LIPOPOLYSACCHARIDE IMPRINTED ELECTROCHEMICAL BIOSENSOR FOR EARLY DIAGNOSIS OF LEPTOSPIROSIS AND TO ESTABLISH LEPTOSPIRAL LPS MEDIATED PATHOGENESIS

Thesis submitted to the
Bharathidasan University in the partial fulfillment
of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOLOGY

Submitted By
P. BOTHAMMAL, M.Sc.,
(Reg. no. 8168)

Under the Supervision of
Dr. K. NATARAJASEENIVASAN M.Sc., Ph.D.,
Professor

DEPARTMENT OF MICROBIOLOGY
CENTRE FOR EXCELLENCE IN LIFE SCIENCES
BHARATHIDASAN UNIVERSITY
(Accredited with 'A+' Grade by NAAC)
TIRUCHIRAPPALLI- 620 024
TAMILNADU, INDIA

MAY – 2022

Lipopolysaccharide Imprinted Electrochemical Biosensor for Early Diagnosis of Leptospirosis and to Establish Leptospiral LPS Mediated Pathogenesis

Thesis submitted to the
Bharathidasan University in the partial fulfillment
of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOLOGY

Submitted By

P. BOTHAMMAL, M.Sc.,
(Reg. no. 8168)

Under the Supervision of

Dr. K. NATARAJASEENIVASAN M.Sc., Ph.D.,
Professor

DEPARTMENT OF MICROBIOLOGY

CENTRE FOR EXCELLENCE IN LIFE SCIENCES

BHARATHIDASAN UNIVERSITY

(Accredited with 'A+' Grade by NAAC)

TIRUCHIRAPPALLI-620 024

TAMILNADU, INDIA

MAY – 2022

BHARATHIDASAN UNIVERSITY

Tiruchirappalli – 620024, Tamil Nadu, India

Dr. K. Natarajaseenivasan, Ph.D
Professor
Department of Microbiology
Centre for Excellence in Life Sciences

CERTIFICATE

This is to certify that the thesis entitled **"Lipopolysaccharide Imprinted Electrochemical Biosensor for Early Diagnosis of Leptospirosis and to Establish Leptospiral LPS Mediated Pathogenesis"** submitted to Bharathidasan University, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** is a record of original and independent research work done by **Ms. P. Bothammal (Register No. 8168)** in the Department of Microbiology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli - 620 024, under my supervision and guidance and the thesis has not formed the basis for the award of any Degree/ Diploma/ Associateship/ Fellowship or other similar title to any candidate of any University.

Signature of the Research Supervisor

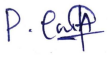
K. Natarajaseenivasan

Tel.: +91 431-2407082, Fax: +91 431-2407045, Mobile: +91 63697 75298
E-mail:natarajaseenivasan@bdu.ac.in

DECLARATION

I, **P. Bothammal**, hereby declare that the dissertation, entitled **"Lipopolysaccharide Imprinted Electrochemical Biosensor for Early Diagnosis of Leptospirosis and to Establish Leptospiral LPS Mediated Pathogenesis",** submitted to the Bharathidasan University, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** is a record of original and independent research work done by me under the supervision and guidance of **Dr. K. Natarajaseenivasan, Professor, Department of Microbiology**, Centre for Excellence in Life Sciences, Bharathidasan University and it has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or other similar title to any candidate in any University.

Signature of the candidate



P. Bothammal

Curiginal

Document Information

Analyzed document	P.Bothammal Ph.D Thesis_For Plagiarism.docx (D138474041)
Submitted	2022-05-30T11:52:00.0000000
Submitted by	Srinivasa ragavan S
Submitter email	bdulib@gmail.com
Similarity	6%
Analysis address	bdulib.bdu@analysis.urkund.com

Sources included in the report

W	URL: https://www.frontiersin.org/articles/10.3389/fcimb.2021.781476/full Fetched: 2022-04-24T09:42:28.6970000	8
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC153370/ Fetched: 2020-11-04T22:05:28.3730000	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4560487/ Fetched: 2021-11-25T16:22:16.3800000	47
W	URL: https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0002290 Fetched: 2020-03-13T07:46:19.8600000	2
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3939550/ Fetched: 2019-10-01T09:25:57.4500000	3
W	URL: https://www.mdpi.com/2076-0817/3/2/280/xml Fetched: 2022-05-30T10:28:11.2670000	1
W	URL: https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0004251 Fetched: 2021-11-05T19:24:45.1270000	2
W	URL: https://emedicine.medscape.com/article/220563-overview Fetched: 2019-10-09T10:07:50.3200000	3
W	URL: https://www.nature.com/articles/s41598-018-27555-2 Fetched: 2020-05-02T20:14:15.1330000	4
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4003590/ Fetched: 2021-06-16T15:45:18.8130000	1
W	URL: https://ncdc.gov.in/WriteReadData/l892s/File558.pdf Fetched: 2019-11-17T20:47:55.8970000	1
W	URL: https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-015-0581-7 Fetched: 2020-02-01T10:46:41.8270000	5
	URL: https://microbewiki.kenyon.edu/index.php/Leptospirosis_induced_by_L._borgpetersenii	

W	URL: https://microbewiki.kenyon.edu/index.php/Leptospirosis_induced_by_L._borgpetersenii Fetched: 2022-01-17T14:35:52.0930000	1
W	URL: https://www.biorxiv.org/content/10.1101/2020.05.18.101857.full.pdf Fetched: 2022-05-30T10:28:28.3030000	2
W	URL: https://www.frontiersin.org/articles/10.3389/fimmu.2020.572999/full Fetched: 2021-02-01T14:15:03.2200000	3
W	URL: https://journals.plos.org/plosone/article%3Fid%3D10.1371/journal.pone.0137130 Fetched: 2021-07-28T11:24:32.7900000	2
W	URL: https://www.mdpi.com/1424-8220/21/7/2552/htm Fetched: 2022-05-30T10:28:15.0200000	2
W	URL: https://link.springer.com/article/10.1007/s00253-022-11901-6 Fetched: 2022-05-30T10:28:18.3270000	1
W	URL: https://onlinelibrary.wiley.com/doi/10.1111/vcp.13129?af=R Fetched: 2022-05-30T10:28:41.5330000	1

ACKNOWLEDGEMENT

First and foremost, I would like to express my deep sense of gratitude and indebtedness to my guide **Dr. K. Natarajaseenivasan**, Professor, Department of Microbiology, Bharathidasan University for initiating the research mind in me and for continued supervision through introducing me to this exciting field of Medical Microbiology. This thesis would not have been possible without his help and advice. I am very much thankful for his inspiration, encouragement, and continuous support, throughout my Ph.D. tenure. His enthusiasm, integral view on research, and his mission for providing high-quality work have made a deep impression on me. I owe him lots of gratitude for having me shown this way of research. Above all, his priceless and meticulous supervision at each and every phase of the work inspired me in innumerable ways. It is a distinguished privilege to work under his guidance and I am sure that the learnings would get reflected in all my future scientific endeavors.

I greatly acknowledge Dr. V. Ganesh, Principal Scientist, EEC Division, CSIR-Central Electrochemical Research Institute, Karaikudi for providing me with such wonderful guidance and support in all the biosensor experiments. His immense knowledge and plentiful experience in the field of biosensors have encouraged me to complete this work with much success. I would like to thank him for his tremendous technical interpretations to accomplish this work fantastically.

I express my sincere gratitude to my Guide-in-Charge Dr. G. Muralitharan, Associate Professor, Department of Microbiology, Bharathidasan University, Tiruchirappalli, for his constant support.

I deeply acknowledge my advisory committee members, Dr. K. Anbarasu, Associate Professor & Head, Department of Marine Biotechnology, Bharathidasan University, and Dr. G. Muralitharan,

Associate Professor, Department of Microbiology, Bharathidasan University for their valuable suggestions and expert opinions throughout my study I also like to thank Dr. K. Emmanuvel Rajan, Professor, Department of Animal Sciences, Bharathidasan University for his constant encouragement throughout my study period.

I place my deep sense of thanks to Lincoln Memorial University, College of Veterinary Medicine (LMU-CVM), the USA for the MoU and funding during my study period. I extend my sincere thanks to Dr. Ashutosh Verma, Associate Professor, LMU-CVM, the USA for his collaboration and support during my research work.

I am grateful to the Indian Council of Medical Research (ICMR), New Delhi, for the financial support through the Senior Research Fellowship.

I would like to thank the Dr. V. Rajesh Kannan, Professor & Head, and Dr. N. Thajuddin, Professor and the former Head of the Department for their immense support and encouragement in the Department to administratively and scientifically. I also extend my thanks to Dr. D. Dhanasekaran, Associate Professor, Dr. G. Muralitharan, Associate professor, Dr. P. Thiyagarajan, Assistant Professor, Dr. M. Prasad, Guest Faculty, Dr. R. Ramya, Guest faculty and the secretarial staff members of the Department of Microbiology for their extended help and support. I also thank Mr. Palanivel, Technical Officer for all the instrumentation facilities and for the store usage during my study.

I would like to sincerely thank our Medical Microbiology Laboratory (MML) research group, Dr. V. Raja, Dr. M. Ganesh, Dr. M. Prasad, Dr. K. Sivasankari, Dr. C. Akino Mercy, and Ms. K. Sumaiya, and Mrs. P. Saranya for their extensive suggestions and help during my research work.

I express my special thanks to Central Electro Chemical Research Institute (CECRI) laboratory seniors Mr. T. Balamurugan and Mr. S. Michelraj for their untired help during my biosensor experiments to complete this new adventure in a successful way.

And I would express my thanks to the esteemed Bharathidasan University for providing facilities through Central Instrumentation Facility, DST-PURSE, and RUSA 2.0.

I sincerely thank KRS hospital for allowing me to collect the human clinical samples and extend my thanks to the laboratory technician for his help during the sample collection.

I would like to thank doctors and compounders from the Government Veterinary Hospital, Edappadi for helping me during the sample collection.

My sincere thanks to Dr. K. Anbarasu, for providing a Fluorescent Microscopy facility for yeast cell analysis.

I am thankful to other research scholars and students from the Department of Microbiology and from the sister neighbor Department research friends for their technical and moral support.

Finally, words are not enough to express my feelings for my family members who work hard and endured a lot for me and for my education. Without their endless encouragement, support, and immense love, I would not have grown into this position. So, I am very much indebted and loveable to them for their unconditional love and affection towards me and my progress.

Abbreviations

5-fluorouracil	……5-FU
Arbitrary units	…….a.u
Area under curve	…….AUC
Atomic force microscopy	……AFM
Bicinchoninic acid	…….BCA
Binding energy	…….BE
Bovine serum albumin	……BSA
Charge transfer resistance	……R_{CT}
Counter electrode	……CE
Cyclic voltammetry	……CV
Deoxyribonucleic acid	……DNA
Dihydrorhodamine	……DHE
Dimethylsolfoxide	……DMSO
Dodecanethiol	……DT
Electrochemical impedance spectroscopy	……EIS
Electron transfer	……ET
Electron volt	…….eV
Ellinghausen-McCullough-Johnson-Harris Medium	…..EMJH
Enzyme-linked immuno-sorbent assay	….ELISA
Glassy carbon electrode	……GCE
Gold nanoparticle	……AuNP
Healthy control	……HC
Heterologous sera	……HES
High osmolarity glycerol	……HOG

Homologous sera	……HS
Immuno-chromatography lateral flow assay	……ICG-LFA
Immunoglobulin G	……IgG
Immunoglobulin M	……IgM
Institutional Ethics Committee	……IEC
Interleukin	……IL
Isoelectric point	…….pI
Kilo Dalton	…...kDa
Limit of detection	……LOD
Limit of quantification	……LOQ
Lipopolysaccharide	.....LPS
Mercaptosuccinic acid	……MSA
Micro capsule agglutination test	……MCAT
microRNA	……miRNA
Microscopic agglutination test	…..MAT
Mitogen activated protein kinase	……MAPK
Multiple cross displacement amplification-lateral flow biosensor	.... MCDA-LFB
Negative control	……NC
Negative predictive value	……NPV
Optical density	……OD
Pathogen associated molecular pattern	……PAMP
Phosphate buffer saline	……PBS
Polyaniline	……PANI
Polymerase chain reaction	……PCR
Positive predictive value	……PPV

Programmed cell death	…….PCD
Reactive oxygen species	…….ROS
Receiver operating curve	…….ROC
Reference electrode	……RE
Ribonucleic acid	……RNA
Saturated calomel electrode	……SCE
Self-assembled monolayer	…….SAM
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis	……SDS-PAGE
Solution resistance	…… R_S
Square wave voltammetry	……SWV
Stress activated protein kinase	……SAPK
Toll like receptor	……TLR
Tumor necrosis factor	……TNF
Water- drop contact angle	……WDCA
Working electrode	……WE
World Health Organization	……WHO
X-ray photoelectron spectroscopy	……XPS
Yeast extract peptone dextrose	…….YEPD
Yeast nitrogen base	……YNB
Zeta potential	……ZP

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

ABSTRACT

Leptospirosis is one of the life threatening infectious diseases in humans and animal husbandry. Rapid point-of-care (POC) assays with minimal sample utilization and low cost are desired in clinical practice. LPS is the major surface antigen, elicits a potent immune response, and the unique antigenic structure and fatty acid composition of LPS aid in the identification at the serovar level. The proposed LPS sensor facilitates the detection of leptospiral LPS concentrations ranging from as low as 10 pM to 1 µM and the optimum concentration for the detection is found to be 100 nM. Upon leptospiral LPS induction miRNA-21 was highly upregulated, this circulating miRNA was used as a biomarker for early leptospirosis detection. The proposed miRNA-based biosensor can detect the miRNA at a very low-level range between 1 aM to 50 nM and the limit of detection was found to be 1.5 aM. The proposed sensor was validated by using structural and electrochemical analysis. Moreover, the developed biosensor is highly selective and clearly discriminates the cross-reactivity. This method is completely a solution-based diagnostic method and therefore it is rapid, simple, and sensitive. Hence this proposed strategy provides a new, ultrasensitive, and rapid point-of-care method for the diagnosis of leptospirosis. Further to analyze the leptospiral LPS mediated pathogenesis in *Saccharomyces cerevisiae.* Leptospiral LPS induces the Hog 1 signaling which is confirmed by phosphorylation of Hog 1 and maintains the cell survival by cellular events such as accumulation of lipid droplets, induction of autophagy related genes, ROS production, and triggering intracellular calcium release. Hence leptospiral LPS is the potential biomarker for diagnosis and study leptospiral pathogenesis.

Key words: Leptospirosis, LPS, biosensor, microRNA, acute diagnosis, electrochemistry, HOG signaling.

CHAPTER I

Introduction

Chapter I: Introduction

This chapter presents the origin of this thesis work and related information in detail. Initially, the introduction part of the thesis deal with background information about leptospirosis and its transmission, its impact on health and economy, and the detection methods available for leptospirosis diagnosis. Further, it deals with the electrochemical detection strategy in human health and its applications. In addition, it specifically describes the definition of the problem, aims, and objective of the thesis work. In the end, it briefly explains the working chapters of the thesis.

1. Introduction

1.1 Leptospirosis

Leptospirosis is an important neglected tropical zoonotic disease, that has worldwide distribution most common in tropical and subtropical regions with high rainfall. *Leptospira* belong to the order Spirochaetales, the family Leptospiraceae, and the genus *Leptospira* (Faine et al., 1999, Levett PN, 2001, Bharti et al., 2003, WHO (2003)). Globally leptospirosis is an important emerging infectious disease affecting 1.03 million cases (95% CI- 4, 34, 000- 1,750,000) per year, from which 58,900 (95% CI- 23,800- 95,900) death occurs (Torgerson, 2015). Leptospirosis has worldwide distribution but is most frequently found in tropics regions like the Caribbean and Latin America, the Indian subcontinent, Southeast Asia, Oceania, and Eastern Europe (Pappas, 2008Incidencesnce range from approximately 0.1 to 1 per 100, 000 per year, in temperate regions is 10 to 100, 000 in the humid tropics.

1.2 Transmission

Transmission of Leptospirosis requires maintaining hosts for their life cycle, rodents were considered primary carriers of leptospires, and cattle and, other domestic animals were widely reported. Humans are usually infected by either direct or indirect contact with the urine of an infected animal. Enter through cuts and abrasions of skin or mucosal surfaces, through conjunctival, or by inhalation of leptospires containing air droplets (Bharti et al., 2003, Haake DA and Levett PN, 2015, Levett PN, 2001). Most the human infections emerged from livestock exposure in tropical and developing countries. Another significant risk factor is associated with exposures occurring through water sources. Many infections result from barefooted walking in damp conditions or gardening with bare hands or drinking contaminated water. Agricultural workers account for more leptospirosis importantly rice field workers often work with barefoot and hands immersed in water for a prolonged period of time (Alavi et al., 2014, Alvarado et al., 2016, Bharti et al., 2003, Haake DA and Levett PN, 2015, Levett PN, 2001, Maze et al., 2018, Natarajaseenivasan et al., 2002 and Sakundarno et al., 2014).

1.3 Clinical features

Human: Leptospirosis has protean clinical manifestations and has similar clinical presentations to other diseases e.g., dengue and other haemorrhagic fever. The clinical presentation of leptospirosis is biphasic with acute or septicemic phase to immune phase. The majority of the cases present with a febrile illness of sudden onset. Other symptoms such as chills, headache, myalgia, abdominal pain, conjunctival suffusion often skin rash to severe with internal organelle impairments such as liver dysfunction, acute renal failure, and haemorrhage (Bharti et al., 2003, Levett PN, 2001 and WHO (2003).

Livestock: Many animals are seronegative carriers, after infection leptospires localized in kidneys and excreted through urine or vaginal discharge. Leptospirosis had a broad spectrum of clinical presentation in cattle. During the acute stage of infection, different symptoms like malaise, depression, anoxia, conjunctivitis, and fever occur. Abortion, stillbirth and mastitis in lactating animals prevail later in the acute stage. If the disease continues, symptoms appear to be more characteristic of leptospirosis, including jaundice, central nervous system (CNS) involvement, liver, and renal failure, etc. (Faine et al., 1999, Lehmann et al., 2014, Wafa et al., 2020).

1.4 Impacts

The estimated annual incidence of human leptospirosis is 1.03 million cases per year, from which 58,900 deaths occur (Costa, 2015). The diagnostic tests incurred costs, between15.3 and 17.2 USD per patient. Pathogenic leptospires are maintained in the renal tubules of certain animals. In dairy cattle, leptospiral infection causes reproductive failure (infertility and abortion) and poor yield of milk (Mori et al., 2017, Yang et al., 2018). In the US alone, an annually estimated loss of USD 7.92 million is reported in beef cattle and sheep industries due to leptospirosis and the vaccination cost of dairy cattle accounts for USD 5.72 million annually (Sanhueza et al., 2020).

1.5 Detection Methods

1.5.1 Culture methods

Leptospires can grow in media containing serum or albumin. Leptospires can be isolated from clinical samples like blood/CSF during the first 7-10 days of illness. The most widely used medium in current practice is Ellinghausen-McCullough-Johnson-Harris (EMJH) with oleic

acid-albumin. Leptospires can grow slowly for 10 to 14 days but live state-maintained for up to 13 weeks (Johnson et al., 1967). Contaminants were prevented/inhibited by the addition of 5-fluorouracil (5-FU). 5-FU is an analog to pyrimidine uracil, inhibiting the growth of many bacteria, by interfering with the biosynthesis of deoxyribonucleic acid, normal ribonucleic acid, and cell wall formation. All leptospires were resistant to 5-FU (Johnson and Rogers, 1964). Culture is difficult, requires several weeks for incubation and specialized media, and has low sensitivity (Bharti et al., 2003).

1.5.2 Molecular methods

The leptospiral genome is approximately 5,000 kb in size and has comprised of two sections (4,400 kb chromosome and 350 kb chromosome). Polymerase Chain Reaction (PCR) is used for direct demonstration of *Leptospira*. PCR can detect the DNA in blood between 5 and 10 days after the onset of the diseasesA real time quantitative PCR using TaqMan chemistry is used to detect leptospires from clinical and environmental samples. This assay is sensitive and can differentiate the pathogenic and non-pathogenic species (Smythe et al., 2002).

1.5.3 Serological methods

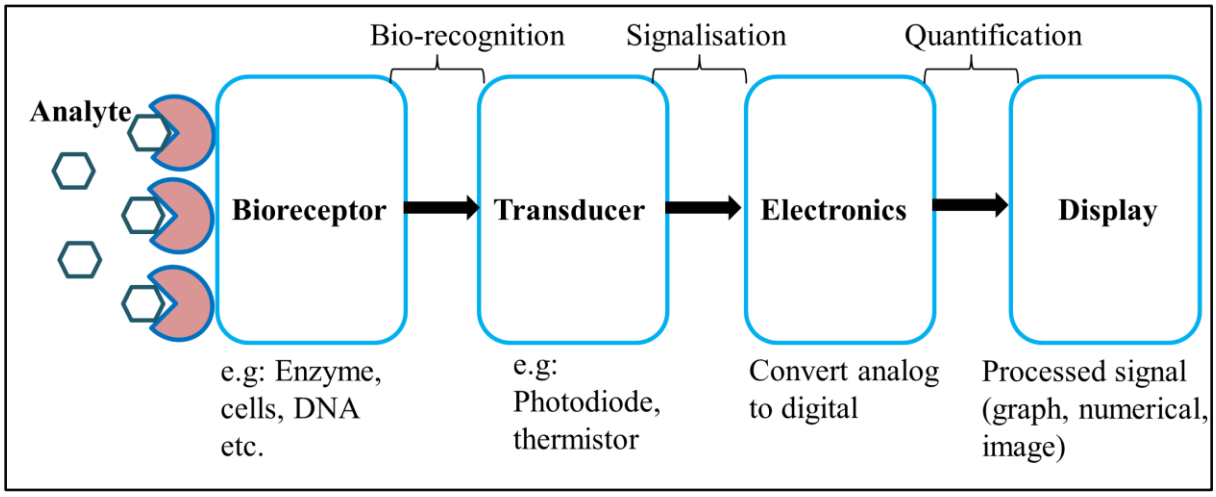
Serological diagnosis is the most frequently used approach for leptospirosis detection. The microscopic Agglutination Test (MAT) is the “gold standard” reference serovar specific test for the detection of leptospires, first introduced by Martin and Pettit in 1918. Live antigens from different leptospiral serogroups are reacted with clinical serum samples. Antibodies are detectable after the onset of symptoms, in blood the detectable antibodies are measured for approximately 5 to 7 days. The agglutination was examined by darkfield microscopy. Although MAT is the gold standard, it is difficult to perform. Also, it involves a large panel of live

leptospiral culture as antigen and moreover needs skilled professional experience and skilled manpower for interpretation of results (Levett, 2001).Enzyme-linked immunosorbant assay (ELISA) detects antibodies from day 6to 8. This method is a broadly reactive genus-specific antigen, thus it is not suitable for serogroup/serovar level identification (Musso and Scola, 2013). A Recent study established the role of circulating stable miRNAs (miR-21-5p, miR-144-3p, and miRlet-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs were able to discriminate acute leptospiral infection from other febrile diseases (Mercy et al., 2020).

1.6 Biosensor

The biosensor is a powerful and innovative analytical device is used in point of care assays like environmental monitoring, food control, drug discovery, and forensics. A biosensor is termed a device that can be used to measure biological or chemical reactions by generating signals proportional to the concentration of an analyte in the reaction. As shown in figure 1.1 typical biosensor consists five parts namely analyte, the substance of interest to be detected; Bioreceptor, a molecule that specificarecognizes the analyte (Antibody, Enzyme, DNA); Transducer, which convert bio-recognition event into another measurable signal either optical or electrochemical; Electronics, where the transduced signal processed and prepares it for display; and finally the display part, which is a user interpretation system. The output signal displayed as graph, number or an image. (Bhalla et al., 2016).

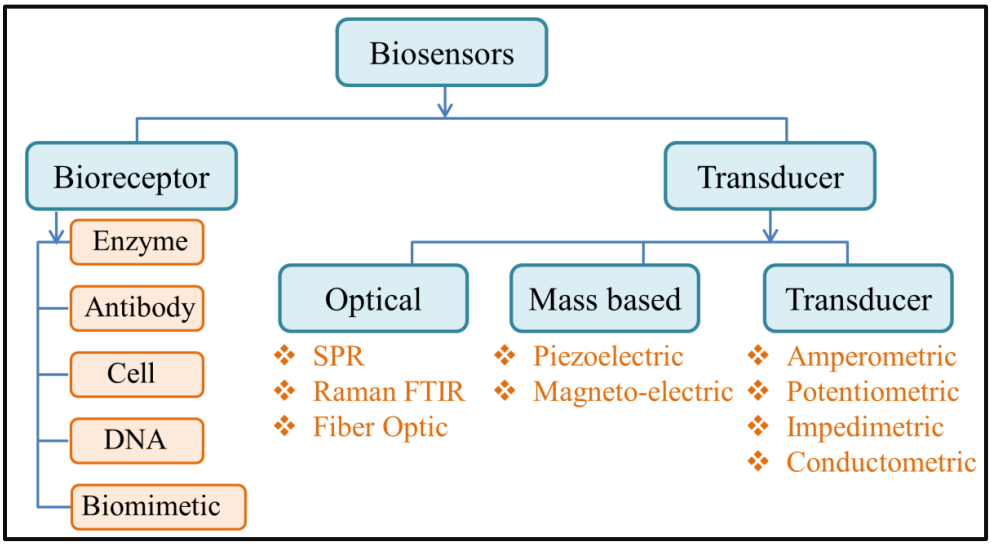
Figure 1.1: Schematic representation of biosensor and its components



1.7 Types of biosensors

Biosensors can be classified based on the Bioreceptor and Transducers. Bioreceptor is a biological recognition element that specifically binds to the target element. Bioreceptors classified into five groups such as Enzyme, antibody/antigen, nucleic acid/DNA, cellular structure/cell, and biomimetic. (Figure 1.2)

Figure 1.2: Classification of biosensor



1.7.1 Enzyme-based biosensors

Enzymes are the widely used bioreceptor because of their specific binding capability and their catalytic activity. The enzymes recognize the target in a similar way to a key fitting a lock. The mechanism for the enzyme-based bioreceptor involves the conversion of analyte into a sensor detectable product, detection of an analyte that acts as an enzyme inhibitor or activator, and evaluation of the modification of enzyme properties upon interaction with the analyte (Karunakaran et al., 2015).

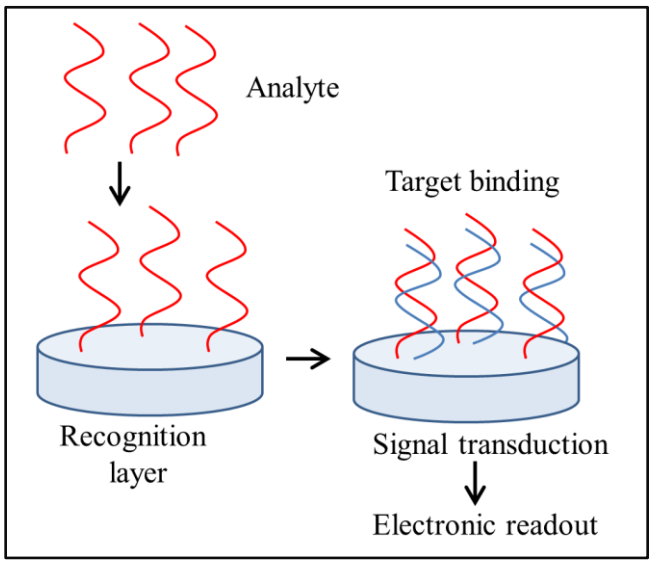
1.7.2 Antibody-based bioreceptors: Immunosensors

Antibody-based bioreceptors used to detect the specific antigen. Immunoassays are the most specific analytical techniques, provide extremely low detection limits. The recognition method is based on the immunoreaction (antigen-antibody interaction). Two types of antibody-based biosensors such as polyclonal and monoclonal. Polyclonal antibodies are less specificity and high sensitivity, they can recognize different types of epitopes. Monoclonal antibodies are identical, produced against to particular type of epitope, and highly specific (Warsinke et al., 2000).

