during the

period of 0-5 h, there was a reduction in recognition of LIC11966 (Fig. 4.18B). The integrity of the spirochete cell wall during proteinase K treatment was confirmed by ELISA using antiserum against LipL31, a previously described cytoplasmic membrane protein. Under the similar condition, an antibody against a known surface-exposed protein OmpL54 showed the reduction in recognition of OmpL54 (Fig. 4.18B). Around 40% reduction in LIC11966 recognition in live *Leptospira* binding was observed after 5 h of protease assay, indicating that LIC11966 to be a surface exposed membrane protein. No significant reduction in reactivity of live spirochete with anti-LipL31 describes that the integrity of spirochete membrane was not altered during the proteinase K assay (Fig. 4.18B).

4.4.15 LIC11966 binds to extracellular matrix components of the host

To evaluate LIC11966 interaction with the ECM components and fibrinogen (a plasma component), ELISA was performed. The r-LIC11966 along with r-Loa22 was allowed to bind with the immobilized ECM components on a 96-well plate with BSA and the highly glycosylated serum protein, fetuin, as negative controls. The r-LIC11966 showed significant binding ($P<0.001$) with all the ECM components tested in contrast to Loa22 (Fig. 4.19). The highest binding of r-LIC11966 was seen with host fibrinogen and collagen (rat tail).

4.4.16 Recognition of recombinant LIC11966 by antibodies of human, bovine and canine leptospirosis serum.

To evaluate the serological recognition of r-LIC11966 in the *Leptospira* infected hosts, ELISA was conducted for the recognition of r-LIC11966 with human (n=50), bovine (n=50) or canine (n=18) serum that tested positive for leptospirosis by the microscopic agglutination test (MAT). As a control, serum samples of human (n=20), bovine (n=20) and canine (n=11)

that had tested negative for leptospirosis by the MAT were included in the serological assay. The calculated sensitivity and specificity of the assay to recognize r-LIC11966 with human,

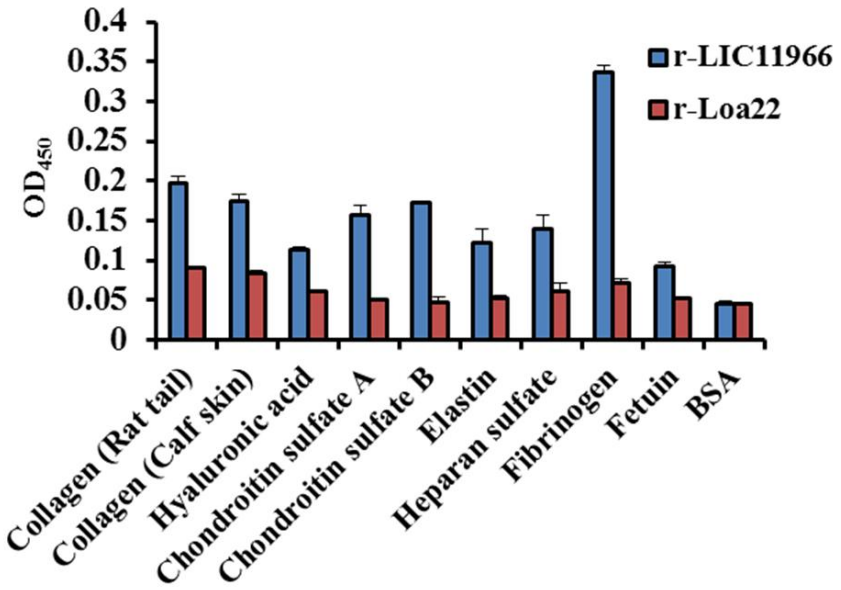

Figure 4.19. The recombinant-LIC11966 binds to host extracellular matrix components. ELISA depicts r-LIC11966 interacts with extracellular matrix (ECM) components, and fibrinogen. Bovine serum albumin (BSA) and the highly glycosylated serum protein, fetuin, were used as controls for non-specific binding. Loa22 was included as a negative control for non-specific binding with the ECM components. r-LIC11966 exhibited significant binding with all ECM components as compared to fetuin or BSA ($P<0.001$) and with a higher binding with fibrinogen and collagen (rat tail). In contrast, Loa22 showed moderate binding with all the ECM components used in this study. Error bars represent the standard errors between two independent experiments.

and bovine, leptospirosis serum was 100% (Fig. 4.20A, and Fig. 4.20C). It was noteworthy that although the average optical density values obtained for the recognition of r-LIC11966 with canine leptospirosis serum samples were lower than those obtained for humans and bovines, the calculated sensitivity and specificity of the assay for canines was found to be

100% (Fig. 4.20E). The specificity and the sensitivity for detecting r-Loa22 by the serum samples were found to be 100% (Fig. 4.20B, Fig. 4.20D, and Fig. 4.20F).

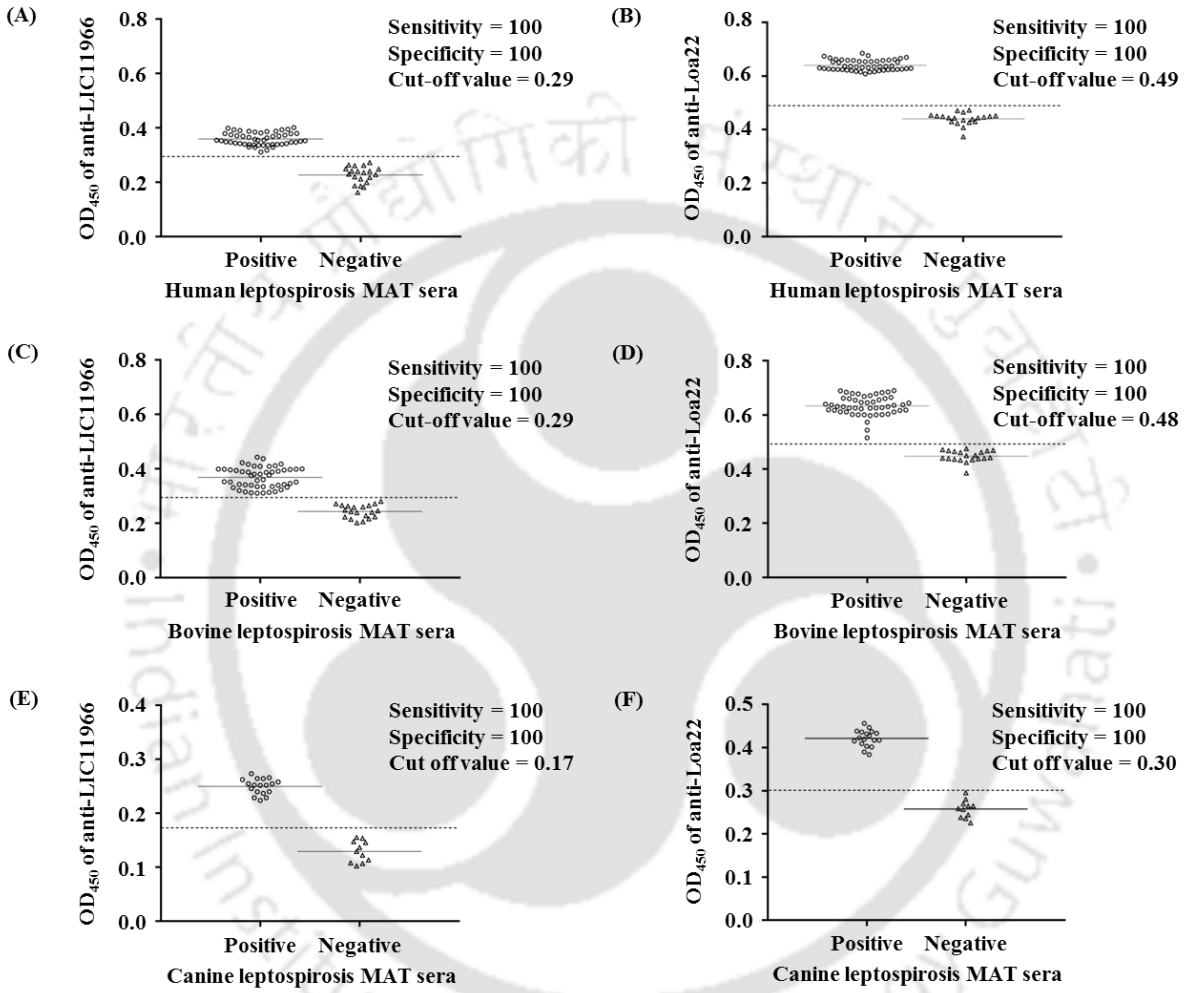

Figure 4.20. Recombinant-LIC11966 is recognized by leptospirosis-positive sera of diverse hosts. ELISA was performed to detect r-LIC11966 using the sera of humans, bovines and canines testing MAT positive (n=50/50/18) and control serum testing MAT negative for leptospirosis. The absorbance obtained from each serum samples were plotted as empty circles or triangles for MAT positive and negative serum samples, respectively. The cut-off value of the assay was derived from the mean of the control group plus 2SDs (standard deviations) for the antigen used (dotted lines). The mean of each group is represented by black horizontal lines. **(A)** ELISA to detect r-LIC11966 (400 ng) using human serum samples (1:100) testing MAT-positive (n=50) or negative (n=20) for leptospirosis. The r-LIC11966 was recognized by infected human sera with 100% sensitivity and specificity. **(B)** ELISA to detect r-Loa22 (400 ng) using human serum samples testing MAT-positive or negative for leptospirosis. The r-Loa22

was recognized by infected human sera with 100% sensitivity and specificity. **(C)** ELISA to detect r-LIC11966 using bovine serum samples testing MAT-positive (n=50) or negative (n=20) for leptospirosis. **(D).** ELISA to detect r-Loa22 using bovine serum samples testing MAT-positive or -negative for leptospirosis. **(E)** ELISA to detect r-LIC11966 using canine serum samples testing MAT-positive (n=18) or negative (n=11) for leptospirosis. **(F)** ELISA to detect r-Loa22 using canine serum samples testing MAT-positive or negative for leptospirosis.

4.4.17 *In silico* analysis of LIC12693 and identification of its orthologs

The gene *LIC12693* which encodes for an outer membrane efflux protein was also characterized in the present study as it was previously found to be downregulated in mammalian host-adapted *Leptospira*. BLAST search analysis was performed using the available LIC12693 sequences from the NCBI database and 27 representative leptospiral sequences were used to construct the phylogenetic tree. The resulting phylogram shows a high level of sequence conservation among the pathogenic species of Leptospira for LIC12693 in comparison to the intermediates and the saprophytes (Fig. 4.21).

4.4.18 Molecular characterization of the hypothetical protein LIC12693

The primers designed for *LIC12693* was used to clone the gene without its signal peptide using genomic DNA of *L. interrogans* serovar Copenhageni. An amplified fragment of 1,590 bp indicated the existence of *LIC12693* (Fig. 4.22A) and this amplified product was then cloned in pET23a vector using *Nhe*I and *Xho*I restriction enzymes (Fig. 4.22B, and Fig. 4.22C). This cloned LIC12693 over-expressed in *E. coli* BL21 (DE3) cells (Fig. 4.22D). The over-expressed r-LIC12693 of 63 kDa was purified by denaturing method (Fig. 4.22E). Exhaustive attempts to purify sufficient amount of r-LIC12693 proved futile.

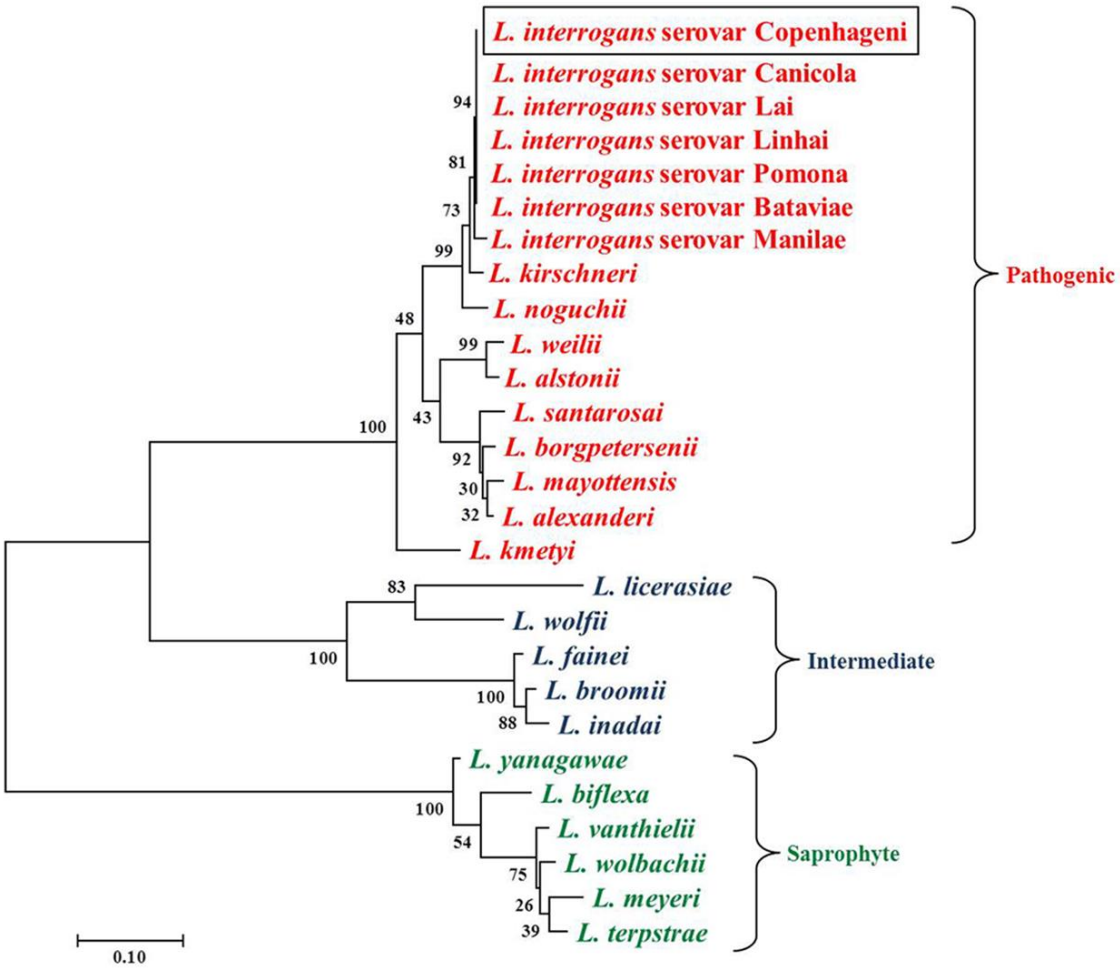

Figure 4.21. Molecular phylogenetic analysis of *Leptospira* spp. based on amino acid sequence of LIC12693 of *L. interrogans* serovar Copenhageni by maximum likelihood method. The amino acid sequence of LIC12693 was retrieved from NCBI protein database and a total of 26 orthologs of LIC12693 were retrieved through NCBI protein BLAST. The sequences were aligned and the phylogenetic tree was constructed using software MEGA version 7.0.26. The tree with the highest log likelihood (-6250.47), inferred following 1000 bootstrap replications, is shown where the bootstrap value greater than 50 shows the reliability of the data. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The resulting phylogram shows the high level of sequence conservation for LIC12693 (black box) among pathogenic *L. interrogans* serovars.