1.7.3 Nucleic acid bioreceptors: Genosensor

Nucleic acid based bioreceptors involve the hybridization of DNA or RNA. A genosensor consists of a substrate modified with probe DNA that can detect complementary DNA sequences (target DNA) through hybridization (Figure 1.3).

Figure 1.3: Principle of genosensor



The target DNA is captured at the recognition layer and the resulting hybridization event is transduced into a usable electric signal (Yang et al., 1997). DNA biosensor provides more specificity towards biosensing applications because of the base pairing interaction with complementary sequences. In electrochemical genosensors the immobilization of DNA probe, electrochemical transduction to detect the hybridization event. Impedance spectroscopy is an electrochemical technique that is used for studying DNA hybridization (Pividori et al., 2000).

1.7.4 Microbial sensors

Microbes have a variety of applications used as biosensing material, able to metabolize a wide range of chemical compounds. Both viable and non-viable cells are used for the fabrication of biosensors. However viable cells attention to fabrication of biosensors, because they metabolise various organic compounds either anaerobically or aerobically resulting in various end products like ammonia, carbon dioxide, acids, etc. that can be monitored using a variety of transducers (D'souza, 2001).

1.8 Classification of biosensors based on transducer

The transducer is an important component of the biosensor that converts biorecognition event into a detectable signal. The detectable signal can be optical (colorimetric, fluorescence, luminescence, and interferometry), electrochemical (potentiometry, conductometry, impedimetry, amperometry, and voltammetry), and mass change (piezoelectric) (Karunagaran et al., 2015)

1.8.1 Optical biosensors

Optical biosensors are analytical devices in which the biorecognition element is integrated into the optical transducer system. Optical sensors detect biological interactions by evaluating the variations induced in the light properties, such as intensity, wavelength, refractive index, or polarization (Sang et al., 2016). It uses various substance biological materials like enzymes, antibodies, nucleic acids, whole cells, receptors, and tissues. Surface plasmon resonance (SPR), evanescent wave fluorescence, and optical waveguide interferometry utilize the evanescent field in close proximity to the biosensor surface to detect the interaction of the biorecognition element with the analyte (Damborsky et al., 2016).

1.8.2 Mass based biosensors

The working principles of mass based biosensors involve responding to a change in the mass of the substance. Mass measurements are associated with a diverse collection of interfacial processes, including chemical and biological sensors, reactions catalyzed by enzymes immobilized on surfaces, electron transfer at and ion exchange in thin polymer films, and doping reactions of conducting polymers (Ward and Buttry, 1990). The advantages of optical biosensors

are electrochemically inactive elements also be sensed and varying wavelengths of the light multiple components can be sensed on a single layer.

1.8.3 Electrochemical biosensors

An electrochemical biosensor contains an electrochemical transducer, measures the current produced from the oxidation and reduction reactions. They can achieve direct conversion of the biological event into an electrochemical signal. These rely on the detection of an electrical property such as resistance, current, potential, capacitance, impedance, and the electrical property are detected and measured using different methods such as potentiometry, conductometry, amperometry, or voltammetry. The principle of the electrochemical biosensor is based on the chemical potential of particular species in solution or the analyte, as measured by comparison to a reference electrode. The current produced from the reaction is related to either the concentration of the electroactive species present or its rate of production/consumption (Karunagaran et al., 2015). Based on the electrical properties the electrochemical sensors are classified as follows:

(a) Potentiometric biosensors: It measures the charge accumulated due to the analyte and bioreceptor interaction at the working electrode relative to the reference electrode under zero current.

(b) Amperometric biosensors: They can be operated in two or three electrode cconfigurations. It measures the current produced due to electrochemical oxidation or reduction of electroactive species at the working electrode when a constant potential is applied to the working electrode with respect to the reference electrode. The current

produced on the working electrode surface is proportional to the analyte concentration present in the solution.

(c) Conductometric biosensors: It quantifies the change in the conductance between the pair of electrodes because of an electrochemical reaction (change in conductivity properties of the analyte). It is used to monitor metabolic processes in living biological systems.

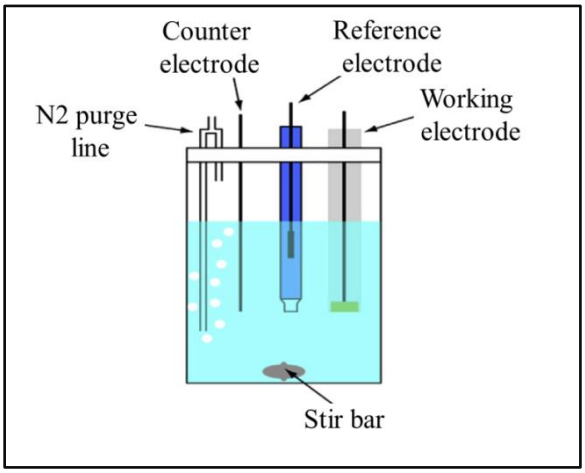
(d) Impedimetric biosensors: Impedimetric biosensors measure the electrical impedance produced at the electrode/electrolyte interface when a small sinusoidal excitation signal is applied. It involves the application of low amplitude AC voltage at the sensor electrode and then the in/out-of-phase current response is measured as a function of frequency using an impedance analyzer

(e) Voltammetric biosensors: Voltammetric biosensors detect analytes by measuring the current during the controlled variation of the applied potential. Voltammetric biosensors are highly sensitive measurements and simultaneous detection of multiple analytes.

1.9 Electrochemical cell

The electrochemical cell, where the experiments are carried out (measure current/potential), consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE) (Karunakaran et al., 2015) (Figure 1.4).

Figure 1.4: Typical electrochemical cell



1.9.1 Working electrode

The reaction of interest is taking place in the working electrode. The WE can be either cathodic (whether the reaction is reduction) or anodic (whether the reaction is oxidation). The different kinds of working electrodes are glassy carbon electrode, Pt electrode, gold electrode, mercury electrode, screen-printed electrode, silver electrode, Indium tin oxide-coated glass electrode, carbon paste electrode, etc. (Zhou et al., 2010).

1.9.2 Reference electrode

The RE is used to produce a constant potential at the electrochemical cell (Inzelt et al., 2013). The reference electrode can minimize the electrode potential changes upon passage of current in the electrode

Reference electrodes are constructed using half-cell components that are stable over time and, with changing temperature, present at well-defined values of activity. The most frequently used reference electrode is the standard hydrogen electrode (SHE), composed of inert solid-like

platinum on which hydrogen gas is adsorbed, immersed in a solution containing hydrogen ions at unit activity. The half-cell reaction is denoted as

$$2H^+ (aq) + 2e^- \longrightarrow H_2(g)$$

The saturated calomel electrode (SCE) has the half-cell potential of E_0= +0.241 V. The Ag/AgCl electrode has the half-cell potential of E_0= +0.197 V. Another major RE is the silver/silver chloride electrode (Ag/AgCl), with potential determined by the reaction.

$$AgCl_{(s)} + e^- \longrightarrow Ag_{(s)} + Cl^-$$

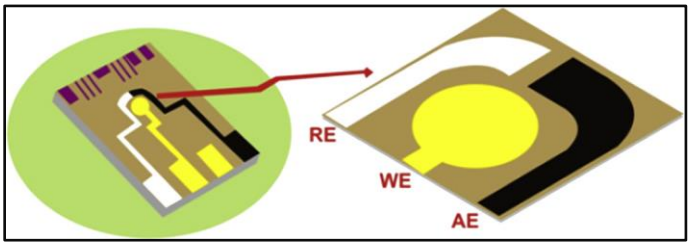
1.9.3 Counter electrode

The CE (auxiliary electrode) is an electrode used to close the current circuit in the electrochemical cell. It is usually made of an inert material (e.g., platinum) and it does not participate in the electrochemical reaction (Thomas and Henze, 2001). The counter electrode functions as a cathode whenever the working electrode is an anode and vice versa.

1.9.4 Lab-on-a-chip

A lab-on-chip (LOC) is a device that integrates several functions in single chip of millimeters to a few square centimeters in size. It is used to analyse extremely small volumes less than pico liters (Figure 1.5).

Figure 1.5: Schematic view of the lab-on-chip model biosensor



Legends: *RE- Reference Electrode, WE- Working Electrode and AE- Auxiliary Electrode*

1.10 Characteristics of a biosensor

A successful biosensor has essential characteristics including selectivity, reproducibility, stability, sensitivity, and linearity (Bhalla et al., 2016). Optimization of these properties is reflected in the performance of the biosensor

1.10.1 Selectivity

Biosensor selectivity depends on the bioreceptor's ability to detect a specific analyte in a sample. The best example is the interaction of antigen with the specific antibody. It is the main characteristic when choosing the bioreceptor in a biosensor platform.

1.10.2 Reproducibility

Reproducibility is that the biosensor can generate identical responses at each time using the same experimental setup. It is characterized by the precision and accuracy of the transducer and electronics in a biosensor.

1.10.3 Stability

Stability is another most crucial feature in biosensor applications where a biosensor requires long incubation steps. The transducers and electronics responses can be temperature-sensitive,

which may influence the stability of a biosensor. So better electronics ensure the stability of the sensor.

1.10.4 Sensitivity

The limit of detection (LOD) or sensitivity is the minimum amount of analyte that can be detected by a biosensor. A biosensor is required to detect analyte concentrations of as low as fg/ml to confirm the analytes in a sample.

1.10.5 Linearity

Linearity is the attribute that the accuracy of the measured response to a straight line. Which is mathematically represented as y=mc, where c is the concentration of the analyte, y is the output signal, and m is the sensitivity of the biosensor. Another way it is associated with the range of analyte concentrations for which the biosensor response changes linearly with respect to the concentration.

1.11 Definition of the problem

Leptospirosis is a re-emerging zoonotic disease, that has been associated with environmental factors including rainfall, flooding, poverty, urbanization, and ecotourism. Leptospirosis frequently leads to mortality, especially in patients with delayed diagnosis and/or patients improperly treated. A problem with developed diagnostic methods is the low sensitivity and specificity during the acute phase of illness since specific antibody responses become detectable

only 4 to 7 days after infection. MAT is the only reference test used for serovar specific diagnosis of leptospirosis. But it requires a live panel of antigens all the time and time consuming. However, PCR based antigen detection methods from blood and tissue can be affected by inhibitory substances of the samples. It is evident that leptospiral lipopolysaccharide (LPS) based diagnosis offers serovar specific detection of leptospirosis. The unique fatty acid profiling in the LPS structure helps to differentiate leptospiral serovar. With this background, it is believed that the development of a rapid, serovar specific electrochemical biosensor will be an immediate requirement to decrease morbidity and mortality during an outbreak situation.

1.12 Aim and Scope of the thesis

The prime aim of the thesis work is to develop the lipopolysaccharide based electrochemical biosensor for rapid, serovar specific detection of leptospirosis using SAM (Self assembled monolayer; 1 do-decanethiol) modified gold electrode. The role of modified electrode surface in terms of structural and electrochemical properties where analysed. To investigate the different leptospiral serovar LPS mediated pathogenesis in Hog1 signaling using *Saccharomyces cerevisiae* as the model organism. Based on these, the objectives of the present thesis work were framed as summarized below.

1.13 Objectives

I. Prevalence of leptospirosis among human and bovine clinical cases

- Collection of clinical samples from clinically suspected cases and healthy controls
- Leptospirosis confirmation by Microscopic Agglutination Test (MAT)
- Risk factor analysis for leptospirosis seroprevalence

II. Evaluation of leptospiral LPS as a potential biomarker for serogroup specific diagnosis of leptospirosis

- Extraction and quantification of leptospiral lipopolysaccharide
- Reactivity of leptospiral LPS by Immunoblotting
- Evaluation of LPS based IgM/IgG ELISA and Dot Blot Assay

III. Development and evaluation of leptospiral LPS imprinted electrochemical biosensor for early diagnosis

- Construction of biosensor matrix
- Structural and functional characterization of developed biosensor
- Electrochemical characterization of LPS based biosensor using clinical samples

IV. Development of ultralow level miRNA based biosensor for leptospirosis detection

- Fabrication of biosensor platform
- Specificity of the biosensor by using different miRNA sequences
- Real sample analysis of the fabricated biosensor

V. Establishment of leptospiral LPS mediated pathogenesis in the HOG signaling pathway

- Activation of Hog1 upon exposure to leptospiral LPS
- Hog1 mediated cellular events upon leptospiral LPS induction
- Leptospiral LPS mediated programmed cell death in *Saccharomyces cerevisiae*

1.14 Layout of the dissertation

This thesis work outlines the development of LPS based electrochemical biosensor for early detection of leptospirosis and analysis of leptospiral LPS mediated pathogenesis in *Saccharomyces cerevisiae* as a model organism.

Chapter I present the background information on leptospirosis, the impact of leptospirosis on human and animal health, and diagnostic methods available for leptospirosis detection. Then it deals with the definition of biosensors and the working principles of different types of a biosensor. Finally, it describes the motivation and scope of this thesis.

Chapter II deals with the theoretical background of experimental details and experimental methodology adopted in this thesis.

Chapter III to determine the seroprevalence of leptospirosis among human and bovine clinical cases in the study population using leptospirosis confirmatory test MAT. Further to analyze the risk factor for the prevalence of leptospirosis in the study area.

Chapter IV deals with the evaluation of the leptospiral lipopolysaccharide as a potential biomarker for serovar specific diagnosis of leptospirosis using serological methods like Enzyme-linked immune sorbent assay (ELISA) and dot blot assay. Both IgM and IgG-based serological diagnosis were performed to determine the serovar specificity.

Chapter V, we tried to develop a leptospiral LPS imprinting based electrochemical biosensor for rapid diagnosis of leptospirosis. Initially, we form the self-assembled monolayer using 1-dodeanethiol on the gold electrode surface. Then the SAM modified surface was functionalized with leptospiral LPS, followed by the identification of specific circulating anti-leptospiral antibodies in the clinical cases. Each addition or formation of each monolayer was confirmed by structural characterization as well as electrochemical characterization.

Chapter VI comprises the fabrication of a biosensor for sensing of ultralow level detection of miRNA in leptospirosis infected serum samples. This was achieved by modification of the GCE surface with PANI and gold nanoparticles. Then the sensor was functionalized with thiolated

probe DNA, which is complementary to the target miRNA-21. All these modifications were confirmed by electrochemical analysis.

Chapter VII discusses the role of leptospiral LPS in *S. cerevisiae* Hog1 signaling cascade and its survival mechanism upon exposure to the various leptospiral lipopolysaccharide in comparison with positive control 0.8 M NaCl. The phosphorylation of stress activated kinase during exposure to LPS provides insight and knowledge about the organization of cellular metabolic products and cell survival during stress conditions.

Chapter VIII describes the overall summary and conclusion of the thesis.

1.15 References

1. Akino Mercy CS, Suriya Muthukumaran N, Velusamy P, Bothammal P, Sumaiya K, Saranya P, Langford D, Shanmughapriya S, Natarajaseenivasan K. MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis. mSphere. 2020 Jul 15;5(4):e00409-20. doi: 10.1128/mSphere.00409-20.
2. Alavi SM, Khoshkho MM. Seroprevalence study of leptospirosis among rice farmers in khuzestan province, South west iran, 2012. Jundishapur J Microbiol. 2014 Jul;7(7):e11536. doi: 10.5812/jjm.11536.
3. Alvarado-Esquivel C, Sánchez-Anguiano LF, Hernández-Tinoco J, Ramos-Nevárez A, Margarita Cerrillo-Soto S, Alberto Guido-Arreola C. Leptospira Exposure and Patients with Liver Diseases: A Case-Control Seroprevalence Study. Int J Biomed Sci. 2016 Jun;12(2):48-52.
4. Bhalla N, Jolly P, Formisano N, Estrela P. Introduction to biosensors. Essays Biochem. 2016 Jun 30;60(1):1-8. doi: 10.1042/EBC20150001.
5. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM; Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003 Dec;3(12):757-71. doi: 10.1016/s1473-3099(03)00830-2.
6. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM; Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003 Dec;3(12):757-71. doi: 10.1016/s1473-3099(03)00830-2.

7. Damborský P, Švitel J, Katrlík J. Optical biosensors. Essays Biochem. 2016 Jun 30;60(1):91-100. doi: 10.1042/EBC20150010.
8. D'Souza SF. Microbial biosensors. Biosens Bioelectron. 2001 Aug;16(6):337-53. doi: 10.1016/s0956-5663(01)00125-7.
9. Faine S, Adler B, Bolin C, Perolat P. "Leptospira" and leptospirosis. Melbourne Vic Australia: MediSci, 1999. 295 p.
10. Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015;387:65-97. doi: 10.1007/978-3-662-45059-8_5.
11. Inzelt G, Lewenstam A, Scholz F, editors. Handbook of reference electrodes. Berlin: Springer; 2013 Apr 16.
12. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic letospires. I. Growth at low temperatures. J Bacteriol. 1967 Jul;94(1):27-31. doi: 10.1128/jb.94.1.27-31.1967.
13. Johnson RC, Rogers P. Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. J Bacteriol. 1964 Dec;88(6):1618-23. doi: 10.1128/jb.88.6.1618-1623.1964.
14. Karunakaran C, Rajkumar R, Bhargava K. Introduction to biosensors. InBiosensors and bioelectronics 2015 Jan 1 (pp. 1-68). Elsevier.
15. Lehmann JS, Matthias MA, Vinetz JM, Fouts DE. Leptospiral pathogenomics. Pathogens. 2014 Apr 10;3(2):280-308. doi: 10.3390/pathogens3020280.
16. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001 Apr;14(2):296-326. doi: 10.1128/CMR.14.2.296-326.2001.
17. Maze MJ, Cash-Goldwasser S, Rubach MP, Biggs HM, Galloway RL, Sharples KJ, Allan KJ, Halliday JEB, Cleaveland S, Shand MC, Muiruri C, Kazwala RR, Saganda W,

Lwezaula BF, Mmbaga BT, Maro VP, Crump JA. Risk factors for human acute leptospirosis in northern Tanzania. PLoS Negl Trop Dis. 2018 Jun 7;12(6):e0006372. doi: 10.1371/journal.pntd.0006372.

18. Musso D, La Scola B. Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect. 2013 Aug;46(4):245-52. doi: 10.1016/j.jmii.2013.03.001.
19. Natarajaseenivasan K, Boopalan M, Selvanayaki K, Suresh SR, Ratnam S. Leptospirosis among rice mill workers of Salem, South India. Jpn J Infect Dis. 2002 Oct;55(5):170-3.
20. Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis. 2008 Jul;12(4):351-7. doi: 10.1016/j.ijid.2007.09.011.
21. Pividori MI, Merkoçi A, Alegret S. Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods. Biosens Bioelectron. 2000 Aug;15(5-6):291-303. doi: 10.1016/s0956-5663(00)00071-3.
22. Sakundarno M, Bertolatti D, Maycock B, Spickett J, Dhaliwal S. Risk factors for leptospirosis infection in humans and implications for public health intervention in Indonesia and the Asia-Pacific region. Asia Pac J Public Health. 2014 Jan;26(1):15-32. doi: 10.1177/1010539513498768.
23. Sang S, Wang Y, Feng Q, Wei Y, Ji J, Zhang W. Progress of new label-free techniques for biosensors: a review. Crit Rev Biotechnol. 2016;36(3):465-81. doi: 10.3109/07388551.2014.991270.
24. Sanhueza JM, Baker MG, Benschop J, Collins-Emerson JM, Wilson PR, Heuer C. Estimation of the burden of leptospirosis in New Zealand. Zoonoses Public Health. 2020 Mar;67(2):167-176. doi: 10.1111/zph.12668.

25. Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, McKay DB. A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. BMC Infect Dis. 2002 Jul 8;2:13. doi: 10.1186/1471-2334-2-13.
26. Thomas FG, Henze G. Introduction to voltammetric analysis: theory and practice. Csiro Publishing; 2001.
27. Wafa EI, Wilson-Welder JH, Hornsby RL, Nally JE, Geary SM, Bowden NB, Salem AK. Poly(diaminosulfide) Microparticle-Based Vaccine for Delivery of Leptospiral Antigens. Biomacromolecules. 2020 Feb 10;21(2):534-544. doi: 10.1021/acs.biomac.9b01257.
28. Ward MD, Buttry DA. In situ interfacial mass detection with piezoelectric transducers. Science. 1990 Aug 31;249(4972):1000-7. doi: 10.1126/science.249.4972.1000.
29. Warsinke A, Benkert A, Scheller FW. Electrochemical immunoassays. Fresenius J Anal Chem. 2000 Mar-Apr;366(6-7):622-34. doi: 10.1007/s002160051557.
30. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control. World Health Organization; 2003.
31. Yang M, McGovern ME, Thompson M. Genosensor technology and the detention of interfacial nucleic acid chemistry. Analytica Chimica Acta. 1997 Jul 21;346(3):259-75.
32. Zhou Y, Qin ZY, Li L, Zhang Y, Wei YL, Wang LF, Zhu MF. Polyaniline/multi-walled carbon nanotube composites with core–shell structures as supercapacitor electrode materials. Electrochimica Acta. 2010 Apr 30;55(12):3904-8.

Techniques and methods

CHAPTER II

Chapter II: Experimental Techniques and Methodologies

This chapter describes a brief explanation of the basic principle and theoretical background of various techniques or methodologies used for forthcoming chapters. The methods will be described in this section divided into three sections. 1) Serological methods like Microscopic Agglutination Test (MAT), Enzyme-Linked Immuno-sorbent Assay (ELISA), and Dot Blot Assay. 2) Structural characterization such as Atomic Force Microscopy (AFM), X-ray Photoelectron Spectroscopy (XPS), Zeta potential, and Contact angle measurement analysis. 3) Electrochemical techniques including Cyclic Voltametry (CV), Electrochemical impedance spectroscopy (EIS), and Square Wave Voltametry (SWV).

2. Leptospirosis diagnostic methods

2.1 Serological methods

Leptospirosis is difficult to distinguish from a number of other diseases because of its clinical symptoms similar to other febrile cases like dengue, typhoid, and malaria. Generally, seroconversion or a four-fold or higher rise in titre in consecutive serum samples is considered to be diagnostic proof of recent or current infection. Antibodies may be genus-specific, serovar-specific, or serogroup-specific to react with antigens. In general, serological tests, a patient's serum is brought into contact with an antigen. The antigen may be live leptospires or other extracts of leptospires (Protein, LPS, peptide, etc.). The detection of titre will depend on the relative concentrations and strength of the reactions between antibodies and antigens. The titre is also sometimes called "positive titre" or "significant titre", if it is above the cut-off point. The cut-off point is determined based on a comparison of the results obtained on culture-positive leptospirosis cases with the serological results from a group of patients with other confirmed

diseases. The titre of antibodies gradually increases during the disease, peaks, and then decreases after recovery. The most used serological methods are MAT, ELISA, and dot blot assay.

2.1.1 Microscopic Agglutination Test

MAT is considered the "gold standard" of test for serodiagnosis because of its serovar/serogroup specificity in comparison with other currently available tests. The MAT is used to determine the agglutinating antibodies in the serum by mixing it in various dilutions with live or killed, formolized leptospires (Goris and Hartskeerl, 2014). Circulating anti-leptospiral antibodies present in the patient's serum cause leptospires to stick together and form clumps. This clumping process is called agglutination and is observed under dark-field microscopy. Agglutinating antibodies will be in both IgM and IgG classes.

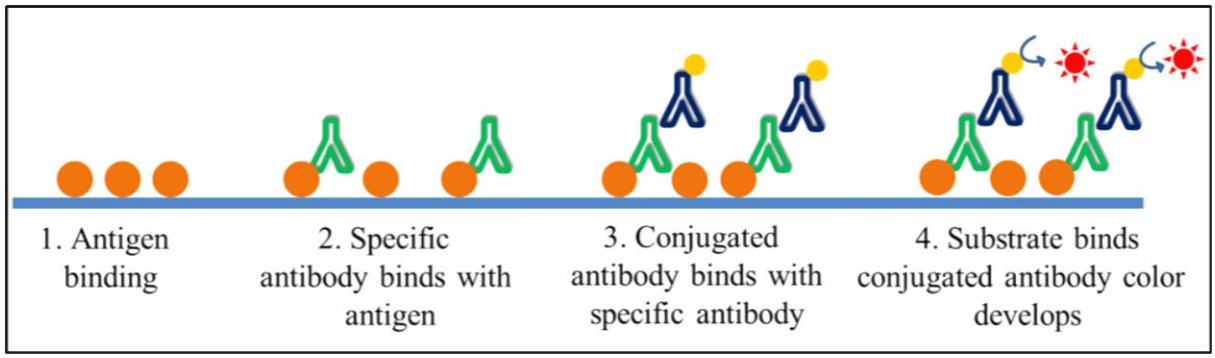
Because of the high specificity, it can be used as a reference test. The major disadvantage is the need for facilities to culture and maintain panels of live leptospires. It is time consuming when a large panel number of leptospires is used. Sometimes antibodies may not be detectable when the causative strain is not represented in the panel or only a low titre is found with a serovar that antigenically resembles the absent causative serovar. Sometimes newly undetermined serovars cause disease, for this reason, it is advisable to include a genus specific screening test such as an ELISA using a broadly reactive antigen (WHO, 2003).

2.1.2 Enzyme Linked Immuno-sorbant Assay (ELISA)

ELISAs are a popular and routine method used for leptospirosis detection. The overall methodology and principle were described in figure 2.1. It can be performed with commercial kits or with antigens produced in house. A broadly reactive genus-specific antigen is generally used to detect IgM, and sometimes IgG antibodies. The presence of IgM antibodies may indicate

current or recent leptospirosis, but it should be remembered that IgM-class antibodies may remain detectable for several years. Genus-specific tests tend to be positive earlier in the disease than the MAT (Palaniyappan et al., 2004; Terpstra et al., 1985).

Figure 2.1 Schematic representation of ELISA and its principle



ELISA standardized method can detect IgM-class antibodies in the early phase of the disease. Only a single antigen is used, namely the genus-specific antigen, which is used to detect both pathogenic and saprophytic leptospires. It is used as commercial kits in remote settings were laboratory facility not available. However, ELISA is less specific than the MAT and weak cross reactions due to the presence of other diseases may be observed. Since it is based on a genus-specific antigen, the ELISA test does not provide information about the infecting serovar (Cumberland et al., 1999; WHO, 2003).

2.1.3 Dot Blot Assay

The Dot Blot test or dot-blot ELISA, dot immunobinding assay, is based on the sensitization of the nitrocellulose membranes (NM) with specific antigens, then subjecting them to the reaction with the primary and secondary antibody usually labeled with peroxidase to develop coloring upon interact the substrate. The principle is similar to ELISA, with the additional advantages of

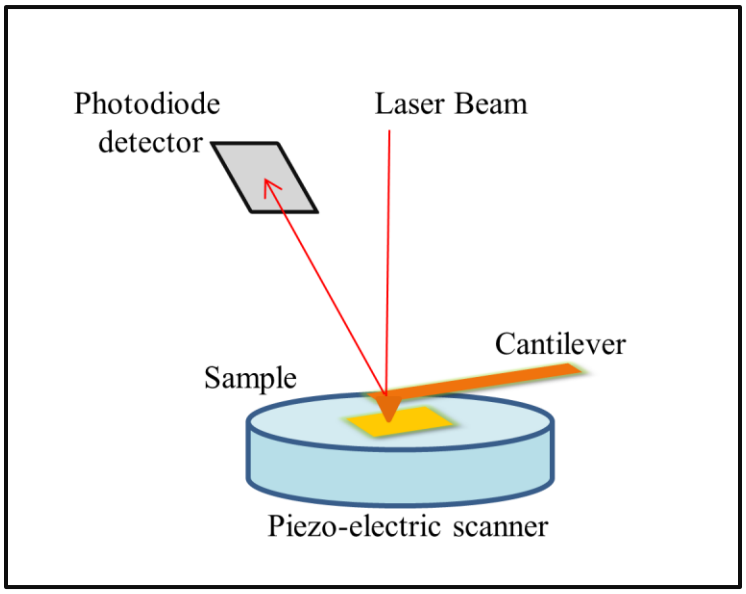
high sensitivity, convenience, and low cost, and does not require sophisticated equipment (Pinheiro et al., 2006).

2.2 Structural Characterization Methods

2.2.1 Atomic Force Microscopy

Atomic force microscope (AFM) is a kind of scanning probe microscopy (SPM) where we can measure the local properties of materials such as height, and friction using a probe. And it can be useful to analyze the surface topographical features of a rigid material, from nanometer to atomic level. AFM uses a mechanical probe to magnify surface features up to 100,000,000 times, and it produces 3D images of the surface (Vahabi et al., 2013). The AFM technique was introduced in 1986 by Binning, Quate, and Gerber with the invention of the scanning tunneling microscope (STM) (Binnig et al., 1986). The major capability of AFM is also to measure non-conductive samples (Maver et al., 2016).

Figure 2.2: Atomic Force Microscopy principle



The principle of AFM is based on the detection of forces acting between a sharp probe and the surface of the sample. The sharp probe known as the AFM tip/AFM sensor, is attached to a flexible cantilever (Figure 2.2). When performing AFM, a tip attached to a flexible cantilever moves across the surface of interest, measuring the surface morphology on the atomic scale. The force between the tip and the sample is measured during scanning, by monitoring the deflection of the cantilever (Cohen et al., 1997, Vahabi et al., 2013).

During the past decade, the AFM has emerged as a powerful tool to obtain the nano structural details and biomechanical properties of biological samples, including biomolecules and cells. Nowadays AFM technique can analyze any kind of sample such as polymers, adsorbed molecules, films or fibers, and powders in the air whether in a controlled atmosphere or in a liquid medium (Vahabi et al., 2013).

2.2.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is generally regarded as an important and key technique for the surface characterization and analysis of biomedical polymers (Keller et al., 1980). XPS technique, also called ESCA (Electron Spectroscopy for Chemical Analysis), provides a total elemental analysis, except for hydrogen and helium, of the top 10–200 Å of any solid surface which is vacuum stable or can be made vacuum stable by cooling (Andrade, 1985).

Studies reported that surface analysis by XPS is accomplished by irradiating a sample with monoenergetic soft x-rays and analyzing the energy of the detected electrons, these photons have limited penetrating power in a solid (1-10 micrometers). They interact with atoms in the surface region, causing electrons to be emitted by the photoelectric effect.

The kinetic energy of emitted electrons as given by

$$KE = h\nu - BE - \phi_s$$

hν is the energy of the photon, BE is the binding energy of the atomic orbital from which the electron originates, and ϕs is the spectrometer work function. Binding energy is referred to as the energy difference between the initial and final states after the photoelectron has left the atom. XPS is also used to identify and determine the concentration of elements on the surface. Variations in the elemental binding energies (the chemical shifts) arise from differences in the chemical potential and polarizability of compounds. These chemical shifts can be used to identify the chemical state of the materials of interest. In addition to photoelectrons emitted in the photoelectric process, Auger electrons are emitted because of the excited ions remaining after photoemission (Chastain and King, 1992).

2.2.3 Zeta potential (ZP)

The biomolecules are adsorbed into the surface through complex mechanisms that comprise electrostatic, van der Waals, hydrophobic and steric interactions, etc. The most important adsorption mechanism is electrostatic interactions. Zeta potential or electrokinetic potential is a measure of the magnitude of electrostatic interactions between charged surfaces (Salgin et al., 2012). In general, when two phases are placed in contact, potential differences occur. The important properties of colloidal systems are determined directly or indirectly by the electrical charge (potential) on the particles (Hunter, 2013). It is the potential at the slipping/shear plane of a colloid particle moving under an electric field. Its measurement brings detailed insight into the dispersion mechanism and colloidal stability of biomolecules. The zeta potential represents the surface charge which occurs in the presence of an aqueous solution when functional groups

dissociate on the surface or ions adsorb onto surfaces from the solution. The ZP reflects the potential difference between the EDL (electric double layer) of electrophoretically mobile particles and the layer of dispersant around the slipping plane (Bhattacharjee, 2016). The zeta potential ranged between -10 and +10 mV is considered neutral, +30 mV is considered strongly cationic, and -30mV is considered strongly anionic respectively (Clogston and Patri, 2011).

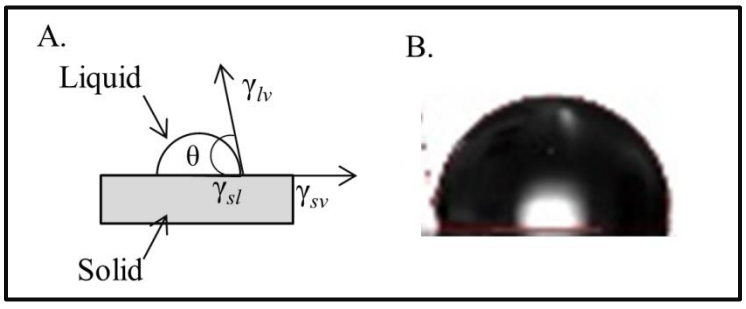
2.2.4 Contact angle measurement analysis

Contact angle measurement is used performed by establishing the tangent (angle) of a liquid drop with a solid surface at the base. The attractiveness of using contact angles is used to estimate the solid vapor and solid liquid interfacial tensions (Kwok and Neumann, 1999). Young in 1805 first time estimates the solid surface tensions from contact angle analysis. The contact angle of liquid drop in the solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions such as solid-vapor (γ_{sv}), and solid-liquid (γ_{sl}), and liquid-vapor (γ_{lv}).

The Young's equation:

$$\gamma_{lv} \cos\Theta_Y = \gamma_{sv} - \gamma_{sl}$$

Figure 2.3: Schematic of a sessile-drop contact angle system (A) and contact angle for DT monolayer (B).



Contact angle experiments were performed using automated axisymmetric drop shape analysis-profile (ADSA-P). ADSA-P is used to determine liquid-fluid interfacial tensions and contact angles from the shape of axisymmetric menisci, i.e., from sessile as well as pendant drops (Figure 2.3) (Kwok et al., 1997).

2.3. Electrochemical studies

Electrochemistry is the study of electricity production from the energy released during the spontaneous chemical reactions and the use of electrical energy to bring about non-spontaneous chemical transformations. The electrode reactions occur between the electric conductor (electrode) and ionic conductor (electrolyte) interface, where the charge distribution differs from the bulk solution. The process is influenced the by structure of the interface region, and the nature of electric and ionic conductors.

2.3.1 Experimental setup

All the experiments were performed using three electrode systems consisting of a working electrode (Gold Disc Electrode), a reference electrode (Saturated Calomel Electrode (SCE)), and an auxillary (counter) electrode (Platinum wire). The electrochemical properties of the monolayer towards the electron transfer process were studied using [Fe(CN)6]3-/4- redox couple as a probe with a supporting electrolyte (NaF). All these electrochemical studies were performed with an Autolab PGSTAT 302 instrument (Metrohm, Autolab-Potentiostat/Galvanostat Instruments (PGSTAT302N), Netherlands) with General Purpose Electrochemical System (GPES 4.9) software.

2.3.2 Fabrication of working electrode

The working electrode is an electrically conductive substrate where oxidation or reduction takes place by applying an electromotive force. It is referred to as the sensor platform. The gold electrode is resistant to oxidation in acid and air. They are widely used in standard electrochemistry cells and used to investigate the oxidation and reduction potentials from organic and inorganic semiconductors. And the gold disc working electrodes are easy to use and clean. It has been very useful, however, for the preparation of modified electrodes containing surface structures known as self-assembled monolayers (SAMs).

Cleaning of the gold electrode

The cleanliness of the sensor surface is important for determining how an electron transfer reaction proceeds. The gold electrode is pre-treated by electrochemical oxidation/ reduction using 0.5 M $HClO_4$, between -0.2 V and +1.5 V for 10 cycles with a scan rate of 0.5 V per second. Then the electrode was rinsed with milli-Q water (having 18.2 Ω resistivity) and ethanol by ultra-sonication for 5 minutes. After this cleaning process, the pre-treated gold electrode was scanned for 10 cycles to obtain a stable cyclic voltammogram. The degree of activation gold electrode can be evaluated by observing a peak potential difference (ΔE_P) using the redox couple consisting of 5mM Ferro/ferricyanide in 0.1 M NaF as the supporting electrolyte.

2.3.3 Electrochemical techniques

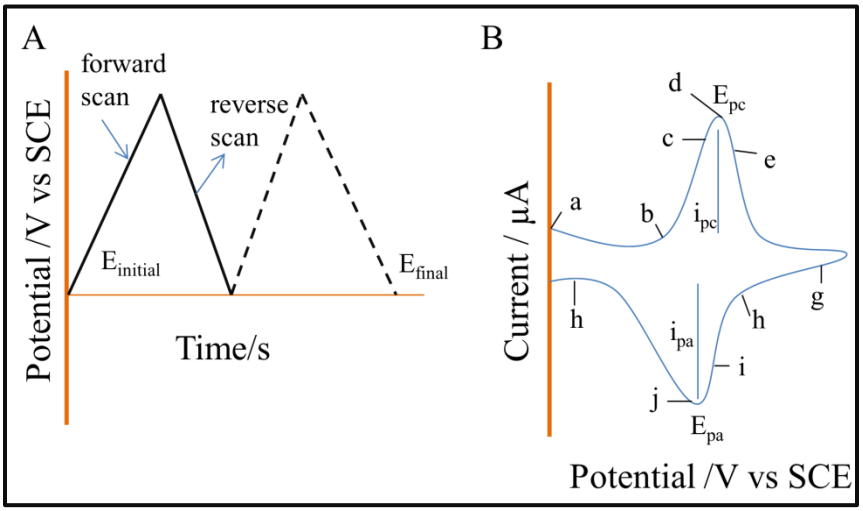
2.3.3.1 Cyclic Voltammetry

Cyclic voltammetry (CV) is the most versatile electroanalytical technique for studying the electroactive species (Heinze, 1984; Mabbott, 1983). It is often performed as the first experiment

in an electrochemical study of a compound, a biological material, or an electrode surface. It has the capability to rapidly observe redox behavior over a wide potential range. A CV consists of cycling the potential of an electrode in an unstirred solution and measuring the resulting current. The potential of this working electrode is controlled versus a reference electrode (SCE). The controlling potential applied across the two electrodes can be considered an excitation potential (Kissinger and Heineman, 1983).

The excitation signal causes to scan of the potential range between +0.80 to -0.20 V versus the SCE, at which the scan direction is reversed, causing a positive scan back to the original potential of +0.80 V. The scan rate is 50mV/s (Figure 2.4 A).

Figure 2.4: (A) An illustration of the triangular potential-wave form of cyclic voltammetry; (B) the corresponding cyclic voltammogram.



The cyclic voltammogram is obtained by measuring the current at the working electrode during the potential scan. The voltammogram display is a current (vertical axis) versus potential (horizontal axis) (Figure 2.4 B). The electrode is switched on at the initial potential (E_i) of 0.80 V and is applied (a) to avoid any electrolysis of $Fe(CN)_6^{3-}$. When the potential is sufficiently

negative to reduce $Fe(CN)_6^{3-}$, the cathodic current is indicated as (b). The electrode reduction process is

$$Fe^{III}(CN)_6^{3-} + e \longrightarrow Fe^{II}(CN)_6^{4-}$$

After reducing of $Fe^{III}(CN)_6^{3-}$, the cathodic current increases sharply until the concentration of $Fe^{III}(CN)_6^{3-}$ diminish at the electrode surface (d). The current then decreases (d-g) as the solution surrounding the electrode is depleted of $Fe^{III}(CN)_6^{3-}$ due to its electrolytic conversion to $Fe^{II}(CN)_6^{4-}$. So, the scan direction switched to positive at -0.15 V (f) for the reverse scan. When the electrode becomes a sufficiently strong oxidant, $Fe^{II}(CN)_6^{4-}$, and accumulated adjacent to the electrode. The electrode oxidation process is

$$Fe^{II}(CN)_6^{4-} \longrightarrow Fe^{III}(CN)_6^{3-} + e$$

This oxidation causes anodic current (i-k). Similarly the anodic current increases sharply until the concentration of $Fe^{II}(CN)_6^{4-}$ diminish at the electrode surface (j). The first cycle is completed when the potential reaches the initial potential + 0.80 V.

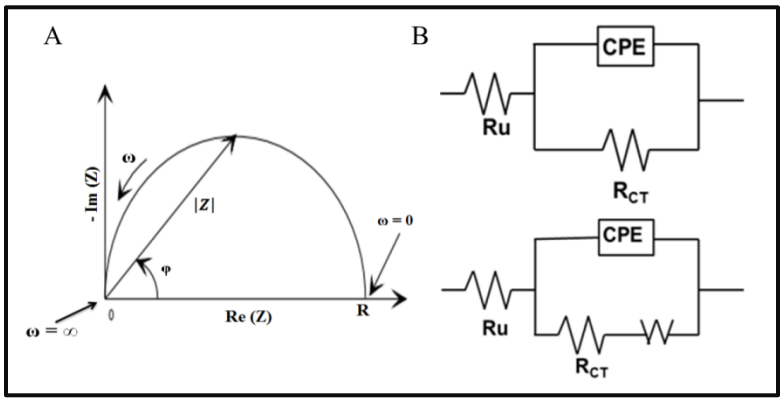
The important parameters of cyclic voltammogram are the magnitudes of cathodic peak current (i_{pc}) and anodic peak current (i_{pa}), and cathodic peak potential (E_{pc}) and anodic peak potential (E_{pa}).

2.3.3.2 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) also named ac impedance. It is used to determine the double-layer capacitance and in polarography (Chang and Park, 2010). It can be also used to characterize the electrode process and complex interfaces. Randle's equivalent circuit is used to describe the electrochemical reaction taking place at the electrode/electrolyte interface. The

charge transfer leads to both faradaic and nonfaradaic components (Lasia, 2002). The faradaic current arises from the electron transfer across the interfaces by overcoming the activation barrier such as polarization resistance (R_p), along with the uncompensated solution resistance. The nonfaradaic current arises from the charging of the double-layer capacitor (C_d).

Figure 2.5: (A) Nyquist plot with impedance vector and (B) Randle's equivalent circuit



In EIS, the response of current or potential is measured when sinusoidal perturbation of excitation signal, current or potential is applied at different frequencies (Figure 2.5). The frequency dependent proportionality between the potential signal and the current response results in the impedance data represented in the form of a Nyquist plot ($Z_{imaginary}$ vs. Z_{real}) or a bode plot (magnitude vs. frequency and phase vs. frequency).

2.3.3.3 Square wave voltammetry (SWV)

SWV provides current output in the form of symmetrical peaks rather than waveforms. In cyclic voltammetry, the minimum faradic current is limited because of the magnitude of the double layer charging current. It is the fastest and most sensitive pulse voltammetry technique. The shape of the curve was derived from the application of potential height (ΔE) (pulse amplitude),

which varies according to the potential step (E_{step}) and *T* duration (Ramaley and Krause, 1969; Mirceski et al., 2018).

2.4 References

1. Andrade JD. X-ray photoelectron spectroscopy (XPS). InSurface and interfacial aspects of biomedical polymers 1985 (pp. 105-195). Springer, Boston, MA.
2. Bhattacharjee S. DLS and zeta potential–what they are and what they are not?. Journal of controlled release. 2016 Aug 10;235:337-51.
3. Binnig G, Quate CF, Gerber C. Atomic force microscope. Phys Rev Lett. 1986 Mar 3;56(9):930-933. doi: 10.1103/PhysRevLett.56.930.
4. Chang BY, Park SM. Electrochemical impedance spectroscopy. Annual Review of Analytical Chemistry. 2010 Jul 19;3:207-29.
5. Chastain J, King Jr RC. Handbook of X-ray photoelectron spectroscopy. Perkin-Elmer Corporation. 1992;40:221.
6. Clogston JD, Patri AK. Zeta potential measurement. InCharacterization of nanoparticles intended for drug delivery 2011 (pp. 63-70). Humana Press.
7. Cohen SH, Lightbody ML, Bray MT, editors. Atomic force microscopy/scanning tunneling microscopy. Plenum Press; 1997 Apr 30.
8. Cumberland P, Everard CO, Levett PN. Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. The American journal of tropical medicine and hygiene. 1999 Nov 1;61(5):731-4.
9. Goris MG, Hartskeerl RA. Leptospirosis serodiagnosis by the microscopic agglutination test. Curr Protoc Microbiol. 2014 Feb 6;32:Unit 12E.5.. doi: 10.1002/9780471729259.

10. Heinze J. Cyclic voltammetry—"electrochemical spectroscopy". New analytical methods (25). Angewandte Chemie International Edition in English. 1984 Nov;23(11):831-47.
11. Hunter RJ. Zeta potential in colloid science: principles and applications. Academic press; 2013 Sep 3.
12. Keller KH, Andrade JD, Baier RE, Dillingham EO, Ely J, Altieri FD. Guidelines for Physicochemical Characterization of Biomaterials. NIH Publication. 1980 Sep;80:2186.
13. Kissinger PT, Heineman WR. Cyclic voltammetry. Journal of Chemical Education. 1983 Sep;60(9):702.
14. Kwok DY, Gietzelt T, Grundke K, Jacobasch HJ, Neumann AW. Contact angle measurements and contact angle interpretation. 1. Contact angle measurements by axisymmetric drop shape analysis and a goniometer sessile drop technique. Langmuir. 1997 May 14;13(10):2880-94.
15. Kwok DY, Neumann AW. Contact angle measurement and contact angle interpretation. Advances in colloid and interface science. 1999 Sep 7;81(3):167-249.
16. Lasia A. Electrochemical impedance spectroscopy and its applications. InModern aspects of electrochemistry 2002 (pp. 143-248). Springer, Boston, MA.
17. Mabbott GA. An introduction to cyclic voltammetry. Journal of Chemical education. 1983 Sep;60(9):697.
18. Maver U, Velnar T, Gaberšček M, Planinšek O, Finšgar M. Recent progressive use of atomic force microscopy in biomedical applications. TrAC Trends in Analytical Chemistry. 2016 Jun 1;80:96-111. doi.org/10.1016/j.trac.2016.03.014.
19. Mirceski V, Skrzypek S, Stojanov L. Square-wave voltammetry. ChemTexts. 2018 Dec;4(4):1-4.

20. Palaniappan RU, Chang YF, Hassan F, McDonough SP, Pough M, Barr SC, Simpson KW, Mohammed HO, Shin S, McDonough P, Zuerner RL. Expression of leptospiral immunoglobulin-like protein by Leptospira interrogans and evaluation of its diagnostic potential in a kinetic ELISA. Journal of medical microbiology. 2004 Oct 1;53(10):975-84.
21. Pinheiro AM, Costa MF, Paule B, Vale V, Ribeiro M, Nascimento I, Schaer RE, Almeida MA, Meyer R, Freire SM. Serologic immunoreactivity to Neospora caninum antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA. Veterinary parasitology. 2005 Jun 10;130(1-2):73-9.
22. Ramaley L, Krause MS. Theory of square wave voltammetry. Analytical chemistry. 1969 Sep 1;41(11):1362-5.
23. Salgin S, Salgin UĞ, Bahadir S. Zeta potentials and isoelectric points of biomolecules: the effects of ion types and ionic strengths. Int. J. Electrochem. Sci. 2012 Dec 1;7(12):12404-14.
24. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. Microbiology. 1985 Feb 1;131(2):377-85.
25. Vahabi S, Nazemi Salman B, Javanmard A. Atomic force microscopy application in biological research: a review study. Iran J Med Sci. 2013 Jun;38(2):76-83.
26. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control. World Health Organization; 2003.

Seroprevalence of Leptospirosis

CHAPTER III

Chapter III: Determination of Seroprevalence of Leptospirosis among Human and Bovine cases in the study population

This chapter deals with the seroprevalence of leptospirosis among human and bovine clinical cases in the study population, Salem, Tamil Nadu, India. The leptospiral serovar prevalence was determined by gold standard MAT using the live panel of 12 different leptospiral reference serovars. This chapter also describes the major risk factor for leptospirosis prevalence in the study site.

3.1 Background and Motivation

Leptospirosis is a neglected zoonotic infectious disease that has a worldwide distribution. Several factors contribute to the particular serovar distribution in each geographical setting. Of the 250 leptospiral serovars few serovars dominated in a particular region. In South East Asia leptospirosis is underreported. In India, leptospirosis is a major health problem reported every year. Early 19th century, Icterohaemorrhagiae was predominant infecting serovars in India, and cities including Bombay and Calcutta (Gupta, 1937; Lahiri, 1941). Late 19th Century Icterohaemorrhagiae and Autumnalis were highly prevalent in Madras city, India (Ratnam, 1984). Between 1998 and 2006 the reported prevalent serovars were Australis, Grippotyphosa, Canicola, and Icterohaemorrhagiae. Male individuals were more seroprevalence rate than female ones. The risk factors were associated with specific serovars, use of stream water, agricultural workers, sewage workers, butchers, and animal handlers who were infected with Grippotyphosa, Icterohaemorrhagiae, and Australis. Bathing in ponds persons were infected with Canicola (Murhekar et al, 1998, Vijayachari et al., 2004 and 2008). In southern regions of Tamil Nadu, the predominant circulating serovar was found to be Autumnalis followed by Icterohaemorrhagiae,

Grippotyphosa, and Australis among rice mill workers and mining workers (Natarajaseenivasan et al., 2006, Parveen et al., 2016).

In Sri Lanka leptospirosis is also named 'rat fever'. The estimated total annual incidence of leptospirosis was found to be 300.6 per 100,000 people and annual death of approximately 730, with an estimated case fatality ratio of 7.0% in Sri Lanka (Warnasekara, 2019). The average incidence amongst Malays was 10.97 per 100,000 population (Banacer et al., 2016). Between 2011 and 2013 the reported dominant circulating serovars among the Rejang basin population were Djasiman (22.1%), Shermani (13.2%), and Pomona (7.9%) (Sutt et al., 2016). In the Philippines, leptospirosis is common in flood-prone areas of urban settings such as Metro Manila. Leptospirosis was first reported in the Philippines in 1932. However human leptospirosis was reported in the late 1960s and 1970s. Several hundred cases were reported between 1932 and 1970. Pyrogenes, Manilae, Icterohaemorrhagiae, Pomona, Javanica, and Grippotyphosa were the serovars detected in this region with the major serovars being Pyrogenes and Manilae. Most of the cases occur during the rainy season between June and November (Yanagihara et al., 2007).

In Europe Icterohaemorrhagiae and Pomona were most prevalent. The epidemiological report also suggested that leptospirosis has a strong association with the season, with higher rates in summer and autumn (ECDC; 2021) Germany, the Netherlands, and the United Kingdom were the highest number of leptospirosis confirmed cases reported in 2015 (ECDC; 2018).

Human leptospirosis incidence was limited in the United States. In the Americas, the annual estimated incidence was 3.9 per 100,000 population (Schneider et al., 2017). In Hawaii, the leptospiral incidence was higher frequency. Between 1974 and 1998, 752 cases were recorded with a mean annual incidence rate was 1.29 per 100,000. Serovar Icterohaemorrhagiae is

predominantly followed by Australis, Ballum, and Bataviae (Katz et al., 2011). Leptospirosis is a highly prevalent infectious disease in both animals and humans in the Caribbean countries. The annual incidence was 50.7 per 100,000 population. The serovar Autumnalis and Icterohaemorrhagiae were highly dominant in the Caribbean region.

Leptospirosis is endemic in Oceania being reported as sporadic cases and outbreaks. The major factors for leptospirosis transmission were climate change and severe flooding (Lau, 2012). In the Oceania region the serovar Hardjo was dominant. The most reported regions were New Zealand, Australia, American Samoa, French Polynesia, and New Caledonia.

In Africa a smaller number of studies are available for serovar distribution, the reported serovars were Canicola, Hardjo, and Icterohaemorrhagiae. It is estimated that 3 to 102 people per 100,000 population are affected (Allan et al., 2015). The leptospiral incidence was reported in Kenya, Seychelles, Tanzania, and the Democratic Republic of Congo (DRC).

Several carrier animals acts as leptospirosis transmission, particular host helps in particular serovar transmission either through direct contact or indirect contact. Leptospirosis occurs in diverse epidemiological settings, in rural populations which affect agricultural workers, and livestock farmers, and in urban settings which affects slum dwellers or sewage workers. Heavy rainfall and flooding are one of the major risk factors for leptospirosis. Serovar prevalence in geographical settings is used to develop preventive measures for leptospirosis transmission from the carrier and to develop a vaccine for the most frequently reported serovar in particular settings.

In this chapter, we describe the prevalence of leptospiral serovars in the study area (Salem) using MAT assay. And the univariate analysis is performed to analyse the risk factor serovar distribution.

3.2 Materials and methods

3.2.1 Study population

The retrospective study of clinical subjects was recruited from, two hospitals and two diagnostic laboratories in the southern province (Salem and Namakkal) of Tamil Nadu. Salem is an endemic place for leptospirosis as per the earlier reports (Natarajaseenivasan et al., 2002, 2004). In total 287 clinical samples were recruited for laboratory investigation from June 2018 to May 2019 (Table 3.1). And the demographic and clinical characteristics of the study population had shown in Table 3.2. Clinical diagnosis by the attending physician was based on signs and symptoms of leptospirosis such as fever, myalgia, jaundice, and conjunctival suffusion. Similarly, the animal (bovine) samples were recruited from clinically suspected dairy cattle which had symptoms including fever, mastitis, and abortion at the Veterinary hospital, Edappadi, Salem Tamil Nadu. Salem and Namakkal are Municipal Corporation surrounded by villages involved in agriculture (especially cultivation of Rice, groundnut, and sugarcane) and cattle, goat sheep rearing. The obtained sera were stored at -80^{0}C until use.

Table 3.1: Different groups of human cases suspected for leptospirosis

Group	Description	No. of Cases (287)
1	Clinically suspected and laboratory confirmed cases of leptospirosis	162
2	Clinically suspected laboratory negative cases for leptospirosis	30
3	Typhoid	04
4	Malaria	17
5	Dengue fever	18
6	Sero-negative healthy controls	56

Table 3.2: Demographic and clinical characteristics of the study participants, N=287, Salem, 2018-19.

		Leptospira seropositive		Controls	
		n	**(%)**	**n**	**(%)**
Demographic Characteristics					
Age	0-10	31	19.1	25	20
	11-20	36	22.2	22	17.6
	21-30	24	14.8	38	30.4
	31-40	53	32.7	22	17.6
	>40	18	11.1	18	14.4
Sex	Male	77	47.5	57	45.6
	Female	85	52.5	68	54.4
Resident in a rural area		96	59.3	72	57.6
Resident in an urban area		66	40.7	53	42.4
Clinical history					
Fever		162	100	110	88
Conjunctival suffusion		47	24.7	13	10.4
Headache		80	49.4	22	17.6
Jaundice		15	9.3	0	0
Liver involvement		8	4.9	2	1.6
Renal involvement		12	7.4	4	3.2

3.2.2 Bovine clinical serum samples

Blood samples were drawn from cattle suspected of leptospirosis (n = 106) at Veterinary Hospitals located in Salem in the state of Tamil Nadu, India. Salem is generally considered as the endemic place for leptospirosis prevalence as previously reported (Natarajaseenivasan, 2002). Clinical suspicion was based on the clinical presentation of the cattle: fever, conjunctivitis, depression, abortion, mastitis, and stillbirth. Control serum samples were obtained from healthy cattle (n = 10) and analyzed for comparison. The collected blood samples were incubated at room temperature until coagulated. Then centrifuged at 3000 rpm for 20 minutes at 4°C and stored at –80°C.

3.2.3 Ethical considerations

The clinical and the sampling procedures followed were approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (Ref No. DM/2007/101/373/Project 2) as well as permitted by the Directorate of Health Services (Ref No. 5796/TV1/07), Tamil Nadu.

The protocol used for this study was approved by the Institutional Animal Ethics Committee (IEC) of Bharathidasan University, Tamil Nadu, India (BDU/IAEC/P30/2018, Dt: 07.08.2018).