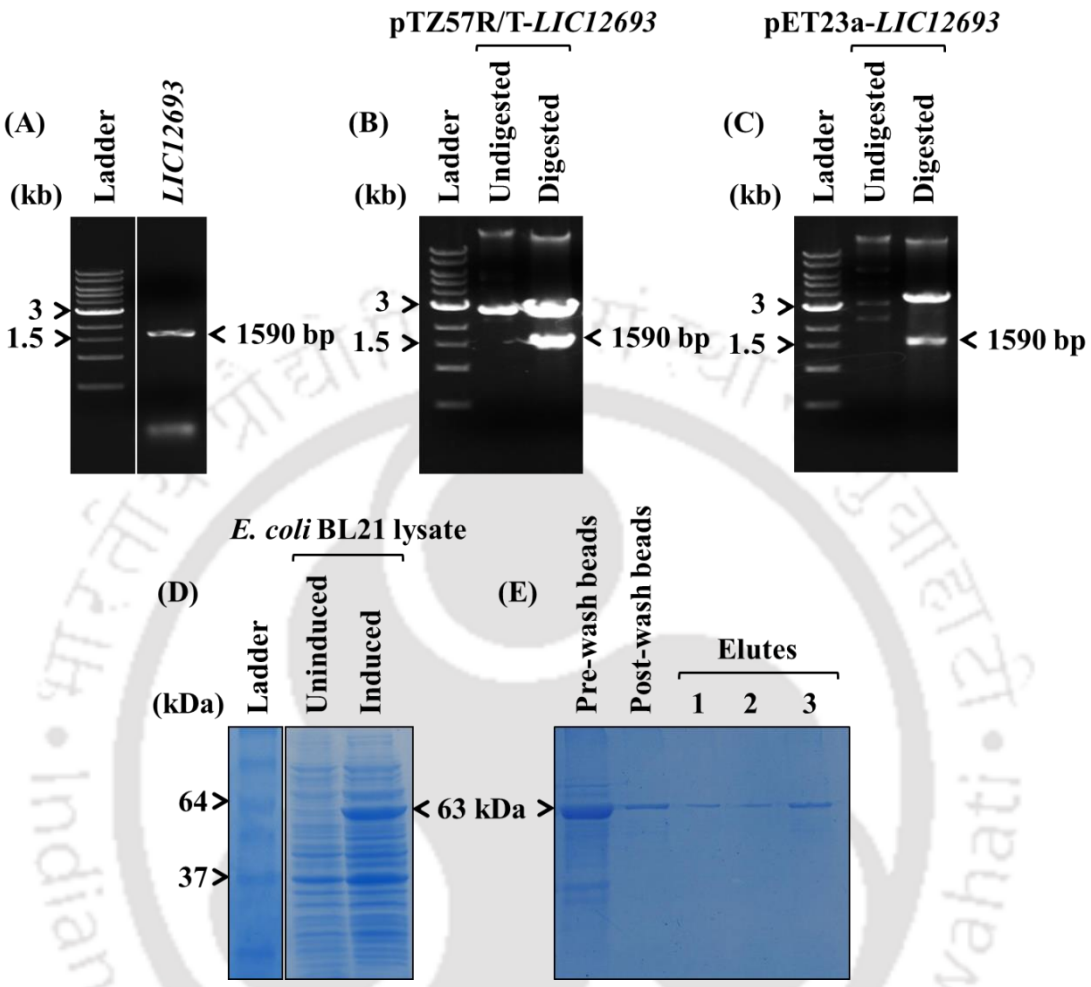

Figure 4.22. Molecular characterization of LIC12693. Cloning of *LIC12693* in pET23a vector. (A) PCR amplification of *LIC12693* gene (1,590 bp) using genomic DNA of pathogenic *L. interrogans* Copenhageni after deleting the signal peptide. **(B)** Cloning of amplified *LIC12693* in PTZ57R/T vector. The double digestion with *Nhe*I and *Xho*I shows a fall-out of 1590 bp. **(C)** Cloning of *LIC12693* in pET23a vector. A fall-out of 1,590 bp using *Nhe*I and *Xho*I confirms the cloning. **(D)** Over-expression of r-LIC12693 in *E. coli* BL21 (DE3) cells. **(E)** Purification of r-LIC12693 using denaturing method.

4.4.19 Evaluation of LIC12693 expression and localization using anti-LIC12693

The polyclonal antibodies generated against r-LIC12693 showed an end-point titer of 5000 as shown in Fig. 4.23A. Immunoblot using anti-LIC12693 antibody detected native LIC12693

in the lysate of pathogenic *L. interrogans* serovars (Copenhageni, Lai and Canicola) (Fig. 4.23B). In contrast, the native protein was not detected in the saprophytic *L. biflexa* serovar Patoc probably due to low sequence identity of LIC12693 ortholog in the saprophytic species of *Leptospira* (Fig. 4.23B). The r-LIC12693 was used as a positive control in the immunoblot experiment. The cellular localization of LIC12693 was analyzed by phase separation of *L. interrogans* serovar Copenhageni proteome using Triton X-114. Herein, the immunoblot analysis performed using polyclonal anti-LIC12693 antibody detected the LIC12693 to be a largely detergent insoluble protein which is a characteristic of many transmembrane proteins (Fig. 4.23C).

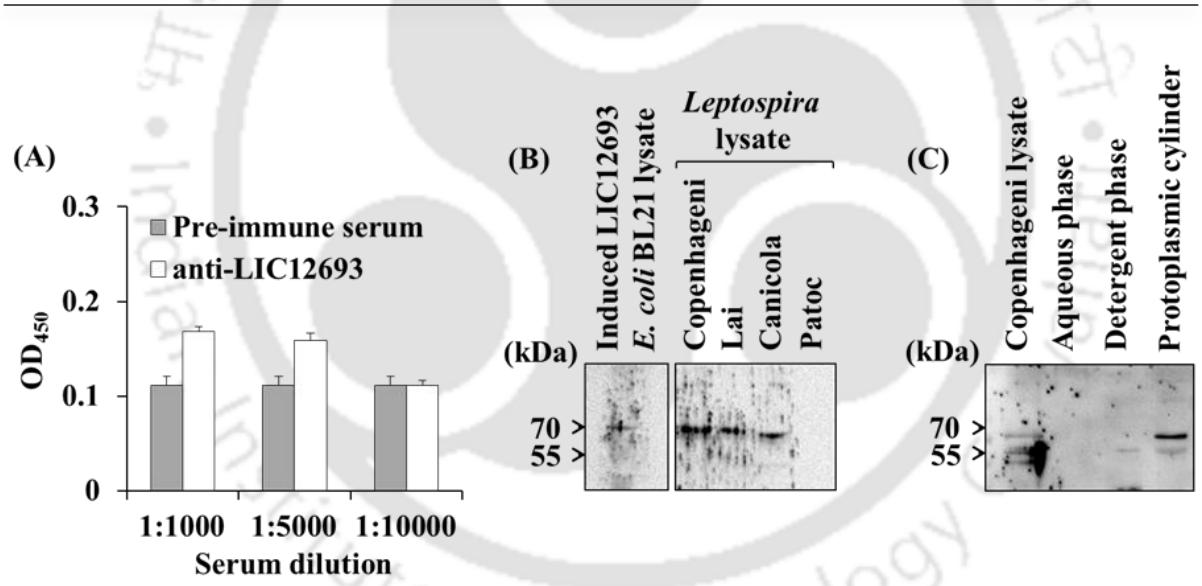

Figure 4.23. Characterization of LIC12693 expression and localization in *L. interrogans* serovar Copenhageni. (A) Immunoassay to determine the titer of antibodies generated against r-LIC12693. The serum obtained before the immunization of r-LIC11966 was used as a control for evaluation of antibody titer. The data is represented as mean ± standard error of two independent experiments. The titer of the antibody of r-LIC12693 was found to be 5000. **(B)** Immunoblot analysis of native and recombinant-LIC12693 antigen. The generated anti-LIC12693 antibody can detect the purified r-LIC12693 and the native LIC12693 in pathogenic *Leptospira* serovars (Copenhageni, Lai, and Canicola). A non-pathogenic *L. biflexa* serovar Patoc was used as negative control in the immunoblot**. (C)** Cellular localization of the hypothetical protein LIC12693. *Leptospira* LIC12693 is a transmembrane protein. Spirochetes whole cell

lysates were subjected to Triton X-114 phase partition into aqueous and detergent phase. The aqueous, detergent phase and protoplasmic cylinder fractions were resolved onto 12% SDS-polyacrylamide gel and subsequently immunoblotted with anti-LIC12693 serum. The native LIC12693 was present in the protoplasmic cylinder fraction, indicating it to be a probable transmembrane protein.