3.2.4 EMJH Medium Preparation

Complete EMJH medium contains two medium like basal medium and albumin fatty acid supplement. The basal medium was prepared by dissolving 0.345 gram of EMJH medium into 135 ml double sterilized distilled water. After autoclaving, the medium was kept at room temperatur overnight to check the sterility of the basal medium. Followed by an albumin fatty acid supplement of 15 ml was added to the basal medium (135 ml) through a 0.22 µM filter. The

fatty acid supplement contains $CaCl_2.2H_2O$ and $M_gCl_2.6H_2O$ (1%), $ZnSO_4.7H_2O$ (0.4%), $CuSO_4.5H_2O$ (0.3%), Vitamin B_{12} (0.02%) and Tween 80 (10%). Prepared mediums are stored at 4ºC.

3.2.5 Leptospiral strains and culture conditions

A panel of pathogenic leptospiral strains (Table 3.3) was obtained from the WHO Reference Centre for Leptospirosis, Regional Medical Research Centre, ICMR, Port Blair, India, and used for the present study. Strains were maintained by regular sub-culturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) in a shaking incubator (100 rpm) at 30°C.

Table 3.3: Panel of leptospiral strains used as antigens in the MAT.

Genospecies	Serogroup	Serovar	Strain
L. interrogans	Australis	Australis	Ballico
	Autumnalis	Autumnalis	N2
	Bataviae	Bataviae	Swart
	Canicola	Canicola	Hond Utrecht IV
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	Hebdomadis	Hebdomadis	Hebdomadis
	Pomona	Pomona	Pomona
	Pyrogenes	Pyrogenes	Salinem
	Sejroe	Hardjo	Hardjoprajitno
L. borgpetersenii	Ballum	Ballum	Mus 127
	Javanica	Javanica	Poi
L. Krischneri	Grippotyphosa	Grippotyphosa	Moskva V

3.2.6 Microscopic Agglutination Test

MAT is a commonly used 'gold standard' serological test for confirmation of leptospirosis. It is serovar specific diagnostic test. It is used to find the seroconversion or four-fold rise in titre of the antibody. Live leptospires are considered as the antigen, the antigens can be used up to one week after 5-7 days of incubation.1-2 x 10^8 leptospires per ml are used as antigen. Initially fill 50 µl of PBS to all the wells in a microtitre plate. Then add another 140 µl of PBS to the wells of column1. Then add 10 µl of serum to the wells of column 1 (which has 1:20 dilution) mix well and discard 100 µl. Similarly, dilute the wells in each row from one well to another, and discard 50 µl of PBS in the final well. Followed by adding 50 µl *Leptospira* culture to all wells. Mix thoroughly and incubate for 2-4 hours at 30ºC. After incubation, the agglutination was observed under the dark field microscope. A minimum titre of 1:400 is the significant titre.

3.2.7 Statistical analysis

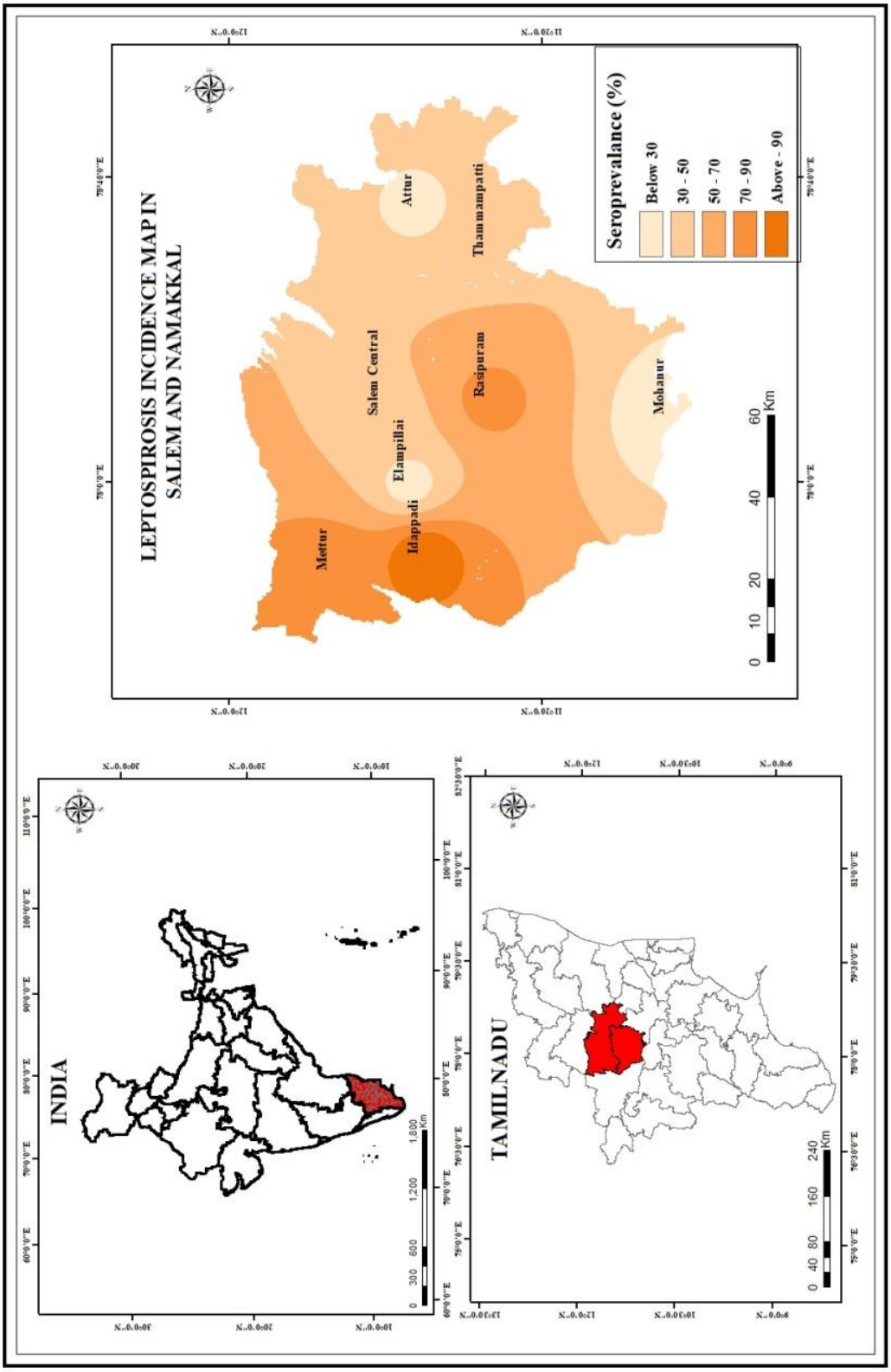
The geographical distributions of the cases from various provinces of India were analyzed using geographic information systems (ArcGIS Version 9.2). The odds ratio and its ±95 % confidence limits were calculated to measure the magnitude of the association of the disease. A Chi-square test was conducted to investigate the association between the disease outcome and the exposure to the most significant risk factors. A 5% level of significance was used to evaluate significance, i.e. p-value ≤ 0.05. The analyses were performed using EpiInfo (version 7.2.2).

3.3 Results and Discussion

3.3.1 Geographic analysis

The geographic analysis showed the seroprevalence of leptospirosis in the study site (Figure 3.1). In Salem, the leptospiral incidence was more highly found in rural areas than in urban areas. The rural area of Edappadi has the highest leptospiral incidence (>90%) recorded during the period between June 2018 and May 2019. The major occupation in this region is associated with agricultural fields such as farming and cattle rearing. In the Salem district, the prevalence reported in descending order of areas like Mettur (85%), Thamampatti (50%), Salem Town (40%), Attur, and Elampillai has less than 30%. In the Namakkal district, the leptospiral seroprevalence was highly seen in Rasipuram (>70 %), and few cases were reported in Mohanur (below 20%).

Figure 3.1: Geographical distribution of leptospirosis cases in the south region of India.



3.3.2 Leptospiral seroprevalence among human cases

A case-control study consisting of 162 leptospiral cases, 69 other febrile cases, and 56 controls were enrolled in this study. In total MAT results revealed that of the 287 clinically suspected human cases 162 presented specific *anti-leptospira* antibodies. Table 3.4 represents the seroprevalence of locally predominating serovars that were mostly prevalent such as Grippotyphosa (40.59%), Ballum (17.9%), Pomona (6.8%), Australis (6.8%), Pyrogenes (6.4%), and Autumnalis (5.5%). Moreover, a MAT result reveals a mixed population of serovar reactivity in a few cases (Table 3.5). Among 162 cases, 44 show the cross reactivity between serogroups. A few leptospiral cases can cross reacts with more than one serovars.

Table 3.4: Seroprevalence of *Leptospira* serovars determined by using Microscopic Agglutination Test (MAT) among human cases.

Serogroups	**MAT titre**				**Total**	**Prevalence**
	80	160	320	>320		**(%)**
Australis	11	3	--	--	16	**6.8**
Autumnalis	12	1	--	--	13	**5.5**
Ballum	20	14	3	5	42	**17.9**
Bataviae	10	1	--	--	11	**4.7**
Canicola	11	3	--	--	14	**5.8**
Grippotyphosa	51	40	3	1	95	**40.59**
Hebdomadis	2	1	--	--	3	**1.28**
Javanica	6	--	--	--	6	**2.56**
Pomona	16	--	--	--	16	**6.8**
Sejroe	4	--	1	--	4	**1.7**
Pyrogenes	15	--	--	--	15	**6.4**
Total	158	63	7	6	234*	**100**
Prevalence (%)	**44.8**	**17.8**	**1.9**	**1.7**	**100**	

* 27% of seropositive patients reacted to >1 serovar, thus 234 does not reflect the case no.

Table 3.5: Cross reactivity among different Leptospira serovars in human cases by MAT assay

Serogroups	Aus	Aut	Bal	Bat	Can	Ict	Gri	Heb	Jav	Pom	Sej	Pyr
Aus	-	9	7	3	9	0	17	2	7	10	0	6
Aut	-	-	9	5	10	0	19	2	6	5	0	9
Bal	-	-	-	9	33	4	42	20	6	6	3	13
Bat	-	-	-	-	7	1	17	4	1	1	0	4
Can	-	-	-	-	-	4	36	24	12	9	3	7
Ict	-	-	-	-	-	-	0	2	1	1	0	0
Gri	-	-	-	-	-	-	-	27	8	19	2	23
Heb	-	-	-	-	-	-	-	-	1	5	1	3
Jav	-	-	-	-	-	-	-	-	-	1	1	2
Pom	-	-	-	-	-	-	-	-	-	-	0	6
Sej	-	-	-	-	-	-	-	-	-	-	-	1
Pyr	-	-	-	-	-	-	-	-	-	-	-	-

3.3.3 Leptospiral seroprevalence among bovine case

A large number of 106 leptospiral suspected cases were enrolled in this study. Out of the total 106 clinically suspected cases, 36 cases were found to have specific anti-leptospiral antibodies as revealed by MAT analysis. Table 3.6 represents the seroprevalence of the most dominant serovars such as Grippotyphosa (36.11%), Pomona (16.6%), and Canicola (11.1%). We have noticed that the serogroup Grippotyphosa has more seropositivity, which is correlated very well with our earlier seroprevalence of human leptospirosis. This confirms that livestock is a transmission host or reservoir host for human leptospirosis in this region. Moreover, MAT results also reveal a mixed population of serovar reactivity in certain cases. Interestingly out of

36 cases, 12 showed cross reactivity between the serogroups. This provides an opportunity to find out alternative methods for the specific detection of leptospirosis.

Table 3.6: Seroprevalence of leptospiral serovars determined by using MAT among bovine cases.

Serogroup	**MAT titre**					**Total**	**Prevalence (%)**
	<80	**80**	**160**	**320**	**>320**		
Single Dominant							
Canicola	3	--	1	--	--	4	11.11
Grippotyphosa	**4**	**2**	**5**	**--**	**2**	**13**	**36.11**
Pomona	3	2	1	--	--	6	16.66
Sejroe	--	--	1	--	--	1	2.77
Subtotal	10	4	8	--	2	24	66.66
Mixed Equals							
Canicola+Grippotyphosa	--	1	1	--	--	2	5.55
Javanica+Sejroe	1	--	--	--	--	1	2.77
Australis+Canicola	--	1	--	--	--	1	2.77
Canicola+Javanica	1	--	--	--	--	1	2.77
Autumnalis+Canicola+Pomona	--	1	--	1	--	2	5.55
Autumnalis+Grippotyphosa+Hebdomadis	--	2	--	--	--	2	5.55
Pomona+Grippotyphosa+Hebdomadis	--	2	1	--	--	3	8.33
Subtotal	2	7	2	1	--	12	33.33
Total	12	11	10	1	2	36	100
Prevalence (%)	33.3	30.5	27.7	2.7	5.5	100	

3.3.4 Association of Risk factors

Bivariable logistic regression of individual risk factors associated with acute leptospirosis was included in Table 3.7. There was a strong association between behaviours involving a single livestock species. For example, having cleaned cattle waste was associated with an OR of 0.9 (95% confidence intervals 0.53-1.5). Also, there was some association between behaviours involving different livestock species. For example having cleaned cattle waste was associated with having cleaned sheep waste with an OR 1.53, 95% confidence interval of 0.7-3.01. Another important variable associated with acute leptospirosis was working in rice fields OR 0.205, 95% confidence interval (CI) 0.124-0.339; walking barefoot OR 2.15 (95% CI 1.29-3.58); Bathed surface water OR 3.003 (95% CI 1.6-5.5), rodents ex and worked as a farmer OR 0.013 (95% CI 0.004-0.04) were significant risk factors ($p<0.001$) for leptospirosis.

Table 3.7: A risk factor for acute leptospirosis among patients with febrile illness, Salem, 2018-19.

Variable	Acute leptospirosis		Control		Bivariable logistic regression	
	n	(%)	n	(%)	OR(95 % CI)	p value
Livestock exposure						
Cleaned cattle waste	42	25.9	35	28	0.9 (0.53-1.5)	0.69
Cleaned sheep waste	28	17.3	15	12	1.53 (0.7-3.01)	0.22
Kept cattle inside the house	30	18.5	18	14.4	1.45 (0.7-2.6)	0.35
Kept sheep inside the house	17	10.5	22	17.6	0.56 (0.27-1.08)	0.08
Milked with cattle	23	14.2	6	4.8	3.28 (1.29-8.3)	0.01
Slaughtered cattle	8	4.9	2	1.6	3.19 (0.67-15.3)	0.14
Slaughtered sheep	15	9.3	10	8.0	1.17 (0.5-2.7)	0.707
Rodents exposure						

Worked as farmer	56	34.6	122	97.6	0.01(0.004-0.04)	<0.0001
Rodents saw in the field	14	8.6	15	12	0.69 (0.32-1.49)	0.35
Rodents saw in the house	8	4.9	10	8	0.59 (0.23-1.56)	0.29
Water exposure						
Drank tap water	34	21.8	72	57.6	0.19 (0.11-0.32)	<0.0001
Bathed in surface water	52	32.1	108	86.4	3.0 (1.6-5.5)	0.0004
Worked in rice fields	48	30.3	84	67.2	0.20 (0.12-0.34)	<0.0001
Walked barefoot	69	42.6	32	25.6	2.15 (1.29-3.58)	0.003

OR: Odds Ratio, CI: Confidence Interval

3.4 Discussion

This study reports an apparent seroprevalence (56.4%) of leptospirosis in Salem. This study raises the risk factor for human leptospirosis in this region mainly due to the occupational associated exposure during their daily work routine. Factors associated with such a high prevalence in humans may be due to the contact with animals like cattle, sheep, and rodents, which also carry leptospiral infections and transmit the leptospires through their contaminated urine. Another important source of infection is the common usage of water sources.

The most reactive serovar in 51.23 % of the tested samples was Grippotyphosa (*Leptospira Krischneri*) which corresponds with the data from the prevalence of leptospirosis in cattle. Our finding demonstrated that the people working with livestock were exposed to pathogenic Leptospira. Early findings reported that the serogroup Autumnalis is the dominant one. In contrast in the present study, Grippotyphosa was the dominant one.

The MAT is relatively serovars-specific but cross reactivity occurs between serovars in the same serogroup or among the genomospecies interaction, thus overestimating the seroprevalence. Exposure to two or more serovars or exposure to one serovar with cross reactivity was found to

be 51.85%. During the acute phase of infection, IgM antibodies may be elevated; later the more specific IgG antibodies could be produced. IgM may contribute to a certain degree of antibody cross-reaction. A serological survey among 162 patients in Salem using the MAT test showed a high prevalence of Grippotyphosa (51.3%), followed by Pyrogenes (11.7 %), Pomona (9.87 %), Australis (8.64 %) and Canicola (8.64%) with the MAT cut-off titre ≥ 1:80. The lower cut-off may explain the higher estimate reported. At a MAT cut-off titre ≤ 1:80, our study shows the seroprevalence of 33.5 %, for 1:80 cut-off titre had 44.8 %, for 1:160 of the cut-off titre had 17.8 % prevalence for leptospirosis. Our study also found that the seroprevalence among females (52.4%) was slightly higher than among males (47.5%).

Our study relates the association between exposures to cattle may be an important source of human leptospirosis in Salem. We also identified work in rice fields and barefoot as an important risk factors. Our findings are also consistent with studies examining risk factors for *Leptospira* seropositivity in Salem (Natarajaseenivasan et al., 2002). However during this study period, Grippotyphosa is the predominant serovar than the previously reported serovar Autumnalis.

3.5 Conclusion

This chapter concludes that seroprevalence of 56.4 % is the strong evidence for exposure to humans in Salem and highlights the potential occupational and public health risks. Prevention and control of leptospirosis in humans in this region involves vaccination and rodent control to prevent the infection transmission from animals and protect people. Improvement of livestock practices (rodent control and confinement), public health education and personal hygiene, and treatment in the acute stage might be reducing the potential for transmission of leptospirosis.

3.6 References

1. Allan KJ, Biggs HM, Halliday JE, Kazwala RR, Maro VP, Cleaveland S, Crump JA. Epidemiology of Leptospirosis in Africa: A Systematic Review of a Neglected Zoonosis and a Paradigm for 'One Health' in Africa. PLoS Negl Trop Dis. 2015 Sep 14;9(9):e0003899. doi: 10.1371/journal.pntd.0003899.
2. Benacer D, Thong KL, Min NC, Bin Verasahib K, Galloway RL, Hartskeerl RA, Souris M, Mohd Zain SN. Epidemiology of human leptospirosis in Malaysia, 2004-2012. Acta Trop. 2016 May;157:162-8. doi: 10.1016/j.actatropica.2016.01.031.
3. European Centre for Disease Prevention and Control. Leptospirosis. In: ECDC. Annual epidemiological report for 2016. Stockholm: ECDC; 2021.
4. European Centre for Disease Prevention and Control. Leptospirosis. In: ECDC. Annual epidemiological report for 2015. Stockholm: ECDC; 2018.
5. Gupta BD. Leptospirosis in India. The Indian Medical Gazette. 1938 Aug;73(8):449.
6. Katz AR, Buchholz AE, Hinson K, Park SY, Effler PV. Leptospirosis in Hawaii, USA, 1999-2008. Emerg Infect Dis. 2011 Feb;17(2):221-6. doi: 10.3201/eid1702.101109.
7. Lahiri MN. A note on the occurrence of Leptospirosis in Bombay. The Indian Medical Gazette. 1941 Nov;76(11):669.
8. Lau CL, DePasquale JM. Leptospirosis, diagnostic challenges, American Samoa. Emerg Infect Dis. 2012 Dec;18(12):2079-81. doi: 10.3201/eid1812.120429.
9. Murhekar MV, Sugunan AP, Vijayachari P, Sharma S, Sehgal SC. Risk factors in the transmission of leptospiral infection. Indian J Med Res. 1998 May;107:218-23.
10. Natarajaseenivasan K, Boopalan M, Selvanayaki K, Suresh SR, Ratnam S. Leptospirosis among rice mill workers of Salem, South India. Jpn J Infect Dis. 2002 Oct;55(5):170-3.

11. Natarajaseenivasan K, Prabhu N, Selvanayaki K, Raja SS, Ratnam S. Human leptospirosis in Erode, South India: serology, isolation, and characterization of the isolates by randomly amplified polymorphic DNA (RAPD) fingerprinting. Jpn J Infect Dis. 2004;57(5):193-197.
12. Parveen SM, Suganyaa B, Sathya MS, Margreat AA, Sivasankari K, Shanmughapriya S, Hoffman NE, Natarajaseenivasan K. Leptospirosis Seroprevalence Among Blue Metal Mine Workers of Tamil Nadu, India. Am J Trop Med Hyg. 2016 Jul 6;95(1):38-42. doi: 10.4269/ajtmh.16-0095.
13. Ratnam S, Sundararaj T, Subramanian S, Madanagopalan N, Jayanthi V. Humoral and cell-mediated immune responses to leptospires in different human cases. Trans R Soc Trop Med Hyg. 1984;78(4):539-42. doi: 10.1016/0035-9203(84)90078-6.
14. Schneider MC, Leonel DG, Hamrick PN, de Caldas EP, Velásquez RT, Mendigaña Paez FA, González Arrebato JC, Gerger A, Maria Pereira M, Aldighieri S. Leptospirosis in Latin America: exploring the first set of regional data. Rev Panam Salud Publica. 2017 Jun 19;41:e81. doi: 10.26633/RPSP.2017.81.
15. Suut L, Mazlan MN, Arif MT, Yusoff H, Abdul Rahim NA, Safii R, Suhaili MR. Serological Prevalence of Leptospirosis Among Rural Communities in the Rejang Basin, Sarawak, Malaysia. Asia Pac J Public Health. 2016 Jul;28(5):450-7. doi: 10.1177/1010539516648003.
16. Vijayachari P, Sugunan AP, Murhekar MV, Sharma S, Sehgal SC. Leptospirosis among schoolchildren of the Andaman & Nicobar Islands, India: low levels of morbidity and mortality among pre-exposed children during an epidemic. Epidemiol Infect. 2004 Dec;132(6):1115-20. doi: 10.1017/s0950268804002948.

17. Vijayachari P, Sugunan AP, Sharma S, Roy S, Natarajaseenivasan K, Sehgal SC. Leptospirosis in the Andaman Islands, India. Trans R Soc Trop Med Hyg. 2008 Feb;102(2):117-22. doi: 10.1016/j.trstmh.2007.08.012.
18. Warnasekara J, Koralegedara I, Agampodi S. Estimating the burden of leptospirosis in Sri Lanka; a systematic review. BMC Infect Dis. 2019 Feb 6;19(1):119. doi: 10.1186/s12879-018-3655-y.
19. Yanagihara Y, Villanueva SY, Yoshida S, Okamoto Y, Masuzawa T. Current status of leptospirosis in Japan and Philippines. Comp Immunol Microbiol Infect Dis. 2007 Sep;30(5-6):399-413. doi: 10.1016/j.cimid.2007.05.003.

LPS as Biomarker for Diagnosis of Leptospirosis

CHAPTER IV

Chapter IV: Leptospiral Lipopolysaccharide as the Potential Biomarker for Serovar Specific Diagnosis of Leptospirosis

This chapter deals with the evaluation of the leptospiral lipopolysaccharide as a potential biomarker for serovar specific diagnosis of leptospirosis using serological methods like Enzyme-linked immune sorbent assay (ELISA) and dot blot assay. Both IgM and IgG-based serological diagnosis were performed to determine the serovar specificity.

4.1 Background and motivation

Bacterial lipopolysaccharide or endotoxin stimulates a wide range of innate immune cells and is involved in various signaling pathways (MAPKs, NF-kβ, etc.) (Beutler and Rietschel, 2003; Ulevitch and Tobias, 1994). In general, LPS comprised of three regions namely: (1) Lipid A – hydrophobic, a real pathogen associated molecular pattern (PAMP) attached to the carbohydrate chain, toxin and stimulates the innate immune system, (ii) core polysaccharide – hydrophilic, which associates with immunogenicity and (iii) O-antigen – hydrophilic, mainly involved in serotype determination. These functional moieties are covalently linked together. Leptospiral LPS is a significant antigen involved in the human antibody response to infection, and host adaptation, and also the IgM antibodies elicited during the acute stage of infection mainly react with LPS, thus acting as a target antigen for diagnosis (Chapman et al., 1991; Evengelista and Coburn, 2010; Patra et al., 2015; Widiyanti et al., 2013). Differences in LPS structure such as fatty acid profiling and o-antigenic variation were usually attributed to the serovar identity (Bulach et al., 2000). LPS acts as both an immunogenic and pathogenic response. It was the main inducer of septic shock and it had a non-specific immune-stimulatory role in the induction of adaptive immune response.

4.2 Leptospiral lipopolysaccharide

Leptospiral lipid A is functionally and structurally different from other Gram negative lipid A. Lipid A is the active component of LPS and is responsible for the toxic activity. Leptospiral LPS has unique fatty acid composition, and a unique methylated phosphate residue (Que-Gewirth et al., 2004). Interestingly leptospiral LPS can be recognized by both TLR4 and TLR2 receptors. It has been reported that activates the production of pro-inflammatory chemokines induced by p38 MAPK phosphorylation through TLR2 activation (Hung et al., 2006) And also increased the levels of IL-6 and TNFα mRNA in the lungs of guinea pigs (Marinho et al., 2009). A study reported that both leptospiral lipopolysaccharide and lipoproteins activate macrophages through CD14 and the Toll-like receptor 2 (TLR2) (Werts et al., 2001).

4.3 Materials and Methods

4.3.1 Extraction of Leptospiral LPS

Leptospira were grown in liquid EMJH medium at 30ºC and harvested at a density of ~5 x 10^8 bacteria/ mL. Leptospiral LPS was extracted following the standard procedure of the hot phenol-water method (Westphal and Jane, 1965). The phenol phase that contained the LPS was used for further purification. The phenol phase was dialyzed extensively against water and insoluble material was removed by centrifugation. LPS was prepared from 6 locally predominant pathogenic leptospiral serovars; Autumnalis, Australis, Canicola, Grippotyphosa, Pomona, Pyrogenes, and Sejroe. Non-pathogenic serovar Andamana was also included for comparison. The extracted LPS were quantified by the phenol/sulfuric acid method using sucrose as a standard (Dubois et al., 1956). The protein was estimated by the Bicinchoninic acid (BCA) method.

4.3.2 Evaluation of Leptospiral LPS enzyme-linked immuno assay (ELISA)

ELISA was carried out using extracted LPS as described previously (Vanithamani et al., 2015). Checkerboard titrations were performed to determine the optimal concentrations of LPS for IgM ELISA and determined as 1 µg. A total of 100 µl of carbonate coating buffer (pH-9.6) containing 1µg of LPS per well was coated on flat-bottom polystyrene microtiter plates at 4°C overnight, followed by blocking with 4% non-fat dry milk. Serum samples (1:200) in duplicate were added and incubated for 1 h at 37°C. Bound IgM or IgG was detected using HRP-conjugated rabbit anti-human IgM and anti-bovine IgG (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:8,000. Plates were developed with o–phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA). The reaction was stopped with the addition of 50 µl of 2 N H_2SO_4, and the optical density was measured at 490 nm (Bio-Rad, USA). The sera samples were divided into homologous sera (HS) and heterologous sera (HES) based on the MAT positivity. Homologous sera are defined as patients' sera that reacted with a specific serogroup in MAT and tested against LPS of the same serogroups. Heterologous sera is defined as patients' sera that reacted with specific serogroup in MAT and against LPS of other serogroups.

4.3.3. Evaluation of Leptospiral LPS by dot blot assay

To validate the specificity extracted LPS Dot blot assay was performed. The optimal concentration for IgM-based detection was determined as 1 µg. The method followed was previously described by Vanithamani et al., 2015. In brief, LPS were dotted on the nitrocellulose membrane and air dried. The membrane was blocked with 5% non-fat milk and incubated at 37°C for 1 hour and washed four times with PBS-T. The membrane was incubated with patients' sera and bovine's sera were diluted in PBS-T (1: 200) for 1 hour at 37°C. The membrane was

incubated with HRP conjugated anti-human IgM or anti-bovine IgG (1:4000; Sigma Aldrich, USA) at 37°C for 1 hour and washed with PBS-T. The membranes were developed with super signal west pico plus chemiluminescent substrate (Thermo Scientific, USA) and the intensity was quantified using Image J software and expressed as arbitrary units (AU).

4.3.4 Statistical Analysis

Data were analyzed and plotted using graph pad prism version 5.0 software (version 5.0c; Graph pad software, San Diego, CA). Cut-off values for each diagnostic were as defined as the corresponding Mean+2SD calculated from the sera of normal healthy controls. Sensitivity and Specificity represent two kinds of accuracy: sensitivity for actually positive cases, and specificity for actually negative cases. The positive and negative predictive values (PPV and NPV respectively) are the proportions of true positive and true negative results. Odds ratios were calculated with 95% confidence intervals. A p-value less than 0.05 was considered significant.

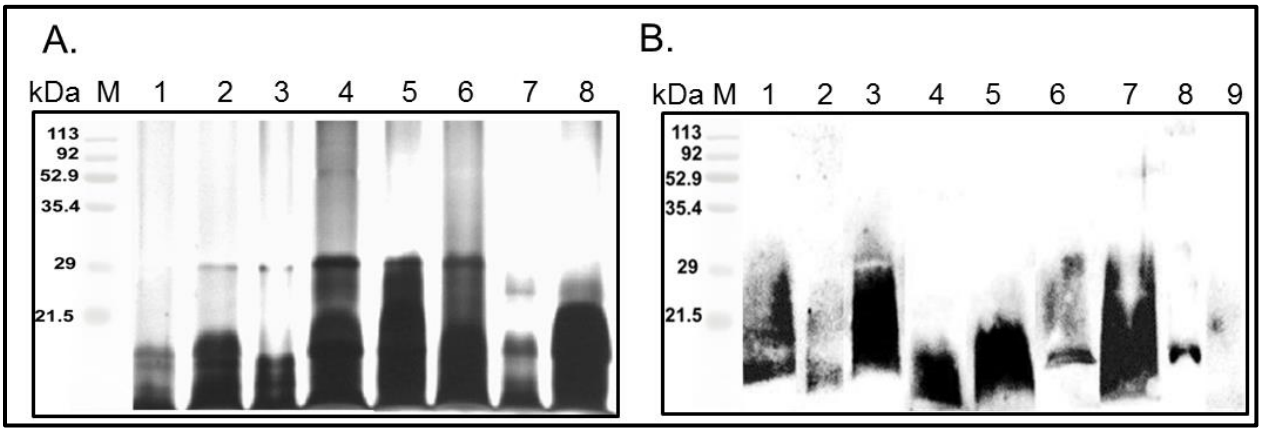
4.4 Results and Discussion

4.4.1 SDS-profile and Immunoblotting of leptospiral LPS

The extracted and purified LPS has the quantity of 750, 630, 890, 980, 740, 1080,400, 230, 890, 740, and 590 (μg ml^{-1}) for Australis, Autumnalis, Bataviae, Canicola, Grippotyphosa, Hebdomadis, Javanica, Pomona, Sejroe, Pyrogenes and Andamana. The apparent molecular mass of extracted LPS lies between 14-28 kDa (Figure 4.1A). There was less or no detectable protein contamination observed in the extracted LPS by the BCA method. Further to analyse of the serogroup reactivity of pooled patients' sera by immunoblots and Figure 4.1B showed reactivity to respective pathogenic leptospiral LPS and there was no reactivity was observed in non-pathogenic serovar Andamana. Serogroup reactivity was observed for all leptospiral LPS

against their homologous and heterologous sera confirming the serogroup's specificity and reactivity.

Figure 4.1: SDS-PAGE profile and Immunoblotting of the extracted LPS

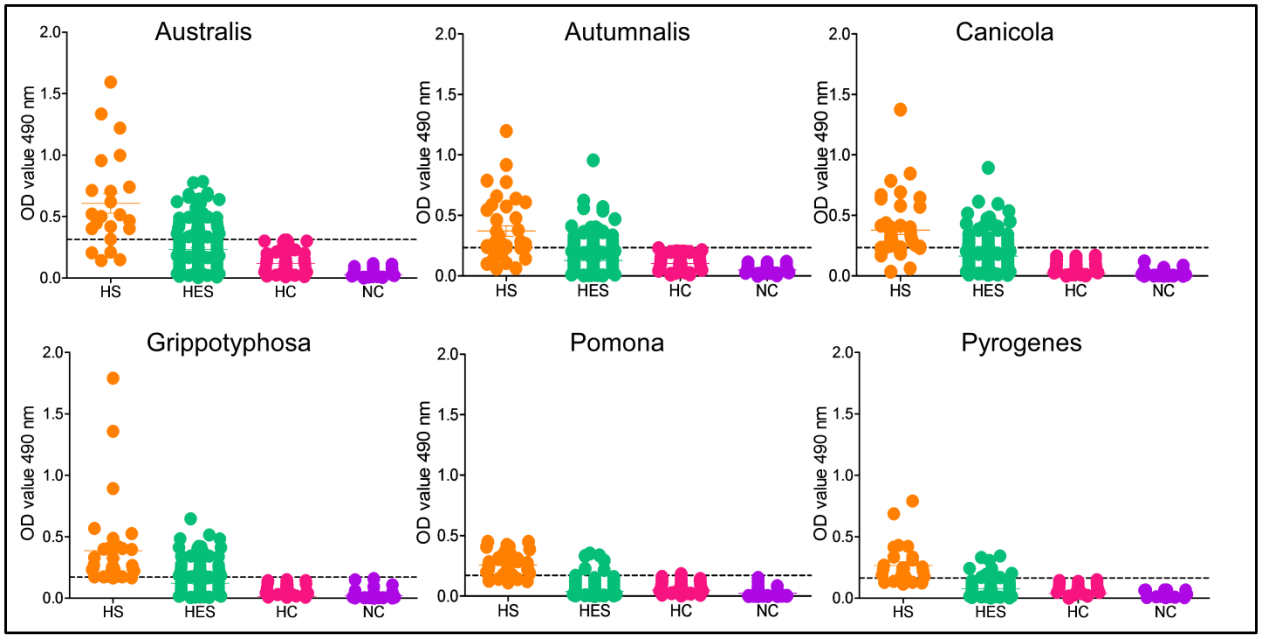


Legends: *(lane 1: Australis, lane 2:Autumnalis, lane 3: Bataviae, lane 4: Canicola, lane 5: Grippotyphosa, lane 6: Javanica, lane 7: Pomona, lane 8: Sejroe, lane 9: Andamana)*

4.4.2 Serodiagnosis for human leptospirosis

The LPS based ELISA offers a reasonable sensitivity and serovar specific reactivity against the serovars. Out of 287 patients' serum samples, 172 were positive using IgM ELISA against the Leptospiral LPS. Both homologous and heterologous titers were obtained. Together the Grippotyphosa serovar reveals the highest titer range followed by the Australis, Canicola, Autumnalis, Pyrogenes, and Pomona. Figure 4.2 shows the reactivity against homologous and heterologous reactivity that occurs in the locally predominating serogroups. Out of 162, MAT confirmed cases 160 were shown to be positive to 160 and only 2 cases failed to react with IgM ELISA. Among 125 MAT negative cases 12 were positive for IgM ELISA. The sensitivity and specificity of the IgM ELISA were determined using MAT positivity as the gold standard. The sensitivity and specificity of the serum samples were 93.9 (%) and 77.5 (%) respectively.

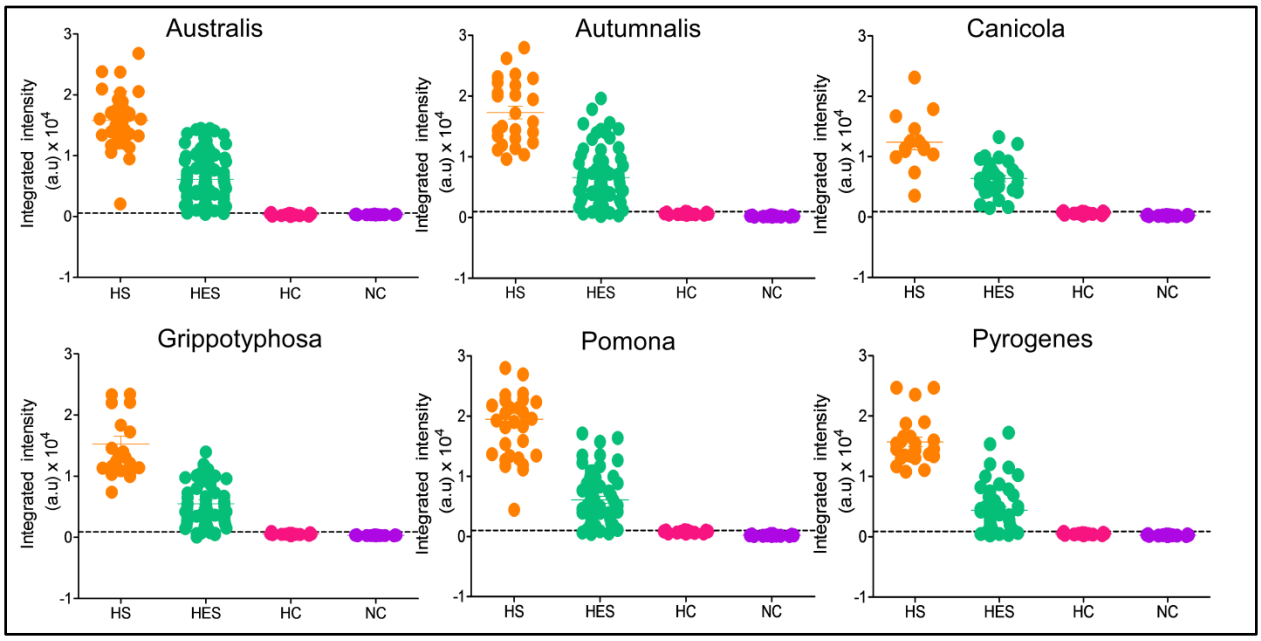
Figure 4.2: Leptospiral LPS specific IgM antibodies among homologous and heterologous patients' sera by ELISA



Legends: *Study groups were indicated on the X-axis (Homologous sera, Heterologous sera, Healthy control, and Negative control) and the optical density at 490 nm on the y-axis. The dashed line represents the cut-off values for each antigen with the absolute cut-off values on the right.*

To validate the specificity extracted LPS Dot blot assay was performed. A total of 287 serum samples from 185 patients diagnosed with leptospirosis were analysed by IgM dot blot with LPS from 6 different serovars of *Leptospira*. The representative reactions of the dot-blot assay are shown in Figure 4.3. The cut-off values for Australis, Autumnalis, Canicola, Grippotyphosa, Pomona and Pyrogenes were 0.0552, 0.0993, 0.0886, 0.0896, 0.1010 and 0.0884 (x 10^5) respectively. Moreover, in this study out of 162 MAT positive cases, 150 were positive to dot-blot assay and 12 cases fail to react in IgM dot-blot assay. Interestingly among 125 MAT negative cases, 35 were shown to be IgM dot-blot positive.

Figure 4.3: Leptospiral LPS specific IgM antibodies among homologous and heterologous patients' sera by IgM dot blot-assay



Legends: *Among 287 patients' serum samples 185 were positive using IgM Dot blot assay. The cut-off values were 552, 993, 698, 886, 896.8, 980, 1010, and 884 for Australis, Autumnalis, Bataviae, Canicola, Grippotyphosa, Javanica, Pomona, and Pyrogenes respectively.*

The overall serodiagnostic accuracy of human leptospirosis was represented in table 4.1.

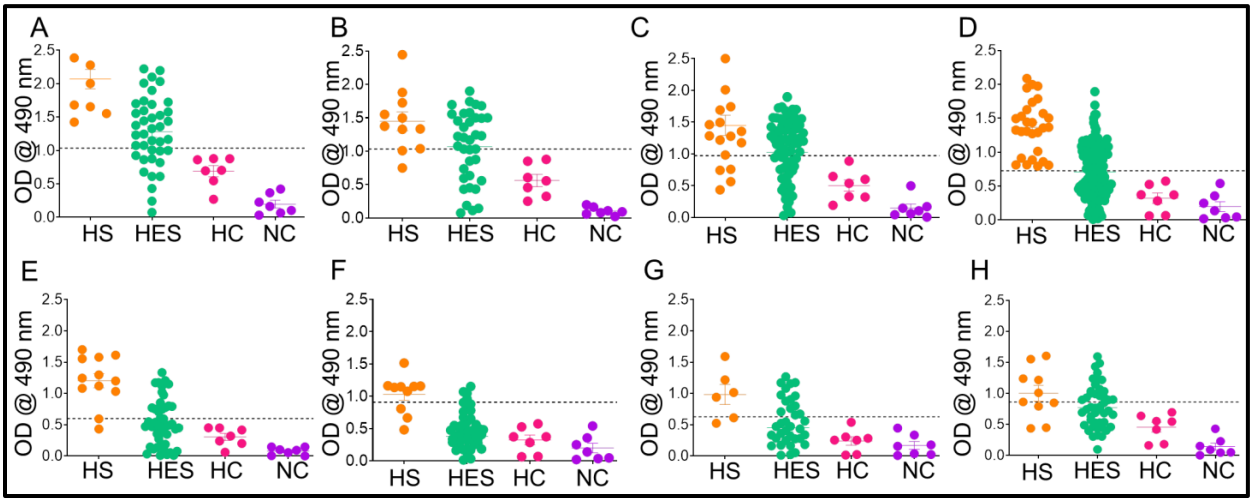
Table 4.1: Diagnostic accuracy of ELISA and Dot Blot assay compared with MAT for detecting Human (IgM) leptospirosis.

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Odds ratio	p-value
IgM ELISA	93.92	77.48	92.06	80.46	6.62	<0.0001
IgM Dot Blot	96.63	73.48	86.79	92.38	3.47	<0.0001

4.4.3 Serodiagnosis for bovine leptospirosis

The IgG-based ELISA for detection of bovine leptospirosis was shown in figure 4.4. Among 106 bovine serum samples, 43 were positive using IgG ELISA. The cut-off OD values for IgG ELISA were 1.035, 1.031, 0.972, 0.725, 0.598, 0.908, 0.863 and 0.626 for Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomadis, Javanica, Pomona and Sejroe respectively.

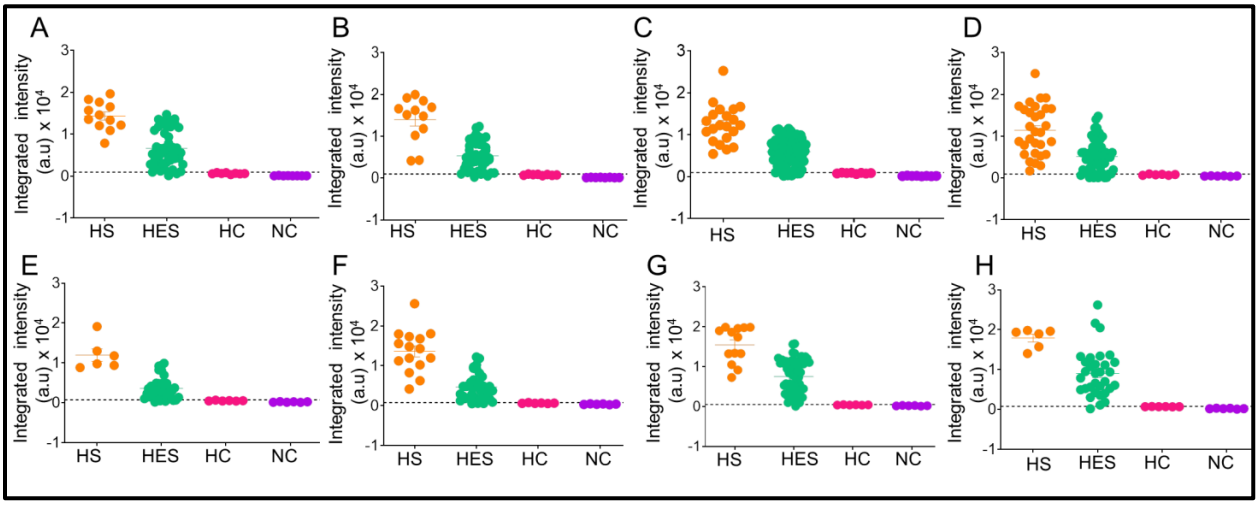
Figure 4.4: Leptospiral LPS specific IgG antibodies among homologous and heterologous bovine sera by ELISA



Legends: *Study groups were indicated on X-axis (Homologous sera, Heterologous sera, Healthy control, and Negative control) and the optical density at 490 nm on the y-axis.*

The results presented in the table shows that the IgG ELISA compared very favorably with the MAT test. Only seven of the samples failed to react with MAT assay, but were reactive in IgG ELISA. Sensitivity and specificity were found to be 90.00% and 89.39 % similarly, PPV and NPV values were 83.27 % and 93.65% respectively.

Figure 4.5: Leptospiral LPS specific IgG antibodies among homologous and heterologous bovine sera by IgG dot blot assay



Legends: *Study groups were indicated on X-axis (Homologous sera, Heterologous sera, Healthy control, and Negative control) and the integrated intensity (a.u units) on the y-axis. The dashed line represents the cut-off values for each antigen with the absolute cut-off values on the right.*

To validate the dot-blot analysis as a diagnostic method for leptospirosis detection, we assessed the accuracy parameters by comparing the results of MAT. The optimal concentrations for IgG-based detection was determined as 1 µg. Of the 36 cases which were positive for leptospirosis by MAT, 33 had a positive dot-blot assay for a sensitivity of 0.83 (83%) (Figure 4.5). Moreover, of the 70 cases which were negative for leptospirosis by MAT, 73 had a negative to dot-blot assay for a specificity of 0.95 (95.71 %). The PPV and NPV were 0.9 (90.9 %) and 0.91 (91.78 %), respectively (Table 4.2). Overall, dot-blot assay results confirm the accuracy of the IgG ELISA.

Table 4.2: Diagnostic accuracy of ELISA and Dot Blot assay compared with MAT for detecting Bovine (IgG) leptospirosis

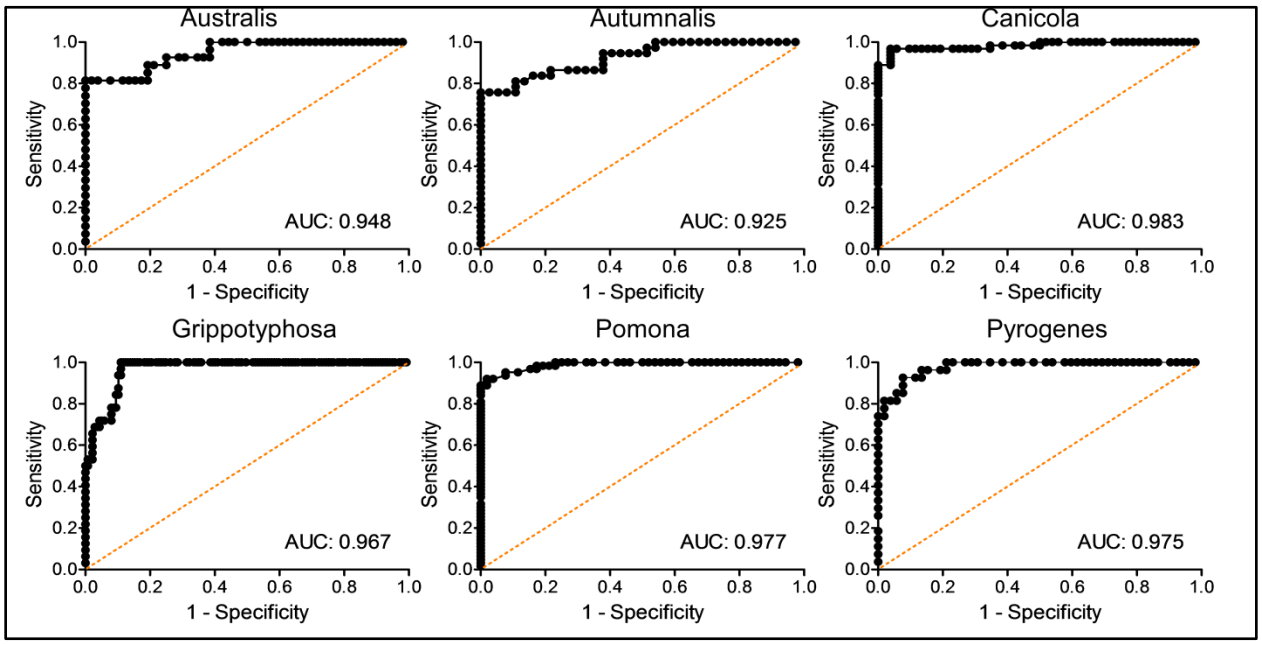
Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV(%)
IgG ELISA	90.0	89.39	83.27	93.65
IgG Dot Blot	83.3	95.71	90.90	91.78

4.4.4 Accuracy of the IgM ELISA for leptospirosis diagnosis

ROC curve analysis has been used to evaluate the ability of a test to discriminate between infected and healthy subjects and to compare the diagnostic performance of two or more tests (Goris et al., 2011; Metz CE., 1978). The ROC curve is obtained by plotting the true-positive rate (sensitivity) as a function of the false-positive rate (1- specificity) that is associated with each cut-off point. The AUC can be used as a measure of the accuracy of the test. If the test cannot distinguish between infected and normal populations, the AUC will be equal to 0.5 and the ROC curve will coincide with the diagonal. On the other hand, if the test is 100% sensitive and specific, then the AUC will be equal to 1 and the curve will reach the upper left corner (Metz CE., 1978, Surujballi et al., 2001, Zweig MH and Campbell G, 1993).

The ELISA data were subjected to ROC curve analysis to compare the diagnostic test (MAT). ROC curve analysis used to optimize the cut-off point yielded an AUC of 0.948, 0.925, 0.983, 0.967, 0.988, and 0.97 for Australis, Autumnalis, Canicola, Grippotyphosa, Pomona and Pyrogenes respectively (Figure 4.6). The optimized cut-off points and the AUC values obtained from the ROC curve analysis were summarized in Table 4.3. At this cut-off point, the sensitivity and specificity estimates were 93.92 % and 77.48 %.

Figure 4.6: ROC curve obtained from ELISA results of the human cases tested.



Legends: *The true positive rate (sensitivity [y-axis]) is plotted against the false-positive rate (1-specificity [x-axis].*

Table 4.3: Summary of the results of ROC curve analysis on different leptospiral serovars

Serovar	**Recommended cut-off**	**AUC**
Australis	0.314	0.948
Autumnalis	0.234	0.925
Canicola	0.215	0.983
Grippotyphosa	0.172	0.967
Pomona	0.172	0.988
Pyrogenes	0.165	0.971

4.5 Conclusion

This chapter concludes that the leptospiral LPS is potent biomarker for diagnosis of early stage of infection using human and bovine clinical samples. For serodiagnosis assay the optimal concentration of target analyte (LPS) determined as 1 µg/mL. From SDS-PAGE profiling we found that the cap like lipid A structure and the ladder like core polysaccharide as well as O-antigen. The diagnostic accuracy of serodiagnosis of human leptospirosis has more than 90 % sensitivity, about 70 % specificity and around 90 % PPV and NPV. Likewise, the diagnostic accuracy of bovine leptospirosis was found to be about 90% of statistics. Together dot blot has more specificity than the ELISA. The AUC values obtained from the ROC curve analyses conducted on the ELISA data were all relatively high, indicating that this assay is very accurate.

4.6 References

1. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol. 2003 Feb;3(2):169-76. doi: 10.1038/nri1004.
2. Bulach DM, Kalambaheti T, de la Peña-Moctezuma A, Adler B. Lipopolysaccharide biosynthesis in Leptospira. J Mol Microbiol Biotechnol. 2000 Oct;2(4):375-80.
3. Chapman AJ, Everard CO, Faine S, Adler B. Antigens recognized by the human immune response to severe leptospirosis in Barbados. Epidemiol Infect. 1991 Aug;107(1):143-55. doi: 10.1017/s0950268800048779.
4. DuBois MK, Gilles JK, Hamilton PA, Rebers P, Smith F. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 1956 28 (3), 350-356 DOI: 10.1021/ac60111a017.
5. Evangelista KV, Coburn J. Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. Future Microbiol. 2010 Sep;5(9):1413-25. doi: 10.2217/fmb.10.102.
6. Goris MGA, Leeflang MMG, Boer KR, Goeijenbier M, van Gorp ECM, et al. (2011) Establishment of Valid Laboratory Cases Definition of Human Leptospirosis. Journal of Bacteriology and Parasitology S5 001: 1–8.
7. Hung CC, Chang CT, Tian YC, Wu MS, Yu CC, Pan MJ, Vandewalle A, Yang CW. Leptospiral membrane proteins stimulate pro-inflammatory chemokines secretion by renal tubule epithelial cells through toll-like receptor 2 and p38 mitogen activated protein kinase. Nephrol Dial Transplant. 2006 Apr;21(4):898-910. doi: 10.1093/ndt/gfi316.

8. Marinho M, Oliveira-Júnior IS, Monteiro CM, Perri SH, Salomão R. Pulmonary disease in hamsters infected with Leptospira interrogans: histopathologic findings and cytokine mRNA expressions. Am J Trop Med Hyg. 2009 May;80(5):832-6.
9. Metz CE. Basic principles of ROC analysis. SeminNucl Med. 1978;8(4):283-298. doi:10.1016/s0001-2998(78)80014-2.
10. Patra KP, Choudhury B, Matthias MM, Baga S, Bandyopadhya K, Vinetz JM. Comparative analysis of lipopolysaccharides of pathogenic and intermediately pathogenic Leptospira species. BMC Microbiol. 2015 Oct 30;15:244. doi: 10.1186/s12866-015-0581-7.
11. Que-Gewirth NL, Ribeiro AA, Kalb SR, Cotter RJ, Bulach DM, Adler B, Girons IS, Werts C, Raetz CR. A methylated phosphate group and four amide-linked acyl chains in leptospira interrogans lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. J Biol Chem. 2004 Jun 11;279(24):25420-9. doi: 10.1074/jbc.M400598200.
12. Surujballi O, Mallory M. Competitive enzyme-linked immunosorbent assay for detection of Leptospira interrogans serovar pomona antibodies in bovine sera. Clin Diagn Lab Immunol. 2001;8(1):40-43. doi:10.1128/CDLI.8.1.40-43.2001
13. Ulevitch RJ, Tobias PS. Recognition of endotoxin by cells leading to transmembrane signaling. Curr Opin Immunol. 1994 Feb;6(1):125-30. doi: 10.1016/0952-7915(94)90043-4.
14. Vanithamani S, Shanmughapriya S, Narayanan R, et al. Lipopolysaccharide Specific Immunochromatography Based Lateral Flow Assay for Serogroup Specific Diagnosis of

Leptospirosis in India. PLoS One. 2015;10(9):e0137130. Published 2015 Sep 4. doi:10.1371/journal.pone.0137130.

15. Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol. 2001 Apr;2(4):346-52. doi: 10.1038/86354.
16. Westphal, O., Jane, K., 1965. Bacterial liopolysaccharides extraction with phenol water and further applications of the procedure. Meth Carbohydr Chem 83-91.
17. Widiyanti D, Koizumi N, Fukui T, Muslich LT, Segawa T, Villanueva SY, Saito M, Masuzawa T, Gloriani NG, Yoshida S. Development of immunochromatography-based methods for detection of leptospiral lipopolysaccharide antigen in urine. Clin Vaccine Immunol. 2013 May;20(5):683-90. doi: 10.1128/CVI.00756-12.
18. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem. 1993;39(4):561-577.

LPS imprinted Biosensor for Leptospirosis Detection

CHAPTER V

Chapter V: Lipopolysaccharide Imprinted Biosensor for Rapid Diagnosis of Leptospirosis- Electrochemical Detection Strategy

In this chapter, we report for the first time a lipopolysaccharide (LPS) based electrochemical biosensor that offers a rapid, highly sensitive, serogroup specific diagnosis of leptospirosis during the acute stage of infection and also to distinguish from other flu like infections. The proposed sensor is fabricated by the immobilization of LPS onto a dodecanethiol (DT) modified gold electrode. A monolayer of DT is attached through covalent bond (Au-S) interaction onto the gold electrode. Thus, leptospiral antibodies from the human serum samples bind to the LPS present on the self-assembled monolayer (SAM) of DT. This biosensor is the first electrochemical sensing platform used for the detection of LPS from Leptospira spp.