4.4.20 Recombinant LIC12693 binds to the laminin and fibronectin

Most of the leptospiral proteins have been shown to bind to the fibrous proteins namely laminin and fibronectin (Vieira et al., 2014). As a preliminary study to evaluate LIC12693 interaction with these ECM components using ELISA, a soluble form of r-LIC12693 was allowed to bind to the immobilized laminin and fibronectin on microtiter plates. The antigen r-LIC12693 exhibited significant binding to both laminin and fibronectin, in a dose-dependent and saturable manner when increasing concentrations of the r-LIC12693 (0-8 µM) were allowed to bind to a fixed amount of laminin and fibronectin (1 µg) (Fig. 4.24). A summary table (Table 4.6) shows the comparative analysis of the four proteins characterized in the present study.

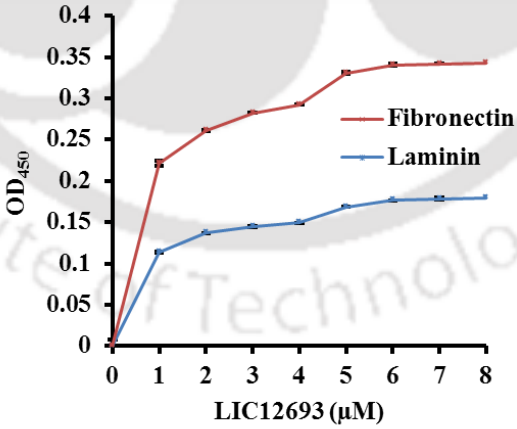

Figure 4.24. Dose-dependent binding of the r-LIC12693 to laminin and fibronectin. Laminin or fibronectin-coated microtiter plates were incubated with increasing concentrations of r-LIC12693 (0-8 µM). Binding of LIC12693 was measured using the anti-LIC12693 serum at an appropriate dilution. The mean absorbance values of r-LIC12693 binding to laminin and fibronectin at 450 nm are shown. Error bars indicate the mean ± standard error from two independent experiments.

Table 4.6. Summary table showing the comparative analysis of the four proteins characterized in the present study

Sr. No.	Copenhageni locus	UniProt ID	Ortholog in Lai	UniProt ID	Copy number[a]	Subcellular localization	Antigenic protein	Putative function
1	LIC20035	Q75G29	LB047	Q8EY10	2008	Surface exposed OMP	Yes	Binds ECM; preferably collagen and chondroitin sulfate (This study), Fas binding outer membrane protein (Du et al., 2018)
2	LIC13341	Q72M50	LA4185	Q8EYM8	ND	Surface exposed OMP	Yes	Binds ECM; preferably laminin and hyaluronic acid (This study)
3	LIC11966 (ErpY-like)	Q72QY9	LA1939	Q8F4V0	4330	Surface exposed OMP	Yes (Eshghi et al., 2009; Padilha et al., 2019) and this study	Binds ECM and fibrinogen (This study)
4	LIC12693	Q72NY5	LA0957	Q8F7I8	377	Transmembrane OMP	NP[b]	Binds to laminin and fibronectin (This study), TolC like outer membrane efflux protein (Caimano et al., 2014; Nally et al., 2007)

[a] Copy number: Estimated by MS (Malmström et al., 2009). ND: None detected

[b] NP: Not performed

4.5 DISCUSSION

LIC20035/LB047 was further characterized as it codes for a conserved hypothetical protein in *Leptospira* and it was getting differentially modulated in the presence of host factors. Additionally, LIC20035 orthologs are more conserved among pathogenic leptospires than the intermediate or saprophytic strains. Many known leptospiral virulent OMPs like Loa22 have orthologs in saprophytic strains of *Leptospira* and therefore absence or presence of orthologs in the saprophytes may not be an accurate criterion for predicting a gene to be virulent (Haake and Zückert, 2015).

The polyclonal antibodies generated against r-LIC20035 were able to recognize native-LIC20035 expression predominantly in the detergent phase of Triton X-114. Similarly, cellular localization experiment using protease-accessibility assay describes *LB047* to be a surface-exposed membrane protein. However, for some surface exposed proteins, the Proteinase K cleavage sites are inaccessible due to steric hindrance by LPS at the surface of *Leptospira* (Pinne and Haake, 2009). A previous study on LIC20035 (demarcated as NT03LIA0039, TIGR locus) from elsewhere (Nally et al., 2007), described LIC20035 to be present in the Triton X-114 fraction of IVCL and was detectable in the immunoblot using antibodies raised against *Leptospira* outer membrane vesicles (OMV). Nevertheless, in the same study LIC20035 was not recognized in the Triton X-114 fraction of spirochetes isolated from infected guinea pig liver (Nally et al., 2007). The spirochete expression of LIC20035 in guinea pig liver may be due to the multiple effects of host factors; however, this is in agreement to the LIC20035 repression in the presence of Epi.

The seroreactivity of LIC20035 against MAT-positive serum samples of humans/bovine demonstrates that humoral immune response was generated against LIC20035 during natural

infection of *Leptospira* in the host. Recognition of LIC20035 by the hosts during infection suggests that LIC20035 could be a useful serodiagnostic antigen. As OMPs are the primary bacterial components that interact with host cells, targeting newly identified OMPs for development of recombinant vaccines could be rewarding. Till date several OMPs of *Leptospira* have been reported, nevertheless, none of them have been commercialized as a highly effective vaccine candidate (Raja and Natarajaseenivasan, 2015). Considering the wide variety of serovars of *Leptospira* and their host specificity, it is tempting to screen more protective antigens in order to develop a multi-component vaccine. Recently, LIC20035 expression was described to be undetectable using serum of rat which was chronically infected with *Leptospira* (Nally et al., 2007). In a recent work, it has been demonstrated that the expression of LB047 in *L. interrogans* serovar Lai was significantly upregulated during infection of macrophages indicating its involvement in the infection of host cells by *L. interrogans*. In the same work, the outer membrane protein LB047 along with LPS of *L. interrogans* was found to induce macrophage apoptosis through the Fas/FasL-caspase-8/3 (Du et al., 2018). This is in line with the present study in which we show that LB047/LIC20035 is an outer membrane protein which is differentially modulated in the presence of host factors. It can also be concluded that the expression of *Leptospira* LIC20035 varies depending upon the host chemical signals. The ECM components of host facilitate adhesions with different microbial proteins and these interactions are essential for microbial pathogenicity (Chen et al., 2008b). Binding assay of r-LIC20035 with host ECM components showed preferential higher affinity for collagen and chondroitin sulfate. The measured affinity of r-LIC20035 for collagen was near to that of LIC13143 (TlyC) (Carvalho et al., 2009) but higher than LIC11352 (LipL32) (Hoke et al., 2008). Additionally, r-LIC20035

binding to chondroitin sulfate is corroborated by the previous reports of *L. interrogans* adhering to chondroitin sulfate (Robbins et al., 2015). The chondroitin sulfate component is one of the most abundant cell surface glycosaminoglycans (GAGs) that mediates bacterial attachment to the host cell surface (Baron et al., 2009). As LIC20035 is found to be surface-exposed membrane protein expressed during infection and its recombinant protein adhere to more than one host ECM components, it is liable to participate in mediating attachment to host tissues as previously reported for other proteins of spirochetes like Len (Stevenson et al., 2007) , and Lsa63 (Vieira et al., 2010d). This study mimics the possible means by which one of the host factors catecholamines is being adapted by *Leptospira* to disseminate in various host tissues.

The leptospiral genome codes for many proteins with unknown functions. The function of such proteins has to be revealed to understand the exact pathophysiology of leptospirosis. Genome sequencing studies from the last two decades have revealed a significant number of potential genes encoding lipoproteins in spirochetes (Fraser et al., 1997; Fraser et al., 1998b; Haake, 2000; Setubal et al., 2006). The majority of these lipoproteins are hypothetical with no assigned function. Among these hypothetical lipoproteins, LIC13341 was chosen for characterization as it is an outer membrane conserved protein in *Leptospira* with no known function. *LIC13341* (*LA4185* ortholog in *L. interrogans* serovar Copenhageni) gene transcript was not detected by RT-PCR or qRT-PCR from the total RNA obtained from *Leptospira* cultured in presence or absence of host factors under *in vitro* condition. This suggests that there can be other unknown host factor(s) or transcription factor(s) which regulate(s) *LIC13341* gene transcription. Attenuation of many virulent genes of *Leptospira* on frequent subculturing under *in vitro* culture condition is also reported previously. To understand the

effect of passaging on the loss of virulent genes, low passage *L. interrogans* was procured and we were able to detect *LIC13341* in all the pathogenic serovars tested. The deduced amino acid sequences of LIC13341 begins with 53-residues long signal peptide that have a positively charged amino-terminal (N-region), a hydrophobic region (H-region) with greater than 6 residues (39^{th} to 48^{th} amino acid), and a carboxyl-terminal region (C-region) demarcated as lipobox with residues Leu49, Phe50, Val51, Ala52, Cys53 (Nascimento et al., 2004b). The bacterial outer membrane-associated proteins may interact with the host and act as a key virulence factor essential in its dissemination, pathogenesis, or protective immunity.