5.1 Background and motivation

Leptospira is a member of the order Spirochaetales and belongs to the family Leptospiraceae, which can be either pathogenic or saprophytic (Bharti et al., 2003). Pathogenic leptospires are present in the renal tubules of infected or carrier animals (Faine et al., 1999). Rodents are the major reservoir of leptospirosis, which is an important emerging global public health problem distributed worldwide. The diagnostic tests incurred costs, between 15.3 and 17.2 USD per patient (Sanhueza et al., 2020). Usually, the infection rate is higher in the tropical regions than in the temperate region (Haake and Levett, 2015). The clinical manifestations vary from mild flu like illness to multi-organs failure and in serious conditions, it may even lead to the death of the infected host (Goris et al., 2011). However, during the early stage of illness, the clinical symptoms of leptospirosis are non-specific and challenging to distinguish from dengue, malaria, influenza, and many other febrile diseases (McBride et al., 2005). Hence, laboratory based tests are needed to confirm the diagnosis to initiate proper treatment and that is vital for controlling

the morbidity and mortality levels (Budihal et al., 2014). The incidence of leptospirosis and its associated mortality rates with an increased outbreak identified leptospirosis as a fatal disease worldwide (Merien et al., 2005).

Even though numerous techniques are used for the diagnosis of leptospirosis, performing a microscopic agglutination test (MAT) to demonstrate four-fold rise in antibody titre and isolation of leptospires from the infected specimen (blood, urine, and/or relevant fluids are employed as target samples) are the direct confirmatory evidence of leptospirosis (Hull-Jackson et al., 2006). Though MAT is deemed as a "gold standard" reference serovar specific test for the diagnosis of leptospirosis, it is difficult to perform. Also, it involves a large panel of live leptospiral culture as antigen and moreover, it needs professional experience and skilled manpower for the interpretation of results (Winslow et al., 1997). As leptospirosis is a critical human health problem in developing or under-developed tropical countries, thus, a precise diagnostic format that can be used for point-of-care analysis is vital. Enzyme-linked immunosorbent assay (ELISA), dipstick assay, Immuno-chromatography Lateral Flow Assay (ICG-LFA), multiple cross displacement amplification-lateral flow biosensor (MCDA-LFB) method, macroscopic agglutination test, and microcapsule agglutination are the other diagnostic formats developed for the diagnosis (Widiyanti et al., 2013; Li et al., 2019). Among these methods, ELISA offers acceptable sensitivity and the option of handling many samples at a time and has been established and evaluated with a few variations starting with the use of crude antigens to conserved peptides, etc. (Terpstra et al., 1985; Kanagavel et al., 2014). A Recent study established the role of circulating stable microRNAs (miRNAs) (miR-21-5p, miR-144-3p, and miRlet-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs were able to discriminate acute leptospiral infection from other febrile diseases (Akino Mercy et al., 2020).

However, the above-mentioned diagnostic techniques are primarily based on the conserved protein antigens/miRNAs and cannot be employed for serogroup level identification. To date there are no serogroup specific diagnostic formats to replace the reference, MAT.

Lipopolysaccharide (LPS), a major component present in the outer membrane of *Leptospira* is one of the major essential immuno-dominant antigens. The antigenic nature of LPS initiates the immuno-modulatory activity during pathogenesis and structural variation between serogroup helps in the serogroup level identification of Leptospira species (Vanithamani et al., 2015; Stromberg et al., 2017). Concerning, validation of such serogroup specific antigens in diagnostics can serve as an alternative to MAT. Electrochemical detection is a desirable unconventional technique for application in primary health care diagnosis due to its considerable advantages for a point-of-care testing facility. Electrochemical immunosensor based on gold-labeled monoclonal anti-LipL32, Loa22 gene based DNA biosensor for the diagnosis of leptospirosis was developed and being a genus-specific technique unable to find the infecting serovar (Jampasa et al., 2019; Verma et al., 2021; Nagraik et al., 2019).

In this context, here, for the first time, we attempt to develop an electrochemical biosensor for serogroup level diagnosis of leptospirosis. Electrochemical sensors offer rapid response, higher sensitivity, simplicity, and ease to use (Hammond et al., 2016). Particularly the sensing layer for LPS detection includes a SAM of dodecanethiol (DT), antibodies, and functionalized LPS that generates the change in the electronic properties upon binding of LPS to specific antibodies. Binding events are then probed by measuring the electron transfer (ET) reaction associated with the redox-active species in a solution using electrochemical impedance spectroscopy (EIS) (Boubour and Lennox, 2000). The charge transfer resistance, RCT is determined by fitting the experimental data to a semicircle and determining its intercept on the x-axis based on the

modified Randle's equivalent circuit. Later, this parameter is correlated with the binding of the target analyte onto the modified electrode surface.

In order to develop a serogroup specific diagnosis, we target to build up a leptospiral LPS-based electrochemical immunosensor using a gold electrode modified with SAM of DT. The binding events were measured using EIS, and our results reveal that the sensor has higher selectivity and sensitivity to the leptospiral specific homologous sera compared to heterologous and sera from other febrile cases. The analytical performances were evaluated systematically for the developed immuno-sensors for their applicability for a serogroup specific diagnosis of leptospirosis. The proposed LPS-based detection strategy allows serogroup specific detection due to the unique structural difference that exists between the serogroups. Until now, MAT is considered a gold standard technique for serogroup level identification of leptospirosis, but it is laborious and difficult to maintain live leptospiral cultures all the time while performing the experiments and inter-laboratory variations may also occur. Hence the proposed LPS-based strategy allows one to overcome the drawbacks of MAT and is ideal an alternative to MAT.

5.2. Experimental procedures and characterization

5.2.1 Electrochemical studies

The binding events that occur during the sensing of these LPSs for the diagnosis of leptospirosis were monitored using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). All these electrochemical studies were performed with an Autolab PGSTAT 302 instrument (Metrohm, Autolab-Potentiostat/ Galvanostat Instruments (PGSTAT302N), Netherlands). The electrochemical properties of the monolayer towards the electron transfer process were studied using K[Fe(CN)6]3-/4- redox couple as a probe. The electrolyte solutions

used for electrochemical measurements were prepared using Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany) having a resistivity of 18.2 MΩ cm. CV and EIS studies were conducted in a three-electrode system at 25ºC consisting of a platinum rod/ wire, a saturated calomel electrode (SCE), and a modified gold electrode as a counter, reference, and working electrodes respectively. Cyclic voltammetry was performed in 1 mM potassium Ferro/ferricyanide aqueous solution containing 0.1M NaF as a supporting electrolyte. Impedance measurements were carried out at the formal redox potential (E1/2 = 0.16 V vs. SCE, as determined from cyclic voltammetry) in an aqueous solution containing equal concentrations of both the oxidized and reduced forms of $[Fe(CN)_6]^{3-/4-}$ (1 mM each) with 0.1M NaF as a supporting electrolyte (Chowdhury et al., 2019). The frequency ranging from 100 kHz to 0.01Hz with an ac amplitude of 5 mV was used for the impedance analysis.

5.2.2 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra were recorded with a MulitiLAB system (Multilab 2000, Twin anode X-ray source, Thermo Scientific, UK). Gold-coated plates were used to prepare the samples that were mounted with a spring clip during the measurement. A monolayer of DT was prepared by immersing the cleaned gold strips into neat DT for 1h. After this, the SAM modified gold surface was thoroughly rinsed with ethanol and Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany). Then, DT monolayer-modified gold electrode was dipped into an aqueous solution of LPS having a concentration ranging from 20 nM – 100 µM for about 1h. After this LPS-DT modified gold electrode was rinsed with Milli-Q water. Further, the LPS-coated DT monolayer-modified gold electrodes were dipped into an aqueous solution of 100 µM bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for about 1h each, after which they were thoroughly rinsed with Milli-Q water. After blocking with BSA, the LPS coated

electrodes were dipped into an aqueous solution of either the homologous or the heterologous patient's sera (contain the specific antibodies against the leptospiral LPS) and sera of healthy controls. In a typical experiment, a few survey scans in the binding energy ranging from -10 eV to 1350 eV were collected at a resolution of 1.0 eV. Then, detailed scans of 10 eV – 30 eV over a single feature/ element were collected at a resolution of 0.1 eV. XPS is primarily used to identify the presence of elements along with their corresponding oxidation states and the confirmation of LPS binding onto the modified electrode surfaces.

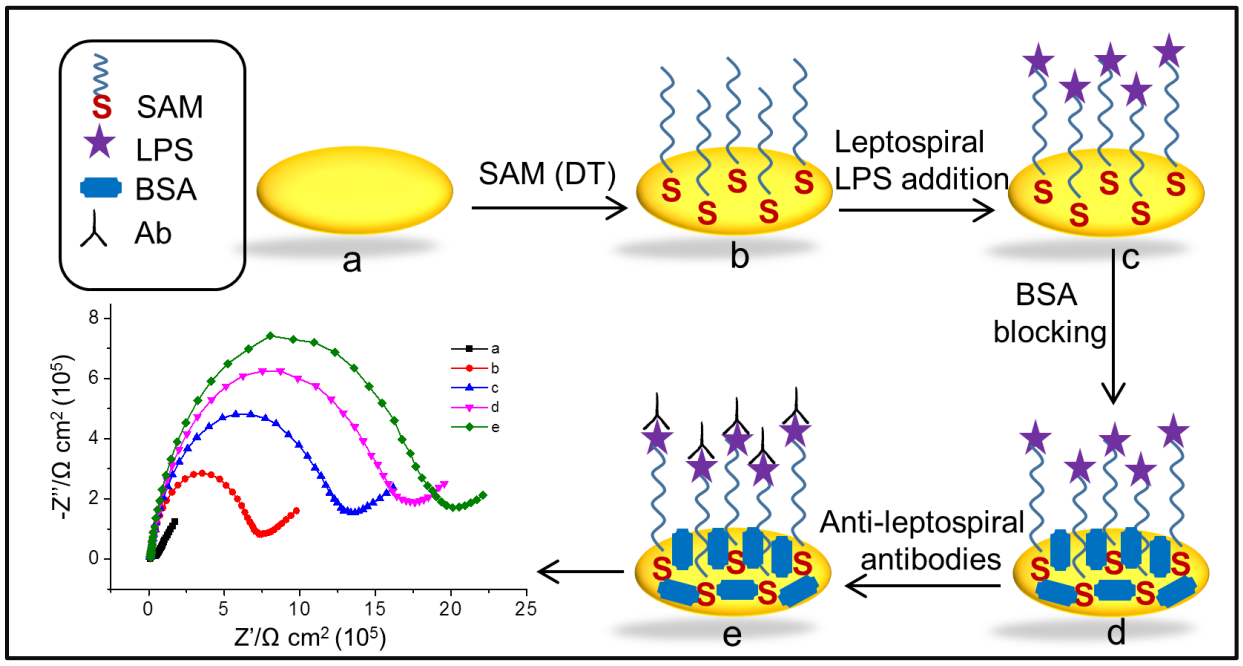
5.2.3 Contact angle measurements and Atomic Force Microscopy (AFM) analysis

Water-drop contact angle (WDCA) measurements were also performed at room temperature by employing the sessile drop technique using a commercial VCA Optima instrument. All the contact angle values were determined from the average of at least three independent measurements performed under the same conditions at different locations on the sample surface. Similarly, the surface topography of bare gold electrode, DT monolayer modified Au, leptospiral LPS immobilized DT electrode and the anti-leptospiral antibody functionalized Au electrode was analyzed using Atomic Force Microscopy [AFM] (5500 scanning probe microscope, Agilent Tech., USA) in a non-contact mode. The surface morphological features and thickness profiles were analyzed using the software provided by them.

5.3. Results

5.3.1 Construction and characterization of the sensing electrode

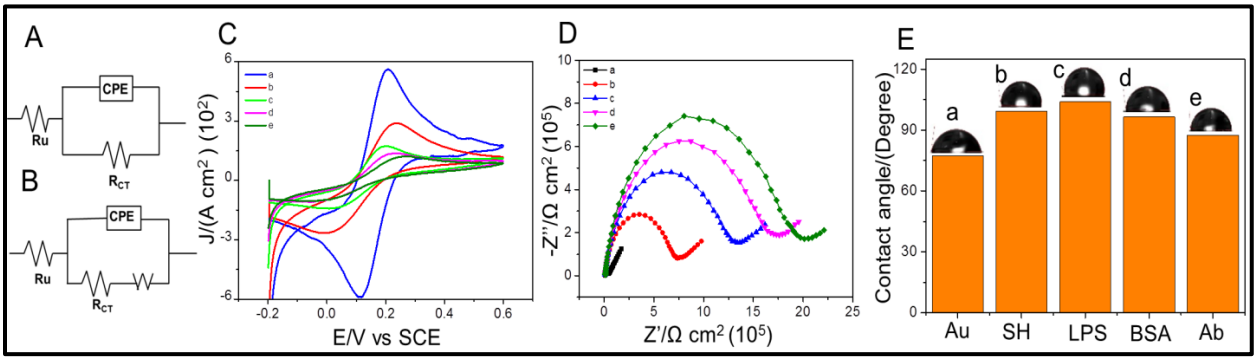
Scheme 5.1 Schematic representation of the biosensor construction process.



***Legends**: (a) bare gold electrode, (b) DT monolayer modification, (c) DT + LPS modification, (d) DT + LPS + BSA addition and (e) DT + LPS + BSA + Anti-leptospiral antibody modification.*

Herein, we demonstrated a simple and efficient way to detect Leptospira specific antibodies using chemically modified gold electrodes. The overall construction process is depicted in Scheme 5.1 A-E. Evaluation of surface modification steps and probing of the binding events towards the development of an electrochemical biosensors for the detection of leptospirosis is carried out using electrochemical techniques namely cyclic voltammetry and impedance measurements using potassium Ferro/ferricyanide as a redox probe.

Figure 5.1: Modified Randle's equivalent circuit diagram of the proposed sensor (A and B). Cyclic voltammograms (C) and Impedance (Nyquist) plots (D) and Water Drop Contact Angle (WDCA) analysis (E).



Legends: *(a) bare Au (Au), (b) DT monolayer modified Au (Au-SH), (c) DT+LPS modified (Au-SH-LPS), (d) DT+LPS+BSA modified (Au-SH-LPS-BSA) and (e) DT+LPS+BSA+Antibody modified (Au-SH-LPS-BSA-HIS) electrode respectively.*

Impedance spectroscopy data were analyzed using an equivalent circuit fitting procedure based on Randle's equivalent circuit model (Figure 5.1A and 5.1B) (sometimes modified one) to determine the charge transfer resistance (RCT), a parameter used to quantify the rate of electron transfer across the interface, in turn, the binding events. Figure 5.1C and 5.1D show the CVs and Nyquist plots of bare Au electrode before modification and after different modification steps used for the fabrication of the sensor along with the corresponding equivalent circuits used for fitting the measured impedance data and for the determination of parameters like RCT. Here Z' represents the real part and Z'' denotes the imaginary part of the impedance at the frequency range between 100 kHz and 0.01 Hz with an alternative current amplitude of 5 mV.

Several factors contribute to the electronic or ionic properties of the surface electrode (Table 5.1), such as the solution resistance (RS), the double layer capacitance (C) associated with

Warburg formed by the ions at the vicinity of the electrode, the charge transfer resistance (RCT) that represents the current flow due to redox reactions at the electrode electrolyte interface upon addition of different elements such as LPS, BSA and anti-leptospiral antibodies and a constant phase element.

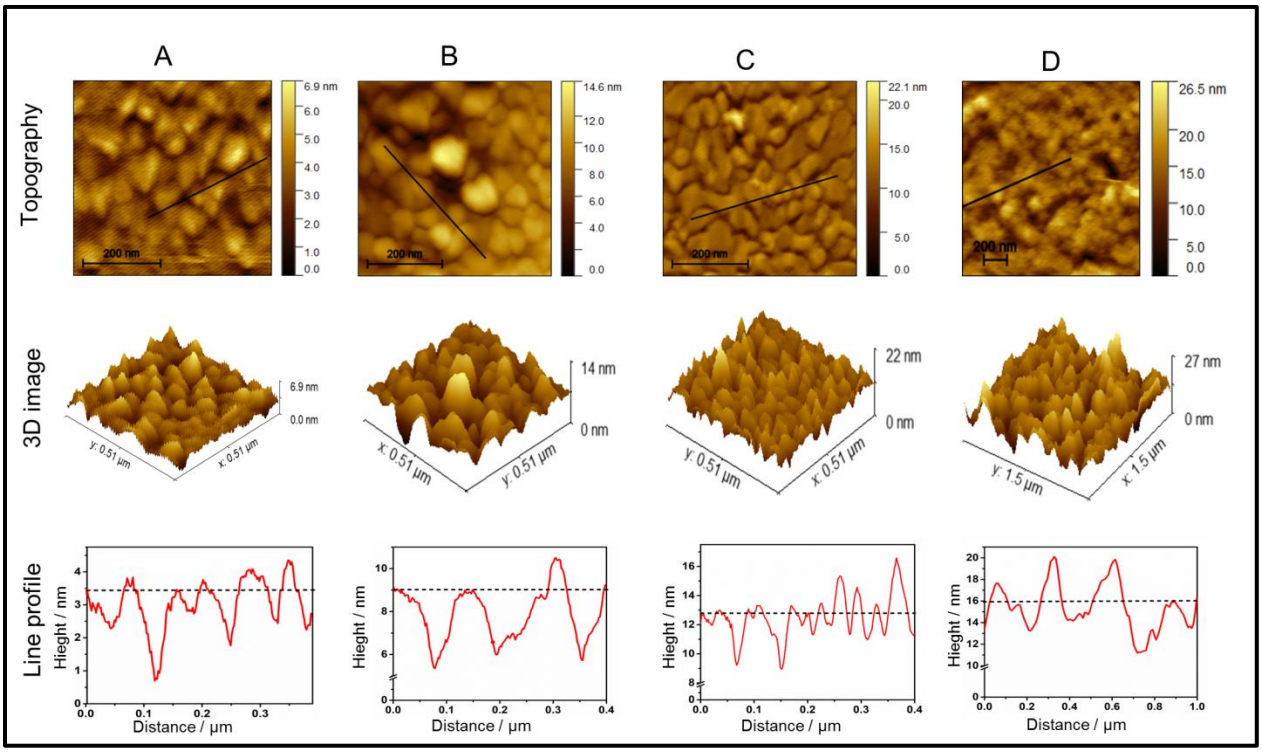
Table 5.1: Elements present in the equivalent circuit used for fitting the impedance data of proposed sensor (Grippotyphosa).

Monolayer	**R_S**	**CPE (x 10^{-7})**	**Freq**	**R_{CT}**	**Warburg(x10^{-5})**	**Chsq**
Bare	342	0.104	0.914	3964	7.793	7.43 x10^{-3}
SAM	444.7	3.649	0.861	1.669 x10^{5}	4.786	1.59 x10^{-2}
LPS	409.7	2.785	0.828	4.19 x10^{5}	2.279	1.48 x10^{-2}
BSA	622.7	6.01	0.835	6.86 x10^{5}	2.241	9.09 x10^{-3}
HS	357.8	3.149	0.818	3.24 x10^{6}	1.179	2.21 x10^{-2}
HES	353.9	6.572	0.814	2.218 x10^{6}	1.097	3.14 x10^{-2}
HC	376.2	3.67	0.807	1.757 x10^{6}	1.586	2.95 x10^{-2}

To illustrate the surface binding after each modification step, WDCA measurements were performed, and the results are shown in Figure 5.1E. WDCA analysis is used to determine the wettability of the functionalized surfaces. Initially bare Au surface without any modification exhibits a contact angle of 83.20º, while the contact angle of SAM-modified surface was measured to be 99.30º, revealing the formation of a monolayer of DT leading to the hydrophobicity of the resultant modified surface. Further, the addition of leptospiral LPS onto the SAM modified surface increases the contact angle to 102.40º indicating the enhancement in the hydrophobic nature of the surface due to the presence of a long alkyl chain from the LPS moiety. In contrast, after attachment of the anti-leptospiral antibody to LPS anchored DT-

modified surface through the antigen-antibody interaction a decrease in the contact angle of 90.00º is noted; revealing its switch over to a slight hydrophilic nature due to the presence of hydrophilic amino acids in the antibody. Thus, these results provide evidence for the modification steps used in our study for the fabrication of sensors.

Figure 5.2: Representative AFM images displaying surface topography, 3D images, and line profiles of various modified electrodes used in this study.



Legends: *Panel A- bare gold electrode, Panel B- DT-monolayer, Panel C- leptospiral LPS modified, and Panel D- anti-leptospiral antibody functionalized electrodes, respectively.*

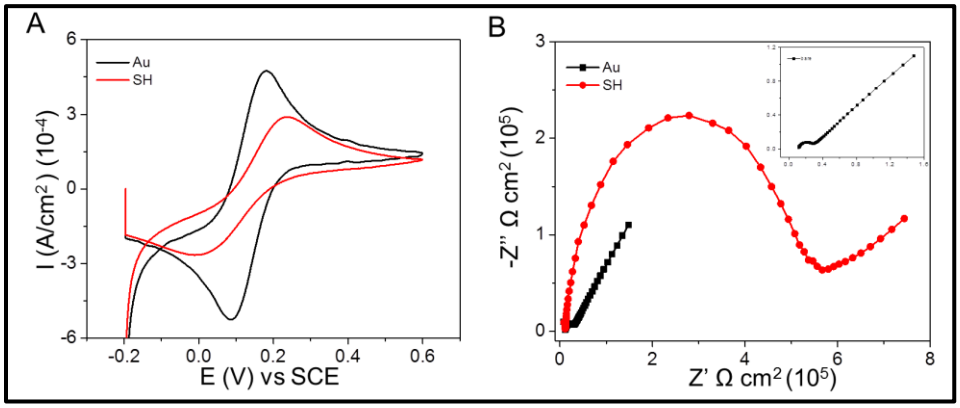
Figure 5.2 displays the representative AFM images of gold electrodes before and after each modification step employed for the fabrication of the biosensor. As seen in Figure 5.2, the bare gold surface showed a smooth surface with an average height of 3.45 nm. After the

formation of a monolayer of DT, the surface height increased to 9 nm, further upon immobilization of LPS, the surface height increased to12.8 nm. Finally, after the functionalization with the antibody, the surface height increased further up to 16 nm. The increasing surface height along with the roughness factor is mainly attributed to the adsorption of molecules in each modification step. These results clearly confirm the functionalization of Au electrodes with the target molecules and reaffirm the formation of different layers on the Au surface during the modification.

5.3.2 Impedimetric response of the chemically modified gold electrodes

Before modification, the electrochemical response of the bare electrode was recorded. The bare Au electrode shows a clear redox peak observed at 0.276 V and 0.201 V in cyclic voltammograms due to the redox reaction occurring on the surface of the gold electrode. Initially, a self-assembled monolayer of DT is formed on the gold electrode, which leads to the formation of a highly organized hydrophobic monolayer that blocks the electron transfer process. A drastic decrease (2-fold in comparison to bare Au) in the redox current is noted in CV after the addition of DT, which indicates that the electron transfer reaction is inhibited (Fig. 5.3A).

Figure 5.3. Electrochemical characterization of the sensing electrode.



Legends: *(A) Cyclic voltammograms and (B) Impedance (Nyquist) plots in an aqueous solution containing 1 mM [Fe(CN)6]3-/4- and 0.1M NaF for bare gold electrode and DT monolayer modified electrode compared to bare Au electrode. (Inset. Nyquist plot of bare Au electrode)*

The Nyquist plot shows, an obvious semicircle up to low frequency with the RCT value of 1.28 x 104 Ω cm^2, revealing the charge transfer controlled process for the redox probe on SAM modified electrode (Figure 5.3B). Figure 5.3B inset shows the Nyquist plot of the bare gold electrode shows a small semicircle with a straight line in the Nyquist plot of impedance spectroscopy and the corresponding RCT value is measured to be 3850 Ω cm^2 which defines the character of a diffusion-controlled process. This EIS data indicates the successful formation of the DT monolayer on the gold electrode and hindering the electron transfer reaction on the surface which correlates well with the CV results. This is due to the inability of the redox species to penetrate the monolayers onto the conductive electrode surface. To examine the surface structure of DT monolayer modified electrode XPS studies were performed.

Figure 5.4: XPS survey spectrum of DT monolayer modified gold slide (A) and S 2p spectrum (B) of the same electrode showing the presence of sulphur peaks.

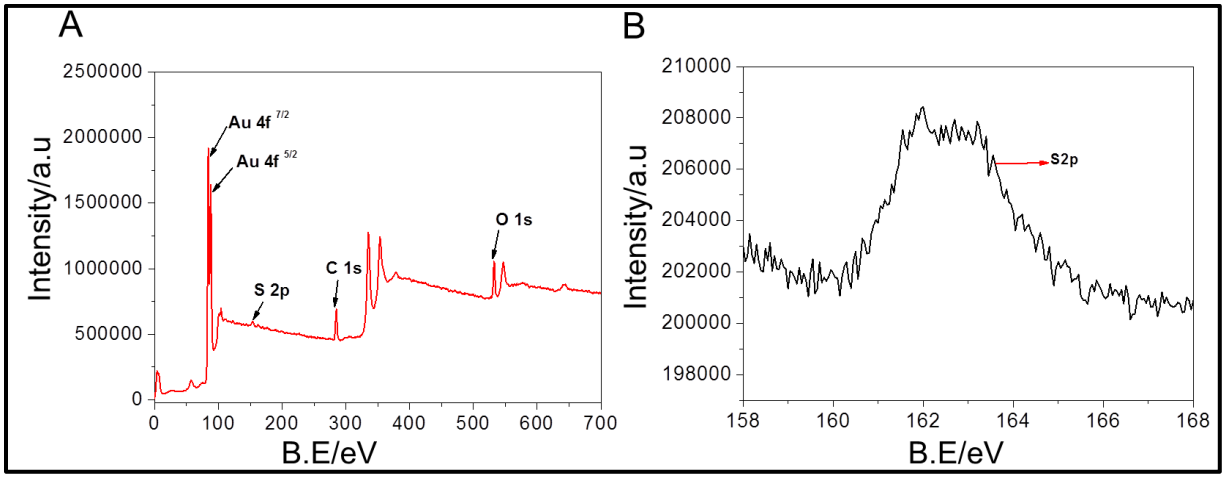
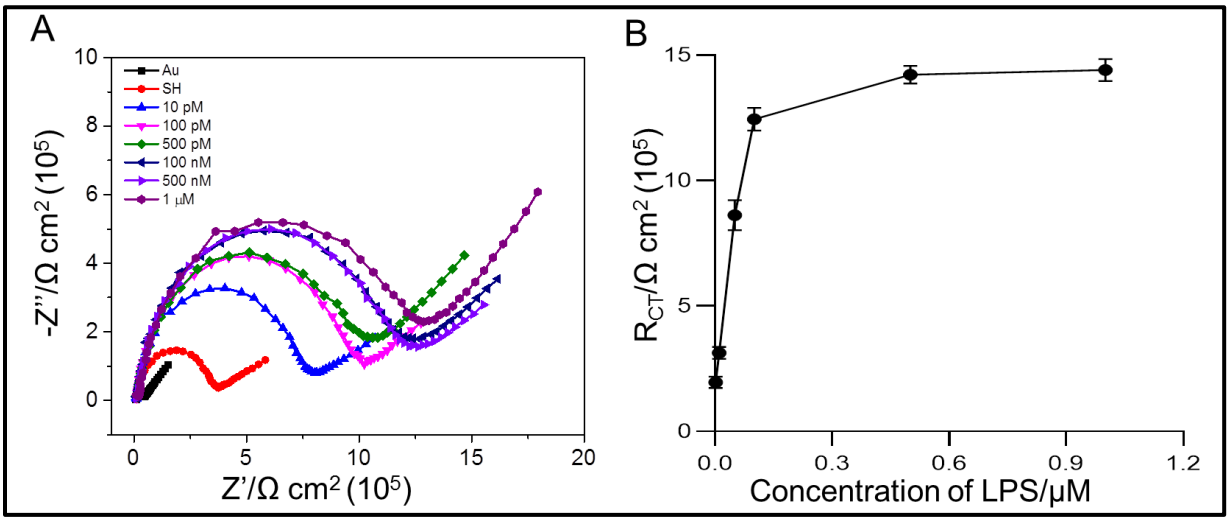


Figure 5.4A depicts the XPS survey spectrum of DT monolayer modified Au electrode and Figure 5.4B reveals the presence of sulphur peaks (S2p) at 162.3 eV corresponding to the sulfhydryl group. DT can interact with Au electrode through sulfhydryl groups forming Au-S interaction leading to the formation of organized SAM.

5.3.3 Functionalization of SAM coated sensing electrode

In order to achieve the serogroup detection of leptospirosis, the SAM coated electrode was functionalized with the addition of leptospiral LPS. Such chemical modification is carried out by anchoring the leptospiral LPS onto the DT monolayer films through hydrophobic interactions between the long aliphatic chains from LPS with the alkyl chains of the monolayer. To detect the optimal concentration of leptospiral LPS for the detection of leptospirosis the impedance responses were recorded for the addition of different concentrations of leptospiral LPS to the SAM coated surface in the range from 10 pM, 100 pM, 500 pM, 100 nM, 500 nM to 1 µM and their corresponding RCT values were measured to be 7.6 x 10^5 Ω cm^2, 9.74 x 10^5 Ω cm^2, 10.09 x 10^5 Ω cm^2, 11.76 x 10^5 Ω cm^2, 11.79 x 10^5 Ω cm^2 and 12.44 x 10^5 Ω cm^2 respectively (Figure 5.5A). The Binding of LPS is identified to be remarkably effective in the concentration range of 100 nM (Figure 5.5B).

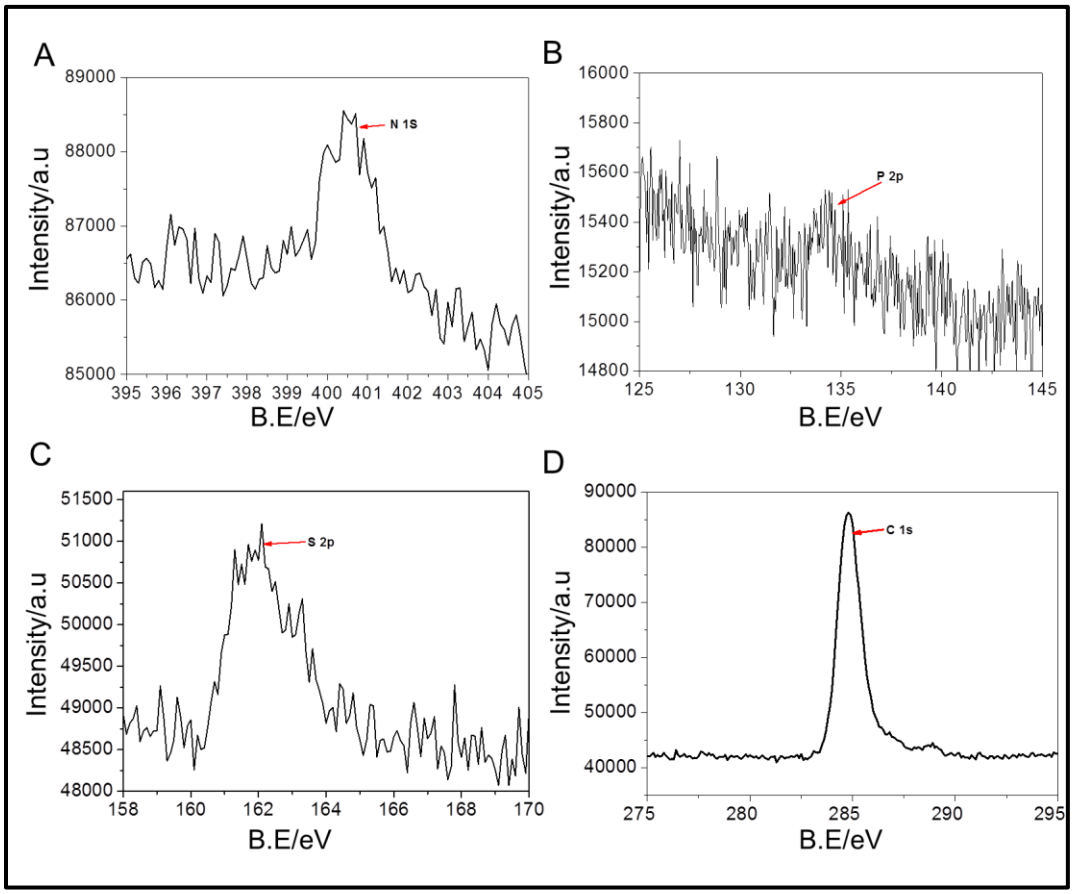
Figure 5.5: Optimization of leptospiral LPS concentration.



Legends: *(A) Impedance plots in an aqueous solution containing 1 mM* $[Fe(CN)_6]^{3-/4-}$ *and 0.1M NaF for different concentrations of Grippotyphosa lipopolysaccharide (Au- Bare Au, SH- DT-monolayer, 10 nM – 1 μM- different concentrations of LPS). The lines show the fitting of measured impedance data points using Randle's equivalent circuit. (B) The plot of the ratio,* R_{CT}/R^0_{CT}*, (where* R^0_{CT} *is the resistance to the electron transfer reaction before LPS addition and* R_{CT} *is the measured values for each addition of LPS) vs. concentration of Grippotyphosa LPS.*

The increase in RCT is probably related to both an increase in negative charges and the thickness of the molecular layer on the electrode surface. However, in some serogroups, the effect of mass transfer persists at the lower frequency range. To further probe into the structure of modified surfaces, XPS analysis of SAM coated gold electrode was carried out. XPS spectra of these modified electrodes display peaks due to nitrogen and phosphorous that appear at 399.1 eV and 132.8 eV due to the presence of LPS apart from the carbon and sulphur peaks noted at 285 eV and 162.9 eV arising from the DT monolayer respectively (Figure5.6).

Figure 5.6: XPS spectra of DT+LPS monolayer modified gold slide showing the presence of nitrogen (A), phosphorous (B), sulphur (C), and carbon (D) peaks respectively.



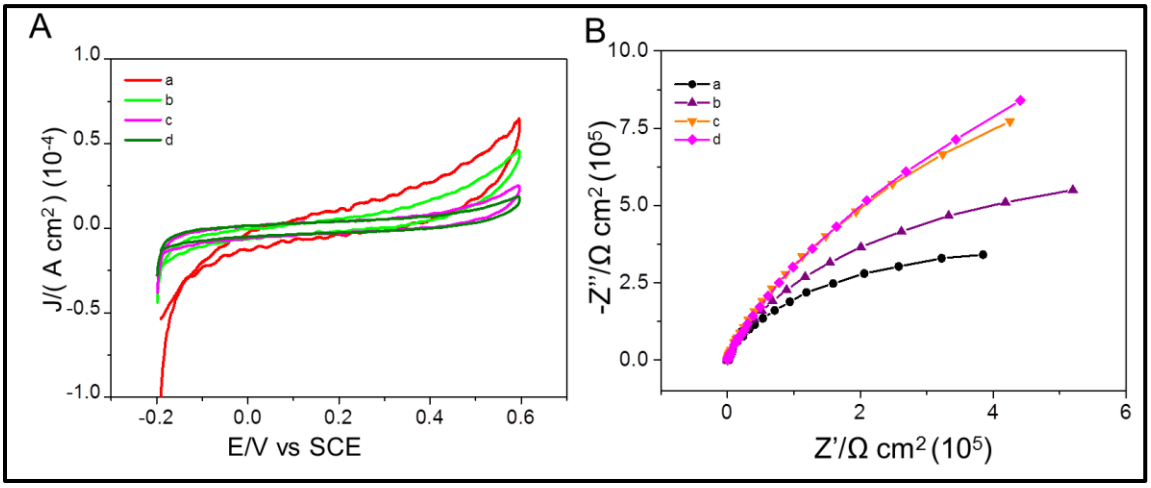
These results clearly suggest the anchoring of LPS onto a monolayer modified gold electrode. In order to saturate the electrode surface, BSA was immobilized and used as a surface covering agent. The addition of BSA, which blocks the surface other than LPS has the RCT value of 4.8 x 10^4 Ω cm^2 and allows the binding of antibodies specific to the target analyte.

5.3.4 Development of serovar specific biosensor

To develop the serovar-specific biosensor and to eliminate the possible cross-reactive binding of other serogroups, independent experiments were conducted using LPS of different leptospiral serogroups. Moreover, to improve the specific binding and to decrease the nonspecific binding

from other sera, homologous sera confirmed by MAT and ELISA analyses were used for the study. The LPS modified electrode was immobilized with the anti-leptospiral antibodies, for that the laboratory-confirmed leptospirosis patient's serum samples were used. EIS response of the sensor is drastically increased further, which indicates a strong affinity between the LPS and anti-leptospiral antibodies. To avoid the hook effect of the antigen-antibody interaction we design the experiments at different dilutions of the patient's serum containing the circulating anti-leptospiral antibodies against the leptospiral LPS.

Figure 5.7: CV and Nyquist plot of the sensor at different antibody dilutions



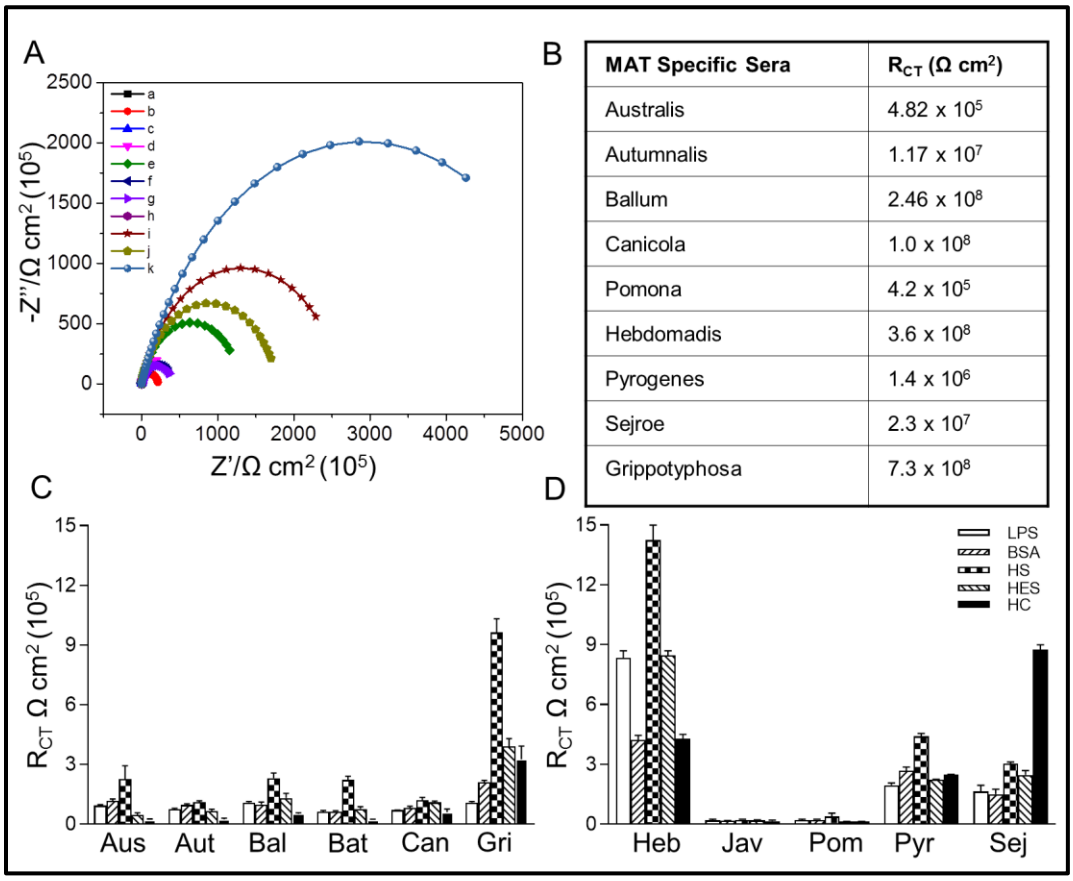
Legends: *(A) Cyclic voltammograms of dilutions of anti-leptospiral antibody in patient's serum against their LPS. (B) Nyquist plots recorded in an aqueous solution containing 1 mM [Fe(CN)6]3-/4- and 0.1 M NaF for different dilutions of anti-leptospiral antibodies. Legends: 1:1000 dilution (a), 1:500 dilution (b), 1:200 dilution (c), and 1:100 dilution (d) respectively.*

The representative RCT values for the dilution of 1:100 (5.682 x 10^7 Ω cm^2), 1:200 (5.475 x 10^7 Ω cm^2), 1:500 (5.089 x 10^7 Ω cm^2) and 1:1000 (4.77 x 10^7 Ω cm^2) respectively was determined (Figure 5.7A and 5.7B). These results indicate the decreasing RCT value while increasing the

dilution of the sera. Upon increasing the dilution of antibody further the RCT values are found to be decreased, confirming the decreasing concentrations of antibody. For all further experiments, a dilution of 1:1000 was used. At the same time, upon addition of healthy control serum samples, there was no significant increase in RCT is noted indicating the selectivity of the biosensor against only leptospiral specific antibodies.

Since the focus of our study is the serogroup specific detection of leptospirosis, we analyzed the interaction between Grippotyphosa LPS molecule to the MAT and ELISA confirmed patients' sera (anti-leptospiral antibodies). Hence the sensing electrode consisting of Grippotyphosa LPS was subjected to various MAT confirmed leptospiral cases which have specific reactivity to Australis, Autumnalis, Ballum, Canicola, Pomona, Hebdomadis, Pyrogenes, Sejroe, and Grippotyphosa. Their corresponding impedance responses were shown in Figure 5.8A from which their RCT values were determined and shown in Figure 5.8B.

Figure 5.8: Serovar specific analysis of the biosensor.



MAT Specific Sera	R_{CT} (Ω cm²)
Australis	4.82 x 10^5
Autumnalis	1.17 x 10^7
Ballum	2.46 x 10^8
Canicola	1.0 x 10^8
Pomona	4.2 x 10^5
Hebdomadis	3.6 x 10^8
Pyrogenes	1.4 x 10^6
Sejroe	2.3 x 10^7
Grippotyphosa	7.3 x 10^8

Legends: *(A and B) Impedance plots in an aqueous solution containing 1 mM* $[Fe(CN)_6]^{3-/4-}$ *and 0.1M NaF for the Grippotyphosa LPS against homologous and heterologous sera (a- bare Au, b- DT monolayer, c- DT+ GrippoLPS, d- DT+ Grippo LPS+BSA, e-j (DT+ Grippo LPS+ BSA+ Heterologous sera, k- DT+ GrippoLPS+ BSA+ homologous sera), and their RCT values. (C and D) Histogram of the RCT values for different serovars namely Australis, Autumnalis, Ballum, Bataviae, Canicola, Hebdomadis, Javanica, Pomona, Pyrogenes, and Sejroe respectively.*

Together the sensing electrode comprising Grippotyphosa LPS has a higher affinity to the Hebdomadis (3.6x 10^8 Ω cm^2) and Ballum (2.46x 10^8 Ω cm^2) compared to the Grippotyphosa

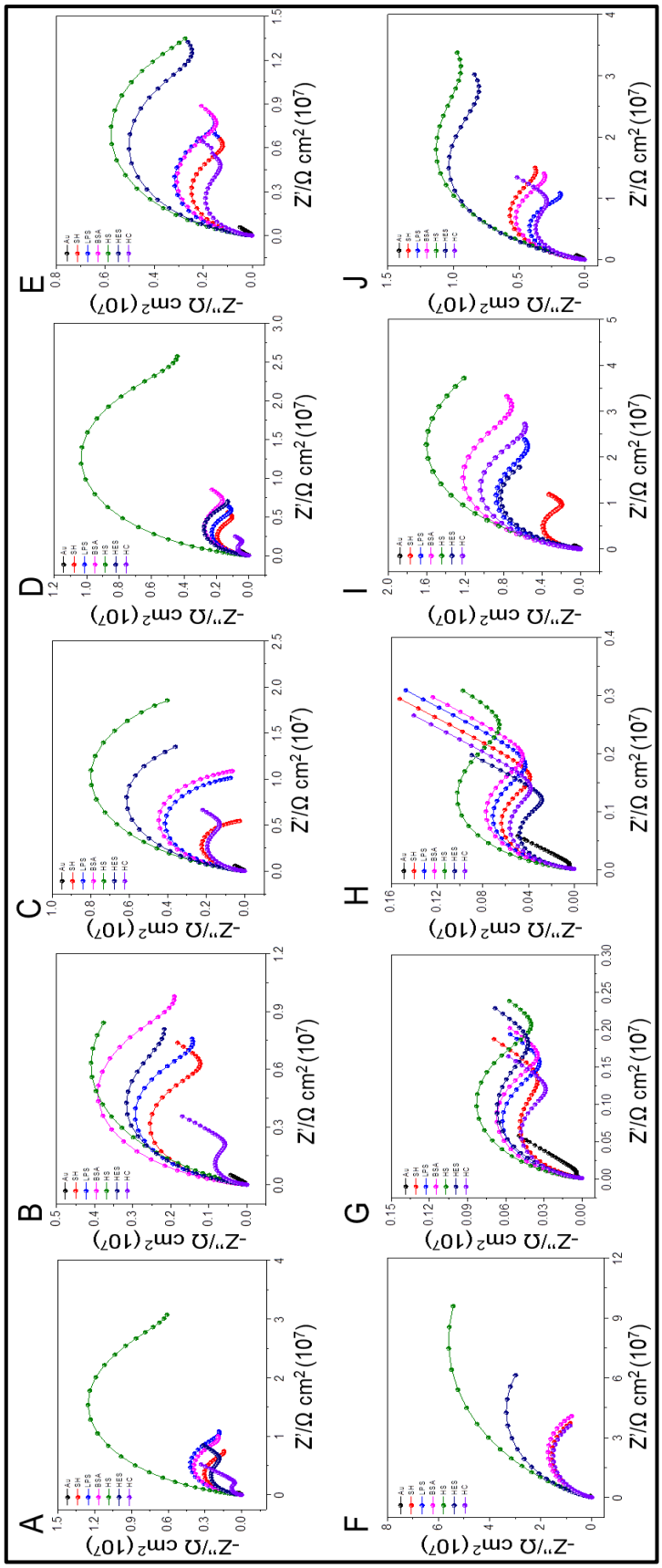
positive case (7.3 x 10^8 Ω cm^2). From these results, the proposed biosensor possesses higher specificity to the homologous sera than the heterologous sera.

A limited number of clinical samples (n=12) were used for the evaluation to assess the ability of biosensors in analyzing the 'real world' samples. As mentioned before the clinical samples were analyzed with serological assays such as MAT and ELISA to confirm the presence of anti-leptospiral antibodies prior to the biosensor experiments. The results summarized in Table 5.2 reveal that the sensor could be able to differentiate the healthy individual sera. The representative histogram (Figure 5.8C and 5.8D) demonstrates the specificity associated with each sensor. From these results, it is clear that the sensor has higher affinity to LPSs of Hebdomadis (8.694 x 10^7 Ω cm^2), Pyrogenes (2.071 x 10^7 Ω cm^2), Sejroe (1.331 x 10^7 Ω cm^2), Ballum (1.033 x 10^7 Ω cm^2) followed by Australis (0.979 x 10^7 Ω cm^2), Canicola (0.707 x 10^7 Ω cm^2), Autumnalis (0.683 x 10^7 Ω cm^2), Bataviae (0.547 x 10^7 Ω cm^2), Pomona (0.165 x 10^7 Ω cm^2) and Javanica (0.142 x 10^7 Ω cm^2) [Figure 5.9A-J].

Table 5.2: RCT values of the sensing electrode consisting of various leptospiral lipopolysaccharide with respect to the homologous and heterologous sera.

Serovar	R_{CT} values of different modified electrodes (x 10^7 Ω cm^2)				
	LPS	BSA	Homologous sera	Heterologous sera	Healthy sera
Australis	0.979	1.092	2.926	0.574	0.193
Autumnalis	0.683	0.893	1.172	0.745	0.156
Ballum	1.033	1.103	2.051	1.538	0.475
Bataviae	0.547	0.666	2.402	0.648	0.169
Canicola	0.707	0.710	1.343	1.158	0.442
Grippotyphosa	1.134	2.199	10.32	4.290	3.920
Hebdomadis	8.694	4.459	15.69	8.694	4.093
Javanica	0.142	0.151	0.188	0.169	0.109
Pomona	0.165	0.177	0.225	0.109	0.128
Pyrogenes	2.071	2.874	4.252	2.230	2.452
Sejroe	1.331	1.251	3.117	2.698	8.991

Figure 5.9: Nyquist plot for serovar specific biosensor

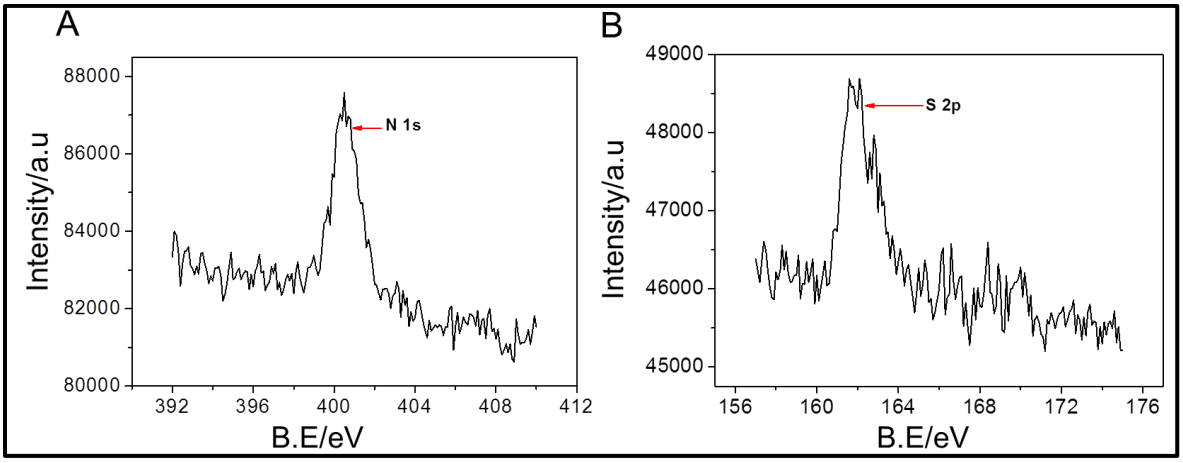


Legends: *Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1M NaF for different serovars namely Australis (A), Autumnalis (B), Ballum (C), Bataviae (D), Canicola (E), Hebdomadis (F), Javanica (G), Pomona (H), Pyrogenes (I) and Sejroe (J) respectively. (Au- bare Au, SH- DT monolayer, LPS- DT + LPS, BSA- DT + LPS + BSA, HS-DT + LPS + BSA + homologous sera, HES- DT + LPS + BSA + heterologous sera, HC-DT + LPS + BSA + healthy sera)*

Then there is a slight increase in RCT value upon the addition of BSA, which indicates the prevention of nonspecific interactions on the LPS functionalized electrode. With further addition of specific anti-leptospiral antibodies (leptospirosis confirmed (MAT) patient samples), a sharp increase in the RCT was observed. The representative RCT values for Hebdomadis, Pyrogenes, Sejroe, Ballum, Australis, Canicola, Autumnalis, Bataviae, Pomona and Javanica were 15.69, 4.252, 3.117, 2.502, 2.926, 1.343, 1.172, 2.402, 0.225 and 0.188 x 10^7 Ω cm^2 respectively. These findings reveal that the antigen-antibody complex was formed on the surface of the sensing electrode. Further, the binding of the anti-leptospiral antibody to the specific leptospiral LPS to form

the antigen-antibody complex occurs on the gold electrode as confirmed by XPS, which shows S 2p peak at 162.5 eV, and N 1s peak at 400.1 eV. Moreover, their corresponding intensity values were increased when compared to SAM and LPS modified electrodes confirming the presence of antibody (Figure 5.10) and reconfirms the binding.

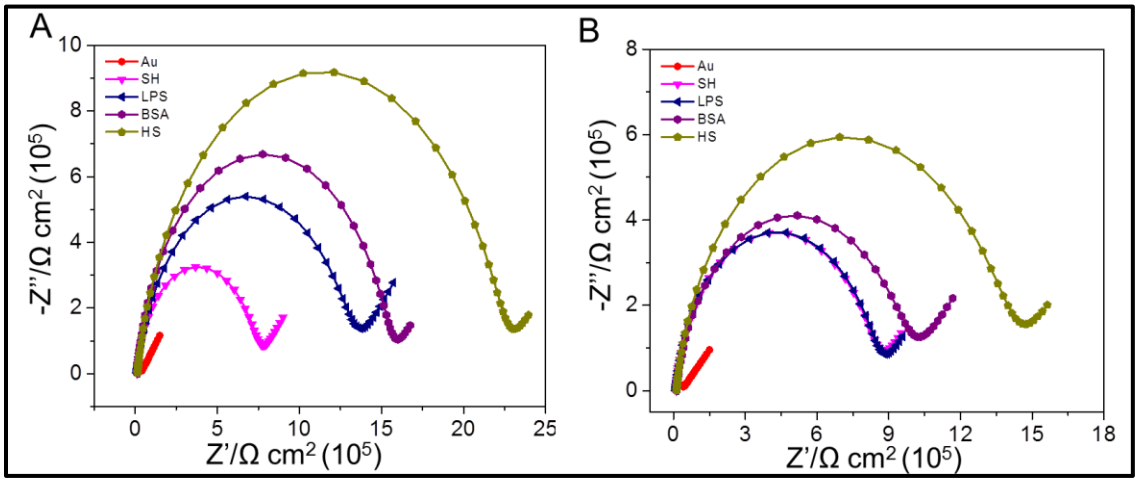
Figure 5.10: XPS spectra of the DT+LPS+antibody modified gold slide showing the presence of nitrogen (A) and sulphur (B) peaks.



5.3.5 Selectivity of the developed biosensor

In order to investigate the selectivity of this particular biosensor, the modified electrode has been tested with sera samples of dengue and typhoid patients, where the RCT values were measured to be 2.3 x 10^6 Ω cm^2 and 1.4 x 10^6 Ω cm^2 and compared the RCT value of Leptospira specific sera of 7.3 x 10^8 Ω cm^2, which was significantly higher.

Figure 5.11: Selectivity of the proposed sensor using different infectious serum samples.

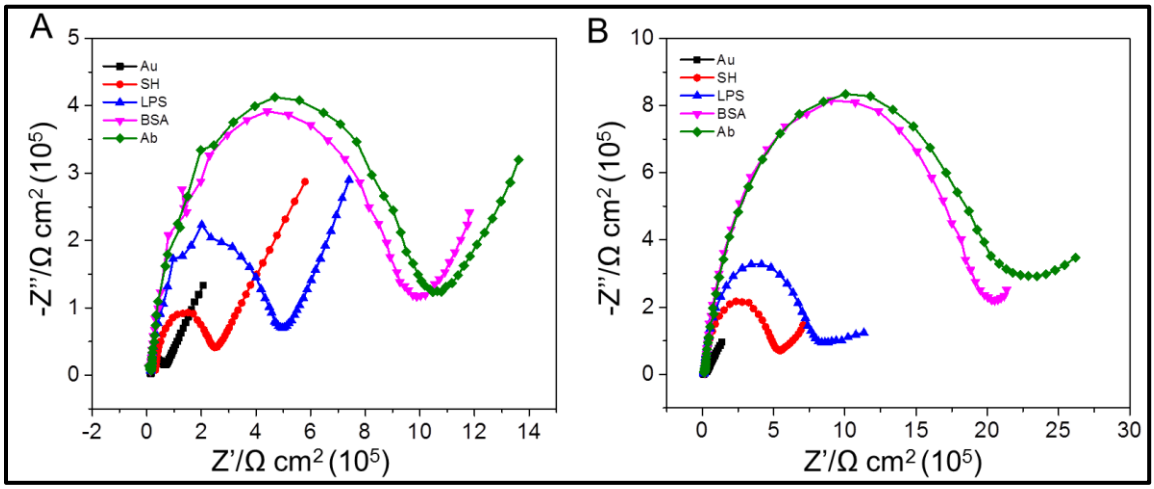


Legends: *Nyquist plots for the leptospiral LPS reactivity against the dengue (A) and typhoid (B) patient's sera. Legends: (Au- bare Au, SH- DT - monolayer, LPS- DT+ LPS, BSA- DT+ LPS+ BSA complex, HS- DT + LPS + BSA + Patient's sera (Dengue or Typhoid).*

As shown in the Nyquist plot (Figure 5.11A and 5.11B), the biosensor responses to the other samples are significantly lower due to the nonspecific interaction with the antibody, which makes the sensor electrode very specific to the target analyte of leptospiral LPS. Further, we also performed the electrochemical response for non-pathogenic leptospiral LPS to the anti-leptospiral antibody (patient's serum) Figure 5.12A, where the RCT value was found to be lower (2.8 x 10^6 Ω cm^2) compared to the pathogenic LPS. Also, the healthy control sera showed

decreased RCT (2.0 x 10^6 Ω cm^2) Figure 5.12B, thus confirming the higher specificity of the proposed biosensor.