We report that LIC13341 is a lipoprotein of *L. interrogans* with a binding ability to host ECM and is a potential candidate marker for a recombinant protein-based serodiagnostic test. The phylogenetic analysis of *Leptospira* using LIC13341 protein sequence shows that the protein is more conserved in pathogenic strains than that of non-pathogenic strains of *Leptospira*. The presence of conserved *LIC13341* gene was clearly demonstrated by PCR and RT-PCR in various serovars of pathogenic *L. interrogans* viz. Copenhageni, Lai, Canicola, Pomona, and Bataviae. The result of RT-PCR was on the same line to that of the immunoblot analysis performed to detect LIC13341 in the lysates of various *Leptospira* serovars. Interestingly, anti-LIC13341 antibody recognized an additional lower band of LIC13341. Such diversity in the mobility of LIC13341 may be due to lipidation, in a manner similar to other *Leptospira* antigens, LenD (Stevenson et al., 2007) and LipL32 (Murray, 2013). It is a known phenomenon that lipoprotein does not bind the same amount of SDS per gram of protein (Haake, 2000). The correlation of mobility and molecular weight fall apart under those circumstances. Moreover, the recombinant LIC13341 protein used is non-lipidated and includes vector fusion.

Cellular localization of LIC13341 in *Leptospira* using protease-accessibility assay and Triton X-114 detergent phase separation assay describes LIC13341 to be a surface-exposed membrane protein. These findings agree with the previous report where LIC13341 (NT03LI4002) was detected by mass spectrometry in the fractioned proteome of *Leptospira* (Nally et al., 2007). Similarly, conditioned *Leptospira* culture, mimicking infection detected LIC13341 by mass spectrometry in whole cell pellet of *Leptospira* (Eshghi et al., 2015b). Moreover, high-throughput RNA sequencing of *Leptospira* demonstrated moderate up-regulation of *LIC13341* transcripts (1.75 fold) in the host in comparison to *in vitro* culture condition (Caimano et al., 2014). Using bioinformatics, cellular location of LIC13341 in *Leptospira* (Eshghi et al., 2015b; Nally et al., 2007) was consistently predicted as a membrane protein that was most likely to occur at the outer membrane, as previously shown for LipL32 (Haake, 2000; Haake et al., 2000b). Therefore, it was interesting to investigate if LIC13341 has a role to mediate *Leptospira*-host interaction.

We demonstrate that r-LIC13341 is an adhesin protein that binds to a wide spectrum of host ECM components. The secondary structure of the r-LIC13341 displayed the sustained integrity of the protein and ideal for the binding study of the ECM components. Moreover, the inclusion of the moderate binding Loa22 antigen to ECM binding assay as previously reported (Ghosh et al., 2018) demonstrates the specific interaction of r-LIC13341 to ECM components. It is suggested that leptospiral attachment to ECM, correlates with virulence since the virulent strains of *Leptospira* attach more effectively than the intermediate and avirulent strains (Ito and Yanagawa, 1987a). Such adhesion of r-LIC13341 to a spectrum of ECM components suggest that LIC13341 may have a role in virulence that aids *Leptospira* to colonize in various tissues of the host as described for other *Leptospira* membrane proteins,

OmpL37 (Pinne et al., 2010) and OmpL1 (Fernandes et al., 2012). The affinity of r-LIC13341 for ECM components laminin and hyaluronic acid was clearly demonstrated in the study. A dose-dependent specific and saturable binding of r-LIC13341 to immobilized laminin and hyaluronic acid fulfills the properties of the typical receptor-ligand interaction. Interestingly, the binding of *L. interrogans* with laminin and hyaluronic acid was not completely inhibited in the presence of r-LIC13341. Such partial inhibition of leptospires binding to laminin and hyaluronic acid by r-LIC13341 suggests the contribution of additional *L. interrogans* binding proteins. Metaperiodate oxidation of laminin or heat-denaturation of r-LIC13341 leads to a sharp decline in receptor-ligand interaction. The critical role of carbohydrate moieties of laminin in the interaction of spirochetes is in agreement to earlier reported data (Barbosa et al., 2006; Cameron, 2003). The calculated binding affinity of r-LIC13341 to laminin was of the same magnitude as that of the previously reported *Leptospira* membrane proteins Lsa25, Lsa33 (Domingos et al., 2012) and OmpL37 (Pinne et al., 2010). An evidence of leptospires binding to hyaluronic acid published elsewhere (Ito and Yanagawa, 1987b) suggest that r-LIC13341 may be one of the receptors that bind to hyaluronic acid. Till date, to our understanding, there is no adhesin in *Leptospira* reported that binds to hyaluronic acid with high affinity. In addition to *Leptospira*, other spirochetes like *T. denticola* (Edwards et al., 2005) and *T. pallidum* (Fitzgerald et al., 1984) have also been found to interact with hyaluronic acid.

We also demonstrate that the antibodies generated during natural *Leptospira* infection in humans and bovines can detect r-LIC13341 with high sensitivity and specificity. However, this recombinant antigen-based serological assay demonstrated relatively lower sensitivity with serum of bovines than humans. Such a difference in sensitivity among the host serum

may be due to the differential expression of LIC13341 during leptospirosis. Moreover, LIC13341 of *L. interrogans* serovar Copenhageni shows only 78% identity with its ortholog in *L. borgpetersenii* serovar Hardjo, the *Leptospira* serovar which is more prevalent in bovine species (Rajan et al., 2017; Tomich et al., 2007).

In conclusion, we have identified a conserved immunogenic LIC13341 lipoprotein encoded by *LIC13341* gene in pathogenic *L. interrogans* serovars. Recombinant LIC13341 protein displays interaction to a spectrum of host extracellular matrices and can be serologically detected by antibodies present in the serum samples of bovine and human naturally infected with *Leptospira*. Such *in vitro* assay to evaluate the possible role of this novel protein, where no function is assigned during genome annotation, may be beneficial to understand leptospirosis. The characterization of new diagnostic antigen marker of *Leptospira* would press forward the present need for diagnosing the under-reported and neglected nature of this disease globally.

Another hypothetical lipoprotein, LIC11966 was characterized in the present study as it was found to differentially downregulated in the presence of physiological osmolarity. The presence of conserved *LIC11966* gene was demonstrated by PCR and RT-PCR in pathogenic serovars of *L. interrogans* viz. Copenhageni and Lai. The result of RT-PCR was on the same line as that of the western blot performed to detect LIC11966 in the lysates of various *Leptospira* serovars. Bioinformatics revealed that LIC11966 was not present in the saprophytic *L. biflexa*, which was also confirmed by our immunoblotting experiments. A study demonstrated that *LIC11966* gene transcripts in *L. interrogans* were strongly down-regulated at physiological osmolarity when compared to low osmolarity conditions. This indicates that LIC11966 responds to host signals encountered by pathogenic *Leptospira*

during the transition from environmental conditions to host tissue conditions (Matsunaga et al., 2007a). In a previous report, LIC11966 annotated as a putative ErpY-like lipoprotein was described as a novel potential virulence factor in *L. interrogans* (Eshghi et al., 2009). To decipher the probable function and localization of this protein, cellular localization studies using Triton X-114 and proteinase-K accessibility assay were performed. Phase partition experiments show that LIC11966 is an outer membrane protein and proteinase K accessibility assay shows that it is surface exposed protein.

An important aspect for the leptospiral pathogenesis in the host is the adhesion to host extracellular matrix and plasma components. The r-LIC11966 was found to interact with all the ECM tested, most notably with collagen and fibrinogen (a plasma component). Previous studies demonstrate that LIC11966 interacts only with fibrinogen (Oliveira et al., 2018). This binding to plasma fibrinogen may interfere with the blood coagulation cascade and contribute to hemorrhage and vascular lesions in leptospirosis (Oliveira et al., 2018). Recent trials to assess the protective efficacy of LIC11966 as a vaccine yielded promising results (Oliveira et al., 2018). The LIC11966 protein along with other membrane proteins like OmpL36, Loa22 etc. could provide cross-protective immunity against leptospirosis (Srikram et al., 2011). All these findings show that LIC11966 is a promising vaccine candidate against leptospirosis.

The potential of LIC11966 to be used as a serodiagnostic tool for the diagnosis of leptospirosis was evaluated in the present study. We demonstrate that antibodies generated during natural *Leptospira* infection in humans, bovines and canines, could detect LIC11966 with 100% sensitivity and specificity. Nevertheless, previous immunofluorescence experiments using liver samples prepared from golden Syrian hamsters infected with *L. interrogans* serovar Pomona failed to detect LIC11966 (Eshghi et al., 2009). Such failure of

detection may either be due to the lack of expression of LIC11966 in serovar Pomona or LIC11966 not getting expressed during hamster infection. However, the same report also shows LIC11966 is expressed during human infection (Eshghi et al., 2009). A recent report demonstrates that LIC11966 is expressed in *L. interrogans* infected swine populations and could be used as a diagnostic tool for swine leptospirosis (Padilha et al., 2019). In conclusion, we have identified a conserved immunogenic LIC11966 lipoprotein in *L. interrogans* serovars. Recombinant LIC11966 can be serologically detected by antibodies present in the serum of human, bovine and canine infected with *Leptospira*.

LIC12693 which is predicted to be an outer membrane efflux protein, annotated as NT03LI3222 was obtained in Triton-X 114 fraction and was found to be expressed during the acute phase of infection (Nally et al., 2007). In the present study, this protein was characterized as it was previously reported to be downregulated in mammalian host-adapted *Leptospira*. Polyclonal antibodies against r-LIC12693 were generated in mice and these specific antibodies were used to evaluate the expression and localization of LIC12693. Cellular fractionation by Triton X-114 extraction and phase partitioning has been broadly applied to determine the localization of the proteins in the leptospiral outer membrane. However, this method has a limited validation in the case of outer membrane-spanning proteins, such as channel-forming outer membrane proteins (porins), which contain a number of amphipathic regions that could result in uncharacteristic interactions with Triton X-114 (Maher and Singer, 1985). In fact, a number of clear examples of incomplete detergent solubilization of known leptospiral outer membrane proteins, including the OmpL1, OmpL54, and OmpL36 have been described, indicating that the complete fractionation into the Triton X-114 detergent phase may not occur for transmembrane outer membrane proteins

and further experiments are needed to determine the localization of leptospiral proteins (Haake et al., 1993; Pinne and Haake, 2009). The unexpected presence of outer membrane proteins in the protoplasmic cylinder fraction has been described previously for several leptospiral OMPs like LipL41 (Shang et al., 1996), LipL21 (Cullen et al., 2003) and LipL46 (Matsunaga et al., 2006). Our study also reveals that LIC12693 partitions in the detergent-insoluble fraction and may be a transmembrane outer membrane protein. We also performed an interaction study of LIC12693 with laminin and fibronectin. Our results show that LIC12693 has the ability to bind to laminin and fibronectin and may possibly help *Leptospira* in adhesiveness in the host tissues (Vieira et al., 2014). The characterization of such novel diagnostic antigen markers for diagnosing leptospirosis may help for the accurate diagnosis of a disease which is mostly underdiagnosed and pose a challenge to many developing countries.

Chapter 5

Conclusion and future prospects

CHAPTER 5

Conclusion and future prospects

5.1 Conclusion

An important focus of the present research is the identification of OMPs that are involved in the pathogenesis of *Leptospira*. Leptospiral OMPs are thought to be important for understanding the host-pathogen interactions due to their location on the bacterial surface. Transcriptomic analysis has shown that *L. interrogans* respond to different host signals such as temperature upshift, osmolarity, iron levels, and serum. The genes that are found to be differentially expressed in the presence of host factors may play an important role in bacterial infection and pathogenesis. This study was aimed at the identification of outer membrane proteins of pathogenic *Leptospira* responding to host signals like catecholamines, temperature upshift and oxidative stress with the potential to serve as virulence factors, new serodiagnostic antigens, and vaccine candidates.

We have developed a novel way of screening antigens which can be tested to be used as diagnostics for leptospirosis. We demonstrate that exposure of *L. interrogans* to different host factors like catecholamines, elevated temperature and osmolarity found in the host, induced a shift in the gene transcription which might facilitate the bacteria to adapt in the mammalian host for a successful establishment of infection. Such genes may be important in the pathogenesis of the bacteria and hence, molecular characterization was performed to elucidate their possible function in the pathogenesis of *Leptospira*.

Bioinformatics and gene expression analysis of *L. interrogans* revealed predicted outer membrane proteins that could be modulated by various host factors. The *LIC20035* transcript was found to be downregulated in the presence of epinephrine, oxidative stress and elevated temperature of 37ºC. The *LIC13341* transcript which was not getting transcribed from the *in vitro* passaged *L. interrogans* serovars being used in the present study indicated a possible loss of virulence due to *in vitro* passaging or unknown transcription/host factors involved in the induction of gene expression. The *LIC11966* transcript was found to be downregulated at the physiological osmolarity of the host. Proteins encoded by these genes were found to be surface exposed outer membrane proteins of *Leptospira*. An outer membrane efflux protein of *L. interrogans*, LIC12693 which was previously reported to be downregulated in mammalian host-adapted *Leptospira* as compared to *in vitro* grown cultures was found to be a transmembrane protein.

Leptospiral proteins LIC20035, LIC13341 and LIC12693 were found to be highly conserved among pathogenic species of *Leptospira*. The LIC11966 is present only in pathogenic species of *Leptospira*. Existence of these proteins in disease causing *Leptospira* species strengthens the possibilities of these proteins to be involved in pathogenesis. These leptospiral proteins are found to bind with wide range of host ECM components which may help *Leptospira* in adherence and dissemination inside the host. The LIC11966 also interacts with plasma fibrinogen suggesting that LIC11966 may interfere with the host fibrin based blood clotting mechanism. *Leptospira* spp. may use these interactions as a possible mechanism for the establishment of infection. Serological assays using these recombinant-leptospiral proteins indicate that these proteins are expressed in diverse hosts during *Leptospira* infection and

hence, these proteins could be targeted as novel serodiagnostic candidates for the diagnosis of leptospirosis.

The present study highlights the modulation of the transcription of the membrane proteins in *L. interrogans* on exposure to host factors under *in vitro* condition and show that *L. interrogans* uses molecular receptors to sense the host catecholamines for facilitating their adaptation in the host. This transcriptional study also gives a preliminary idea that *L. interrogans* possibly modulates the proteins of LPS synthesis in response to catecholamines, thereby indicating the role of LPS in the virulence and bacterial survival inside the host. Molecular characterization of these host factor modulated genes encoding hypothetical proteins revealed that LIC20035, LIC13341, LIC11966, and LIC12693 are outer membrane adhesins which could be targeted as novel serodiagnostic candidates for the diagnosis of leptospirosis. However, the biological relevance of these *in vitro* interactions must be confirmed through further experiments like inhibition of the binding by antibodies or purified protein, or gain of function after expression in saprophytic *L. biflexa*. The characterization of such antigen markers may help for the accurate diagnosis of a disease which is mostly misdiagnosed in the present scenario.

5.2 Future prospects

The current work highlights the means by which one of the host factors catecholamines is being adapted by pathogenic *Leptospira* to disseminate in various host tissues. Future work on host-pathogen chemical signaling is needed for the development of wide-range of disease intervention strategies. The exact mechanism of interaction of catecholamines with the bacterial histidine kinase (HK) receptors needs to be elucidated. Since catecholamines and autoinducer AI-3 bind to the same HK receptors, it will be interesting to identify the

interaction between microbial endocrinology and quorum sensing signaling pathway in *Leptospira*. One of the genes significantly modulated by catecholamines, *LA3307* (*rfe*), needs to be further characterized. The transcriptional analysis performed for the *rfe* gene should be checked at the translational level through western blotting in leptospiral cultures grown in the presence of catecholamines and its antagonist. The effect of tunicamycin (inhibits the function of rfe in some organisms) on the rfe expression in *Leptospira* can also be studied. Catecholamines have been shown to upregulate *rfe* and *rfb* gene clusters, thereby pinpointing to the fact that the LPS synthesis gets affected when *L. interrogans* transitions from the environment to the host tissues. The effect of polymixin-B (affects the LPS synthesis) on the growth pattern and LPS biosynthesis of *L. interrogans* can also be analyzed in the future. Nonetheless, it will be an interesting topic of future study to understand the exact effect of catecholamines on the LPS synthesis pathway. The characterization of novel hypothetical membrane proteins like *LB047*/*LIC20035* and *LA3263*/*LIC10878* responding to host factors will pave the way for the identification of new serodiagnostic antigens and possible vaccine candidates. The other genes responding to host factors namely *LB186*/*LIC20148* (*hol*), *LB191*/*LIC20151* (*hbpA*), *LA0616*/*LIC12966* (*lipL41*), and *LA3961*/*LIC13166* (*ompL36/fcpA*) have been previously well characterized. However, it will be interesting to evaluate the possible role of these genes with respect to leptospiral response to host factors during infection. This will go a long way in the management of this under-reported and neglected zoonosis. The reverse genetic technique to disrupt the genes of interest though double homologous recombination, could help us elucidate the possible role of these hypothetical proteins in the survival and persistence of *L. interrogans* in the host during infection.

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Appendix

I. REAGENTS FOR SDS-PAGE

1. **Acrylamide/ Bisacrylamide solution (30% w/v) 100 mL**

   Acrylamide/Bisacrylamide premix powder (29:1)…...30 g

   Distilled water to make……..100 mL

2. **1.5M Tris-Cl, pH 8.8 1000 mL**

   Tris base……...121.1 g

   Distilled water……..800 mL

   Adjust pH to 8.8.

   Distilled water to make……1000 mL

3. **1 M Tris-Cl, pH 6.8 1000 mL**

   Tris base……..121.1 g

   Distilled water…….800 mL

   Adjust pH to 6.8.

   Distilled water to make……...1000 mL

4. **Ammonium persulfate (10% w/v) 10 mL**

   Ammonium persulfate…….1 g

   Distilled water to make……10 mL

5. **SDS (10% w/v) 10 mL**

   SDS……. 1 g

   Distilled water to make……10 mL

6. **Tris-glycine Electrophoresis buffer (10X, pH 8.3) 1000 mL**

   Tris base……30.3 g

   Glycine……..144 g

   SDS……...10g

   Distilled water to make……..1000 mL

7. SDS Gel loading buffer (5X) 1 mL

Tris-Cl (1M, pH 6.8)……...0.25 mL

SDS……..80 mg

Bromophenol blue……..1 mg

Glycerol (80%, v/v)…….0.5 mL

Dithiothreitol (1 M)……..0.1 mL

Distilled water……...0.25 mL

8. Coomassie Brilliant Blue staining solution 100 mL

Coomassie Brilliant Blue R-250…….0.05 g

Methanol……..50 mL

Glacial acetic acid……10 mL

Distilled water to make……100 mL

9. Destain solution 100 mL

Methanol……30 mL

Glacial acetic acid……..10 mL

Distilled water to make……..100 mL

II. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. Gel loading buffer (6X) 1 mL

Bromophenol blue……..2.5 mg

Xylene cyanol…….2.5 mg

Glycerol……...0.3 mL

Distilled water to make……1 mL

2. TAE (50X) 1000 mL

Tris base…….242 g

Glacial acetic acid……..57.1 mL

EDTA (0.5 M, pH 8.0)……..100 mL

Distilled water to make……..1000 mL

3. Ethidium bromide (10 mg/mL) 10 mL

Ethidium bromide……..0.1 g

Distilled water to make……...10 mL

III. REAGENTS FOR WESTERN BLOTTING

1. Transfer buffer (10X, pH 8.0) 1000 mL

Tris base…….2.41 g

Glycine……...11.16 g

Adjust pH to 8.0.

Methanol……200 mL

Distilled water to make……..1000 mL

2. Tris-buffered saline (TBS) 1000 mL

Tris-Cl (1 M, pH 7.5)……20 mL

Sodium chloride……8 g

Distilled water to make……1000 mL

3. Blocking buffer 10 mL

Skim milk……..0.5 g

0.05% TBS-Tween 20 to make…….10 mL

IV. REAGENTS FOR ELISA

1. Phosphate buffered saline (PBS) 1000 mL

Sodium chloride……...8 g

Potassium chloride……..0.2 g

Disodium hydrogen phosphate……...1.44 g

Dihydrogen potassium phosphate……...0.24 g

Distilled water……..800 mL

Adjust pH to 7.4.

Distilled water to make……1000 mL

2. Blocking buffer **10 mL**

Bovine serum albumin…… 0.3 g

0.05% TBS-Tween 20 to make……10 mL

V. REAGENTS FOR PROTEIN PURIFICATION

❖ **For purification of LIC20035, LIC12693, and Loa22**

1. 10X Stock solution A **100 mL**

Sodium phosphate, monobasic……2.76 g

Sodium chloride……29.2 g

Distilled water to make……....100 mL

2. 10X Stock solution B **100 mL**

Sodium phosphate, dibasic……..2.84 g

Sodium chloride……29.2 g

Distilled water to make……....100 mL

3. 5X Native purification buffer **100 mL**

Sodium phosphate, monobasic……..3.5 g

Sodium chloride……14.6 g

Distilled water……80 mL

Adjust pH to 8.0.

Distilled water to make……...100 mL

4. 3M Imidazole (pH 6.0) **100 mL**

Imidazole……20.6 g

10X Stock solution A……..8.77 mL

10X Stock solution B…….....1.23 mL

Distilled water……80 mL

Adjust pH to 6.0.

Distilled water to make……...100 mL

5. Denaturing binding buffer (pH 7.8) 100 mL

Urea…….48.1 g

10X Stock solution A……..0.58 mL

10X Stock solution B…….....9.42 mL

Distilled water…….80 mL

Adjust pH to 7.8.

Distilled water to make……....100 mL

6. Denaturing wash buffer (pH 6.0) 100 mL

Urea…….48.1 g

10X Stock solution A……..7.38 mL

10X Stock solution B…….....2.62 mL

Distilled water…….80 mL

Adjust pH to 6.0.

Distilled water to make……...100 mL

7. Denaturing elution buffer (pH 4.0) 100 mL

Urea…….48.1 g

10X Stock solution A……..10 mL

Distilled water……..80 mL

Adjust pH to 4.0.

Distilled water to make……....100 mL

8. Native wash buffer (pH 8.0) 100 mL

5X purification buffer…….20 mL

3M Imidazole (pH 6.0)……....670 µL

Distilled water…….80 mL

Adjust pH to 8.0.

Distilled water to make……....100 mL

9. Native elution buffer (pH 8.0) 100 mL

5X purification buffer…….20 mL

3M Imidazole (pH 6.0)……....8.33 mL

Distilled water……...80 mL

Adjust pH to 8.0.

Distilled water to make……...100 mL

10. Dialysis buffer 1000 mL

Tris (1M, pH 8)……50 mL

Sodium chloride (5M)…….20 mL

Distilled water…….....700 mL

Adjust pH to 8.0.

Distilled water to make……...1000 mL

❖ **For purification of LIC13341 and LIC11966**

11. Native lysis buffer 100 mL

Tris (1M, pH 8)……..5 mL

Sodium chloride (5M)……6 mL

Glycerol……..10 mL

Triton X-100……...1 mL

Distilled water……....70 mL

Adjust pH to 8.0.

Distilled water to make……...100 mL

12. Native wash buffer 100 mL

Tris (1M, pH 8)……..5 mL

Sodium chloride (5M)……6 mL

Glycerol……..10 mL

Imidazole (3M, pH 6.0)……..0.333 mL

Distilled water……....70 mL

Adjust pH to 8.0.

Distilled water to make……...100 mL

13. Native elution buffer **100 mL**

Tris (1M, pH 8)……5 mL

Sodium chloride (5M)…….6 mL

Glycerol……10 mL

Imidazole (3M, pH 6.0)……...8.333 mL

Distilled water…….....70 mL

Adjust pH to 8.0.

Distilled water to make……...100 mL

14. 50 mM sodium phosphate buffer (pH 7.4)

100 mM sodium phosphate, monobasic……..19 mL

100 mM sodium phosphate, dibasic……81 mL

Distilled water to make……100 mL

VI. REAGENTS FOR COMPETENT CELLS PREPARATION

1. Calcium chloride (0.1 M) **100 mL**

Calcium chloride……..1.11 g

Distilled water……...80 mL

Allow to dissolve.

Distilled water to make…….100 mL

2. Calcium chloride (0.1 M) with 15% Glycerol **100 mL**

Calcium chloride……1.11 g

Glycerol……..15 mL

Distilled water to make……...100 mL

VII. COMMON LABORATORY REAGENTS

1. EDTA (0.5 M, pH 8.0) 100 mL

Disodium EDTA×2H_2O……18.61 g

Distilled water……80 mL

Adjust pH to 8.0 with sodium hydroxide.

Distilled water to make……..100 mL

2. Sodium chloride (5 M) 100 mL

Sodium chloride……29.2 g

Distilled water……...80 mL

Allow to dissolve.

Distilled water to make……100 mL

3. Sodium hydroxide (10 N) 100 mL

Sodium hydroxide……40 g

Distilled water……80 mL

Allow to dissolve.

Distilled water to make……..100 mL

Research output

Publications from thesis work

1. **Ghosh KK**, Prakash A, Shrivastav P, Balamurugan V, Kumar M. Evaluation of a novel outer membrane surface exposed protein LIC13341 of Leptospira as adhesin and serodiagnostic candidate marker for leptospirosis. *Microbiology* 2018; 164(8):1023-1037.

2. **Ghosh KK**, Prakash A, Balamurugan V, Kumar M. Catecholamine-Modulated Novel Surface-Exposed Adhesin LIC20035 of Leptospira spp. Binds Host Extracellular Matrix Components and Is Recognized by the Host during Infection. *Applied and Environmental Microbiology* 2018;84(6):e02360-02317.

Publications from other collaborative research work

1. Dixit B, **Ghosh KK**, Fernandes G, Kumar P, Gogoi P, Kumar M. Dual nuclease activity of a Cas2 protein in CRISPR–Cas subtype I-B of Leptospira interrogans. *FEBS Letters* 2016;590(7):1002-1016.

Presentations in conferences

1. **Karukriti Kaushik Ghosh** and Manish Kumar (2018). Catecholamines as chemical messengers for host-*Leptospira* interaction. Research Conclave, IIT Guwahati, Assam.

2. **Karukriti Kaushik Ghosh** and Manish Kumar (2016). Modulation of gene expression of Leptospira interrogans exposed to catecholamines and its role in host immune evasion. 57th Annual Conference of Association of Microbiologists of India and International Symposium on "Microbes and Biosphere" at Gauhati University, Assam.

3. **Karukriti Kaushik Ghosh,** Prateek Shrivastav, Rajeev Kumar Sharma and Manish Kumar (2016). *In vitro* expression analysis of selected outer membrane proteins of *Leptospira interrogans* Copenhageni strain Fiocruz L1-130 for diagnostics and vaccination. Global Symposium on Animal Health: Newer techniques and their application" at Khanapara, Guwahati, Assam.

4. **Karukriti Kaushik Ghosh** and Manish Kumar (2015). Characterization of hypothesized outer membrane protein of *Leptospira interrogans* Copenhageni. 8th Indo Global Summit and Expo on Vaccines, Therapeutics and Healthcare 2015" at Hyderabad, Telangana (Best Poster award).

Conferences/workshops attended

1. 9th TCS Annual Event and Flow Cytometry Workshop on "Flow Application in Basic, Applied and Clinical Biology" (FABACTCS) at IIT Guwahati, Assam (2016).

2. National conference on Recent Developments in Medical Biotechnology and Structure-Based Drug Designing at IIT Guwahati, Assam (2015).

3. Conference on Bioinformatics and Computer-Aided Drug Design at IIT Guwahati, Assam (2015).

4. Symposium cum Workshop on Advances in Computational Biology and Computer-Aided Drug Design at IIT Guwahati, Assam (2015).

5. National conference on Recent Advances in Cancer Biology and Therapeutics at IIT Guwahati, Assam (2014).

Society memberships

1. The International Leptospirosis Society (ILS)
2. The American Society for Microbiology (ASM)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Curriculum Vitae of Graduating PhD Student

Name:	Ms. Karukriti Kaushik Ghosh	IITG Roll No.	136106014
Gender:	Female		
Department:	Department of Biosciences and Bioengineering (BSBE)		
Thesis Title:	Analysis of Genes Encoding Outer Membrane Protein of *Leptospira interrogans* and Its Modulation Due to Host Factors: An Approach for Understanding Host-Pathogen Crosstalk		
Thesis Supervisor(s):	Dr. Manish Kumar		

Month / Year of Joining	Date of Thesis Submission	Date of Thesis Viva-Voce Exam
July / 2013	26.02.2019	19.06.2019
Broad Area of Research:	Molecular Medical Microbiology	

Prior Degree(s) Obtained			
Degree	Discipline	Institute/ University	Month & Year of Completion
M. Sc.	Microbiology	RTM Nagpur University	June, 2012
B. Sc.	Microbiology, Zoology, Chemistry	RTM Nagpur University	June, 2010

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List of Publications in Journals	
1.	**Ghosh KK**, Prakash A, Shrivastav P, Balamurugan V, Kumar M. Evaluation of a novel outer membrane surface exposed protein LIC13341 of *Leptospira* as adhesin and serodiagnostic candidate marker for leptospirosis. *Microbiology* 2018; 164(8):1023-1037.
2.	**Ghosh KK**, Prakash A, Balamurugan V, Kumar M. Catecholamine-Modulated Novel Surface-Exposed Adhesin LIC20035 of *Leptospira spp*. Binds Host Extracellular Matrix Components and Is Recognized by the Host during Infection. *Applied and Environmental Microbiology* 2018;84(6):e02360-02317.
3.	Dixit B, **Ghosh KK**, Fernandes G, Kumar P, Gogoi P, Kumar M. Dual nuclease activity of a Cas2 protein in CRISPR–Cas subtype I-B of *Leptospira interrogans*. *FEBS Letters* 2016;590(7):1002-1016.

List of Publications in Conference Proceedings	
1.	**Karukriti Kaushik Ghosh** and Manish Kumar (2018). Catecholamines as chemical messengers for host-*Leptospira* interaction. Research Conclave, IIT Guwahati, Assam
2.	**Karukriti Kaushik Ghosh** and Manish Kumar (2016). Modulation of gene expression of *Leptospira interrogans* exposed to catecholamines and its role in host immune evasion. 57th Annual Conference of Association of Microbiologists of India and International Symposium on "Microbes and Biosphere" at Gauhati University, Assam
3.	**Karukriti Kaushik Ghosh,** Prateek Shrivastav, Rajeev Kumar Sharma and Manish Kumar (2016). *In vitro* expression analysis of selected outer membrane proteins of *Leptospira interrogans* Copenhageni strain Fiocruz L1-130 for diagnostics and vaccination. Global Symposium on Animal Health: Newer techniques and their application" at Khanapara, Guwahati, Assam
4.	**Karukriti Kaushik Ghosh** and Manish Kumar (2015). Characterization of hypothesized outer membrane protein of *Leptospira interrogans* Copenhageni. 8th Indo Global Summit and Expo on Vaccines, Therapeutics and Healthcare 2015" at Hyderabad, Telangana **(Best Poster award)**

After your PhD, Are you taking up any job/research position? If yes, please provide details.

I am planning to pursue Postdoctoral research in the field of leptospirosis.

Date: 19.06.2019