Figure 5.12: Selectivity of the proposed sensor by using non-pathogenic LPS and healthy serum samples



Legends: *Nyquist plots in an aqueous solution containing 1 mM [Fe(CN)6]3-/4- and 0.1M NaF for the non-pathogenic leptospiral LPS reactivity against the patient's sera (A) and pathogenic leptospiral LPS reactivity against the healthy sera (B). Legends: (Au- bare Au, SH- DT- monolayer, LPS- DT+ LPS monolayer, BSA- DT+ LPS+ BSA complex, Ab- DT + LPS + BSA + Patient's sera and HS- DT + LPS + BSA + Healthy sera.*

5.4. Discussion

In the outer membrane of leptospires, LPS is the major antigenic component present, and it is thought of as the best immunogenic molecule because they are serogroup specific (Trueba et al., 1990). Since the laboratory diagnosis for leptospirosis mainly depends upon the serological techniques, the serogroup-specific MAT is the most widely used reference method these days. Generally, MAT detects both anti-leptospiral, IgM, and IgG and most of the agglutinating

antibodies confronted in MAT are IgM that is produced against the LPS in the acute phase of illness (Priya et al., 2011; Toyokawa et al., 2011). Even though MAT is considered the gold standard test for the diagnosis of leptospirosis, it is complex to control, perform, and interpret. Mostly it is performed with locally circulating predominant leptospiral serovars as live antigens and MAT forms the bases for serological diagnosis and for the classification of leptospires. Especially during outbreak conditions, investigation of such a large number of serum samples by a complicated technique like MAT may compromise the quality of results. Apart from these drawbacks, MAT also suffers from other major issues like cross-contamination of the antigen cultures, interference of the culture medium to affect the MAT titers, and the maintenance of a large number of strains that are hazardous for laboratory workers (Ramadass et al., 1992).

Under critical circumstances, the use of an alternate rapid serological diagnostic test is highly warranted. Serological tests like the microcapsule agglutination test (MCAT), Lepto Dipstick, Lepto Lateral Flow, and Lepto Dri Dot have been evaluated as rapid tests for leptospirosis as alternative techniques. But the majority of them are genus-specific and they mainly suffer from low sensitivity and specificity during the acute stage of the disease (Sehgal et al., 1997 & 2003; Vijayachari et al., 2002). An attempt was made to develop a serogroup specific diagnosis of leptospirosis by ICG-LFA, but in this array the Ballum LPS showed cross-reactivity with other serovars.

Further to improvise the rapidity of the technique and make it applicable in outbreak situations, a point of care serogroup specific diagnosis, LPS based electrochemical biosensor is proposed and demonstrated in this work for the first time and evaluated for the diagnosis of leptospirosis. This particular sensor is fabricated by immobilizing LPS onto the DT-modified gold electrode. This

test could potentially be assessed and employed as a point of care alternative test for MAT to detect the locally prevalent infecting serogroups in endemic regions and offers many advantages.

5.5. Conclusion

In conclusion, our results clearly demonstrate the rapid diagnostic method for the specific diagnosis of acute leptospirosis using electrochemical impedance measurements by employing LPS based serovar detection strategy. The proposed biosensor could sense the wider concentration of leptospiral LPS ranging from 10 pM to 1 µM. EIS measurements of LPS-based sensor offer a simple and promising method to develop an extremely effective and sensitive serogroup specific diagnosis of acute leptospirosis. This method has several significant advantages for the prevention of leptospirosis diseases, whereby this specific sensor could be directly employed to identify the serogroup specific leptospiral agglutinins. Further, the proposed sensor could also help for the timely initiation of antibiotics that may be critical for the treatment of leptospirosis.

5.6 References

1. Akino Mercy CS, Suriya Muthukumaran N, Velusamy P, Bothammal P, Sumaiya K, Saranya P, Langford D, Shanmughapriya S, Natarajaseenivasan K. MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis. mSphere. 2020 Jul 15;5(4):e00409-20. doi: 10.1128/mSphere.00409-20.
2. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM; Peru-United States Leptospirosis

Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003 Dec;3(12):757-71. doi: 10.1016/s1473-3099(03)00830-2.

3. Boubour E, Lennox RB. Insulating properties of self-assembled monolayers monitored by impedance spectroscopy. Langmuir. 2000 May 2;16(9):4222-8.
4. Budihal SV, Perwez K. Leptospirosis diagnosis: competancy of various laboratory tests. J Clin Diagn Res. 2014 Jan;8(1):199-202. doi: 10.7860/JCDR/2014/6593.3950.
5. Chowdhury AD, Takemura K, Li TC, Suzuki T, Park EY. Electrical pulse-induced electrochemical biosensor for hepatitis E virus detection. Nat Commun. 2019 Aug 19;10(1):3737. doi: 10.1038/s41467-019-11644-5.
6. Faine S, Adler B, Bolin C, Perolat P. Leptospira and leptospirosis, Melbourne. Australia: MediSci. 1999;259.
7. Goris MG, Wagenaar JF, Hartskeerl RA, van Gorp EC, Schuller S, Monahan AM, Nally JE, van der Poll T, van 't Veer C. Potent innate immune response to pathogenic leptospira in human whole blood. PLoS One. 2011 Mar 31;6(3):e18279. doi: 10.1371/journal.pone.0018279.
8. Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015;387:65-97. doi: 10.1007/978-3-662-45059-8_5.
9. Hammond JL, Formisano N, Estrela P, Carrara S, Tkac J. Electrochemical biosensors and nanobiosensors. Essays Biochem. 2016 Jun 30;60(1):69-80. doi: 10.1042/EBC20150008.
10. Hull-Jackson C, Glass MB, Ari MD, Bragg SL, Branch SL, Whittington CU, Edwards CN, Levett PN. Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis. J Clin Microbiol. 2006 May;44(5):1853-5. doi: 10.1128/JCM.44.5.1853-1855.2006.

11. Jampasa S, Lae-Ngee P, Patarakul K, Ngamrojanavanich N, Chailapakul O, Rodthongkum N. Electrochemical immunosensor based on gold-labeled monoclonal anti-LipL32 for leptospirosis diagnosis. Biosens Bioelectron. 2019 Oct 1;142:111539. doi: 10.1016/j.bios.2019.111539.
12. Kanagavel M, Shanmughapriya S, Anbarasu K, Natarajaseenivasan K. B-cell-specific peptides of leptospira interrogans LigA for diagnosis of patients with acute leptospirosis. Clin Vaccine Immunol. 2014 Mar;21(3):354-9. doi: 10.1128/CVI.00456-13.
13. Li S, Liu Y, Chen X, Wang M, Hu W, Yan J. Visual and rapid detection of Leptospira interrogans using multiple cross-displacement amplification coupled with nanoparticle-based lateral flow biosensor. Vector-Borne and Zoonotic Diseases. 2019 Aug 1;19(8):604-12.
14. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. Curr Opin Infect Dis. 2005 Oct;18(5):376-86. doi: 10.1097/01.qco.0000178824.05715.2c.
15. Merien F, Portnoi D, Bourhy P, Charavay F, Berlioz-Arthaud A, Baranton G. A rapid and quantitative method for the detection of Leptospira species in human leptospirosis. FEMS Microbiol Lett. 2005 Aug 1;249(1):139-47. doi: 10.1016/j.femsle.2005.06.011.
16. Nagraik R, Kaushal A, Gupta S, Dhar P, Sethi S, Kumar D. Optimized DNA-based bioassay for Leptospira interrogans detection: a novel platform for leptospirosis diagnosis. 3 Biotech. 2019 Jul;9(7):284. doi: 10.1007/s13205-019-1815-4.
17. Priya CG, Bhavani K, Rathinam SR, Muthukkaruppan VR. Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis. J Med Microbiol. 2003 Aug;52(Pt 8):667-673. doi: 10.1099/jmm.0.05120-0.

18. Ramadass P, Jarvis BD, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic Leptospira species by DNA hybridization. Int J Syst Bacteriol. 1992 Apr;42(2):215-9. doi: 10.1099/00207713-42-2-215.
19. Sanhueza JM, Baker MG, Benschop J, Collins-Emerson JM, Wilson PR, Heuer C. Estimation of the burden of leptospirosis in New Zealand. Zoonoses Public Health. 2020 Mar;67(2):167-176. doi: 10.1111/zph.12668.
20. Sehgal SC, Vijayachari P, Subramaniam V. Evaluation of leptospira micro capsule agglutination test (MCAT) for serodiagnosis of leptospirosis. Indian J Med Res. 1997 Dec;106:504-7.
21. Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. J Med Microbiol. 2003 Oct;52(Pt 10):897-901. doi: 10.1099/jmm.0.05064-0.
22. Stromberg LR, Mendez HM, Mukundan H. Detection methods for lipopolysaccharides: past and present. Escherichia coli-recent advances on physiology, pathogenesis and biotechnological applications. InTech. 2017 Jul 12:141-68.
23. Terpstra WJ, Ligthart GS, Schoone GJ. ELISA for the detection of specific IgM and IgG in human leptospirosis. J Gen Microbiol. 1985 Feb;131(2):377-85. doi: 10.1099/00221287-131-2-377.
24. Toyokawa T, Ohnishi M, Koizumi N. Diagnosis of acute leptospirosis. Expert Rev Anti Infect Ther. 2011 Jan;9(1):111-21. doi: 10.1586/eri.10.151.
25. Trueba GA, Bolin CA, Thoen CO. Evaluation of an enzyme immunoassay for diagnosis of bovine leptospirosis caused by Leptospira interrogans serovar hardjo type hardjo-bovis. J Vet Diagn Invest. 1990 Oct;2(4):323-9. doi: 10.1177/104063879000200413.

26. Vanithamani S, Shanmughapriya S, Narayanan R, Raja V, Kanagavel M, Sivasankari K, Natarajaseenivasan K. Lipopolysaccharide Specific Immunochromatography Based Lateral Flow Assay for Serogroup Specific Diagnosis of Leptospirosis in India. PLoS One. 2015 Sep 4;10(9):e0137130. doi: 10.1371/journal.pone.0137130.
27. Verma V, Kala D, Gupta S, Kumar H, Kaushal A, Kuča K, Cruz-Martins N, Kumar D. Leptospira interrogans Outer Membrane Protein-Based Nanohybrid Sensor for the Diagnosis of Leptospirosis. Sensors (Basel). 2021 Apr 6;21(7):2552. doi: 10.3390/s21072552.
28. Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. Epidemiol Infect. 2002 Dec;129(3):617-21. doi: 10.1017/s0950268802007537.
29. Widiyanti D, Koizumi N, Fukui T, Muslich LT, Segawa T, Villanueva SY, Saito M, Masuzawa T, Gloriani NG, Yoshida S. Development of immunochromatography-based methods for detection of leptospiral lipopolysaccharide antigen in urine. Clin Vaccine Immunol. 2013 May;20(5):683-90. doi: 10.1128/CVI.00756-12.
30. Winslow WE, Merry DJ, Pirc ML, Devine PL. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J Clin Microbiol. 1997 Aug;35(8):1938-42. doi: 10.1128/jcm.35.8.1938-1942.1997.

miRNA based Biosensor for Leptospirosis Diagnosis

CHAPTER VI

Chapter VI: Detection of Ultra low level miRNA based biosensor for the diagnosis of Leptospirosis

In this chapter, we aimed to develop a simple electrochemical biosensor for ultralow level detection of microRNA (miRNA) in human serum samples in particular leptospirosis infected cases. The biosensor had 3 major fabrication processes. Initially, the cleaned glassy carbon surface was electropolymerized with PANI followed by gold nanoparticles. Then the AuNP modified sensor was functionalized with thiol attached probe DNA (anti-miRNA 21). Finally, the sensor was subjected to hybridization with target miRNA 21 and leptospirosis serum. The developed sensor was confirmed electrochemically through cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and square wave voltammetry (SWV). The simple electrochemical strategy offers a fast and accurate diagnosis of infectious diseases at an early stage of infection.

6.1 Background and Motivation

A great challenge in modern society is associated with the increasing population along with health problems and diseases. Depending on the infection the host can induce an immune response in terms of antibodies, hormones, or proteins against the infectious agent. And these biomolecules are found in either blood or body fluids and acts as potential biomarkers for the detection of disease as a whole molecule or their DNA/RNA. Detection during the acute stage of infection is necessary to control the spread of the infection and help for therapeutic intervention, thereby decreasing the cost and controlling the infection. Several diagnostic tools available for disease detection still have several limitations. With the emerging prognostic value of miRNAs, research has been a focus on POC analysis using miRNA as a diagnostic tool.

MicroRNAs (miRNAs) are an abundant class of noncoding genes of small RNAs around 22 nucleotides long, regulating the gene expression. miRNAs were up regulated and down regulated in several types of cancer (Lee and Dutta, 2009; Zhao et al., 2014, Tan et al., 2018). For instance, miR-155 and miR-21 are up-regulated in breast cancer (Mattiske et al., 2012; Cardoso et al., 2016); miR145 is down regulated in several types of cancer including bladder cancer, colon cancer, breast cancer, ovarian cancer and prostate cancer (Xu et al., 2014; Cui et al., 2014); miR-91is down regulated in breast cancer. miRNAs have present in body fluids such as blood serum, plasma, urine, and saliva (Cortez et al., 2011; Condrat et al., 2020). Circulating miRNAs are highly stable blood, which is associated with an RNA binding protein Nucleophosmin 1, or present inside the microparticles and exosomes (Li et al., 2012; Correia et al., 2017).

Earlier studies reported that three miRNAs (miR-let-7b-5p, miR-144-3p, and miR-21-5p) were observed at increased levels in vivo during exposure to leptospiral LPS (Akino Mercy et al., 2020). Herein we fabricated a highly sensitive and selective electrochemical biosensor to detect miR-21-5p in leptospirosis infected individuals. We developed the PANI-AuNP modified GCE electrode for our sensing purpose. Using conducting polymers received great attention due to their superior electrical conductivity, good adhesion property, high conductivity, and stability (Wang et al., 2011). The deposition of polyaniline (PANI) on glassy carbon electrodes (GCEs) by electrochemical cyclic voltammetry (CV) method. PANI forms a porous coating and stable long time as they are not mechanically damaged (Das Neves and De Paoli, 1998; Dinh et al., 1998). To enhance the performance of the biosensor the electroactive surface of the sensor was functionalized with gold nanoparticles through electrodeposition. Electrodeposition of gold nanoparticles provides a rapid and cheap alternative method to chemical synthesis (Dai et al., 2004; Mohanty, 2011). Another reason for modifying the electrode surface with gold

nanoparticles was the binding of the thiolated probe on the gold nanoparticles. The fabricated sensor allows the detection of ultra-low levels of microRNA.

6.2 Materials and methods

6.2.1 Apparatus and Reagents

The electrochemical measurements were performed with a potentiostat/galvanostat from Metrohm Autolab, PGSTAT320N, controlled by NOVA 1.11 software. The three electrode system consists (i) glassy carbon electrode as the working electrode (3mm diameter), (ii) silver/ silver chloride as a reference electrode, and (iii) platinum wire as the counter electrode. Ultrapure Milli-Q water (18.0 Ωm) or 0.1% diethylpyrocarbonate (DEPC) treated autoclaved water to be used for buffer preparation and to increase the stability of the miRNA by decreasing the RNase activity. All the reagents were used without further purification.

The oligonucleotide probes were thiol modified anti-miRNA-21 (complementary to target miRNA 21) and miRNA 21, single mismatch and non-complementary sequences were obtained from the Invitrogen. The stock solution of oligonucleotide (anti-miRNA) was prepared in SSPE buffer (0.02 M EDTA, 2.98 M NaCl and 0.2 M phosphate buffer). The stock solution of miRNA-21 was prepared in Tris-Cl (0.02 M Tris, 0.14 M NaCl, 0.001 M MgCl2, 0.005 M KCl and 0.001 M CaCl2 (pH 7.4)).

6.2.2 Fabrication of electrochemical biosensor

6.2.2.1 Electropolymerization of aniline on the GCE surface

Prior formation of monolayer or addition the GC surface was cleaned by washing with distilled water, followed by CV electrochemical treatment with 0.5 M H2SO4 for 15 cycles (50 mV/s; -

0.1 to 1.5 V). Then the GCE surfaces were thoroughly polished with 0.3 and 0.05 µm α- Al2O3 powders on a soft cloth and were sonicated with double distilled water for 5 min to remove possible contaminants. Finally, the GC surface was washed with ultrapure water. The PANI coating was formed on GCE surfaces by dipping the electrode in 0.5 M H2SO4 electrolyte containing 0.1 M aniline monomers and processing CV from -0.2 V to 1.2 V at a scan rate of 50 mV/s for 10 cycles. After the polymerization of PANI, the fabricated PANI/GCEs were washed with double distilled water to remove unpolymerized aniline monomers remaining in the PANI coatings if any.

6.2.2.2 Electrodeposition of gold nanoparticles on GCE-PANI electrode

The resulting PANI-GCE electrode was further modified with electrodeposition of gold nanoparticles. The AuNP–PANI-GCE electrode was obtained by CV scanning from 1.5 V to -0.1 V in 0.5 M H2SO4 electrolyte containing 0.25 mM HAuCl4 at a scan rate of 50 mV/s.

6.2.2.3 Immobilization SH- DNA probe (Anti-miRNA 21) on the GCE-PANI-AuNP sensor

Further, the sensor area was modified with the thiolated DNA probe (anti-miRNA 21) which is complementary to the target miRNA-21. This was achieved by dipping the electroactive surface into the 1 nM solution for –SH DNA probe solution for 20 mins at room temperature. After that, the surface was cleaned with doubly distilled water to remove the unbinding probe in the surface

To achieve the selective binding, the surface area was blocked Mercaptosuccinic acid (MSA) (0.01 %) for 20 mins, which increased the selective binding of the further target analyte to the probe, not with the surface.

6.2.2.4 Hybridization of target analyte (miRNA-21) to the GCE-PANI-AuNP sensor

To achieve the goal of our aimed work the sensor area was subjected to hybridization with our target miRNA-21. The electrode was dipped into the different concentrations of miRNA solution for about 20 mins. For real sample analysis, the sensor area was dipped with leptospirosis confirmed serum samples. Similarly, the sensor selectivity was achieved by using single mismatch RNA, non-complementary RNA, and healthy serum samples.

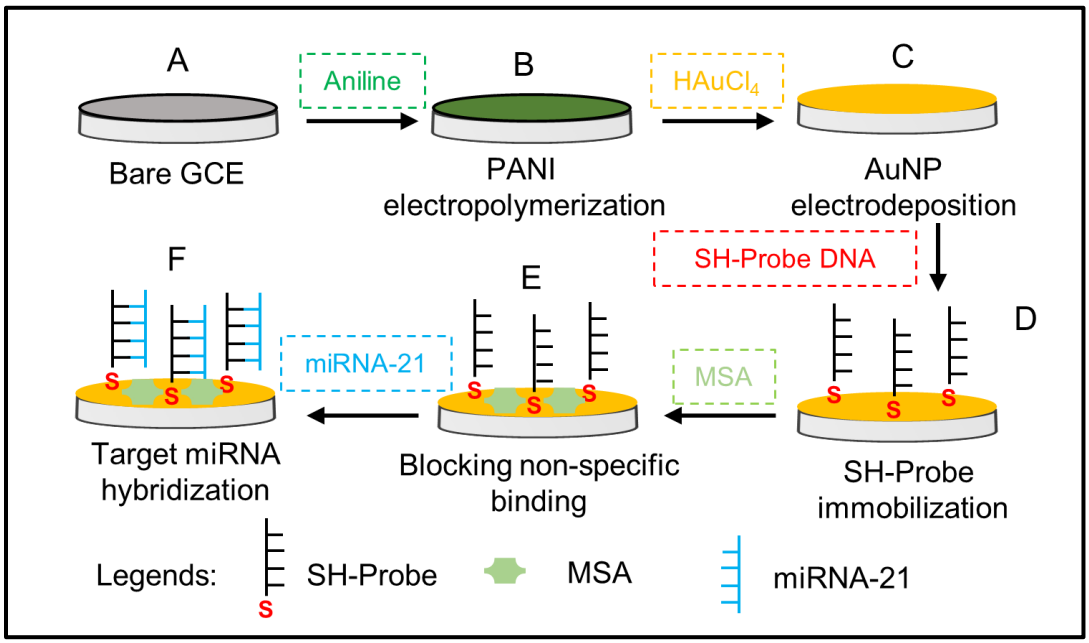
6.2.3 Electrochemical analysis

All the electrochemical experiments were recorded using the AUTOLAB PG STAT instrument. The electrical properties of before and after modification of GCE surfaces were determined by three electrochemical analysis namely Cyclic voltammetry (CV), Electrochemical Impedance Spectroscopy (EIS), and Square wave voltammetry (SWV). All the electrochemical characterization was performed using the redox probe solution 5.0 mM of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ prepared in PBS buffer (pH 7.4). CV analysis was recorded with the potential between -0.2 and 0.6 V, at a scan rate of 30 mV/s. EIS assays were performed between the frequency of 0.1 and 100,000 Hz with the reference potential of 0.2 V, using a sinusoidal potential perturbation with an amplitude of 0.05 V and 60 data points. Then the EIS data were fitted into Randles equivalent circuit, using Zsimpwin software. SWV experiments were conducted between the potential range of -0.2 and 0.8 V, with a frequency of 20 Hz and step height of 0.05 V.

6.3 Result and Discussion

6.3.1 Construction of Biosensor

Scheme 6.1: Schematic illustration of mi-RNA biosensor fabrication process

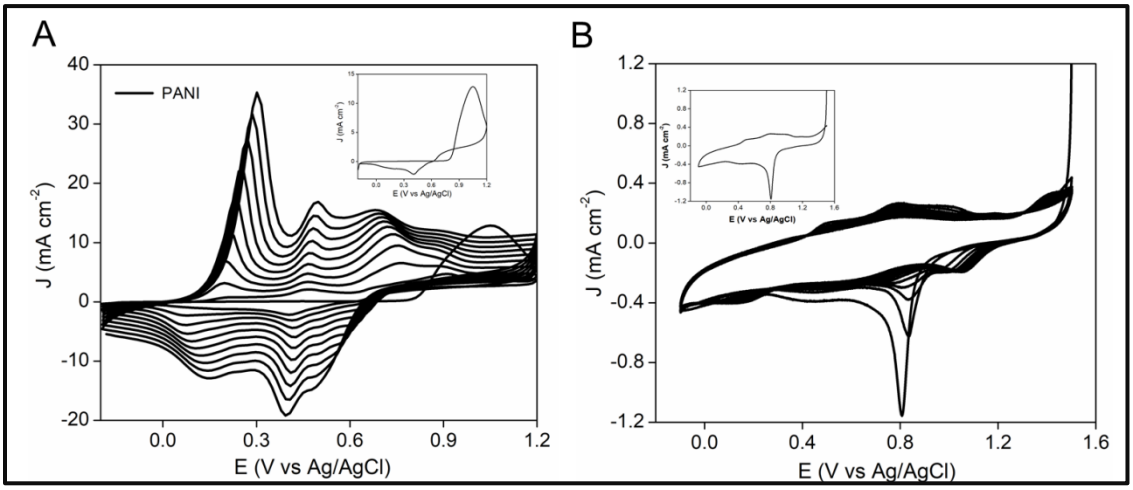


The schematic illustration of biosensor assembly as shown in Scheme 6.1. The steps involved in biosensor construction were (1) GCE electrode pretreatment; (2) electropolymerization of PANI; (3) *in situ* gold nanoparticle electropolymerization; (4) immobilization of probe DNA (anti-miRNA 21); (5) non-specific blocking and (6) target (miRNA 21) RNA binding.

Initially, the GCE electrode was cleaned by polishing with alumina slurry followed by washing with distilled water and after electrochemical cleaned using H_2SO_4 by CV analysis. PANI coatings have a porous and branched structure that can increase the specific surface area. The voltammograms measured during the synthesis of the PANI coatings on the surface of GCE are shown in Figure 6.1A In the CV the oxidation peaks at 0.235 V indicate that the transformation of leucoemeraldine form (fully reduced state) to emeraldine salt (neutral salt). Then the oxidation

peaks about 0.513 V refers that the state transformation from emeraldine to pernigraniline (fully oxidized state). The oxidation peaks about 0.706 V are related to the polymerization of aniline.

Figure 6.1: (A) Electropolymerization of PANI and (B) gold nanoparticle



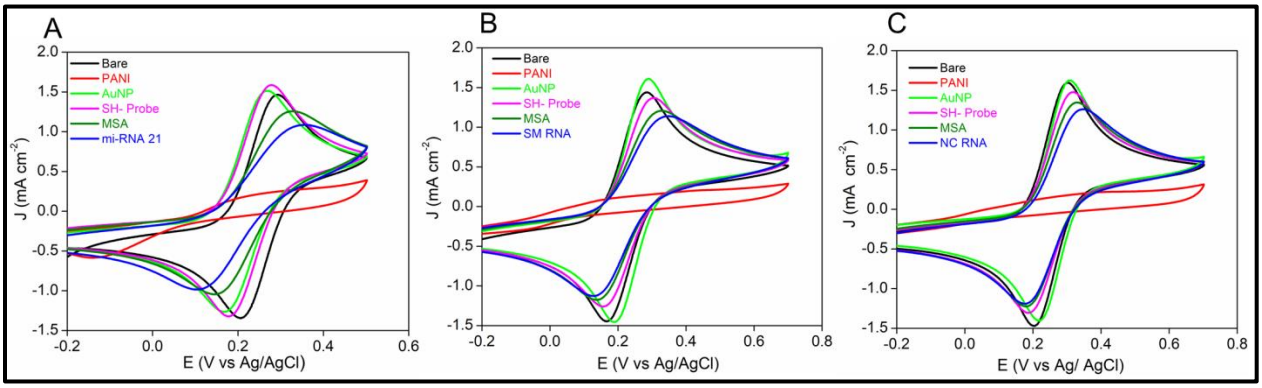
Further, the GCE-PANI electrode was modified with gold nanoparticles by *in situ* electrodeposition of 0.1 M H_2SO_4 electrolyte containing 0.25 mM of $HAuCl_4$ through 20 cycles of CV analysis of a potential range of 1.5 V to 0.1 V at a scan rate of 50 mV/s (Figure 6.1B). Anodic peak current at 1.3 V is associated with the Au oxidation and in the backward scan, the peak at 0.89 V is the subsequent reduction of oxides previously formed. Then the GCE-PANI-AuNP electrode was subjected to immobilization of SH-Probe DNA (anti-miRNA 21) by dipping the modified electrode into 10 nM of probe DNA solution for about 20 mins. The probe DNA was covalently bonded with AuNP on the GCE-PANI electrode with its thiol group in the 5' end. It had a complementary sequence to the target analyte miRNA-21. Next, the electrode surface was blocked with MSA, because its –SH bond allows to covalently bind with free AuNP areas present in the modified electrode. This step was performed to avoid direct interaction or binding of the target analyte or any other biomolecule to the GCE-PANI-AuNP electrode.

Finally, the hybridization of target miRNA-21 to the probe DNA was performed by incubating the modified working electrode into the solution of different concentrations of target miRNA for about 20 mins at room temperature.

6.3.2 Electrochemical characterization of modified sensor

Before and after modification of the biosensor were electrochemically characterized using CV, EIS and SWV.

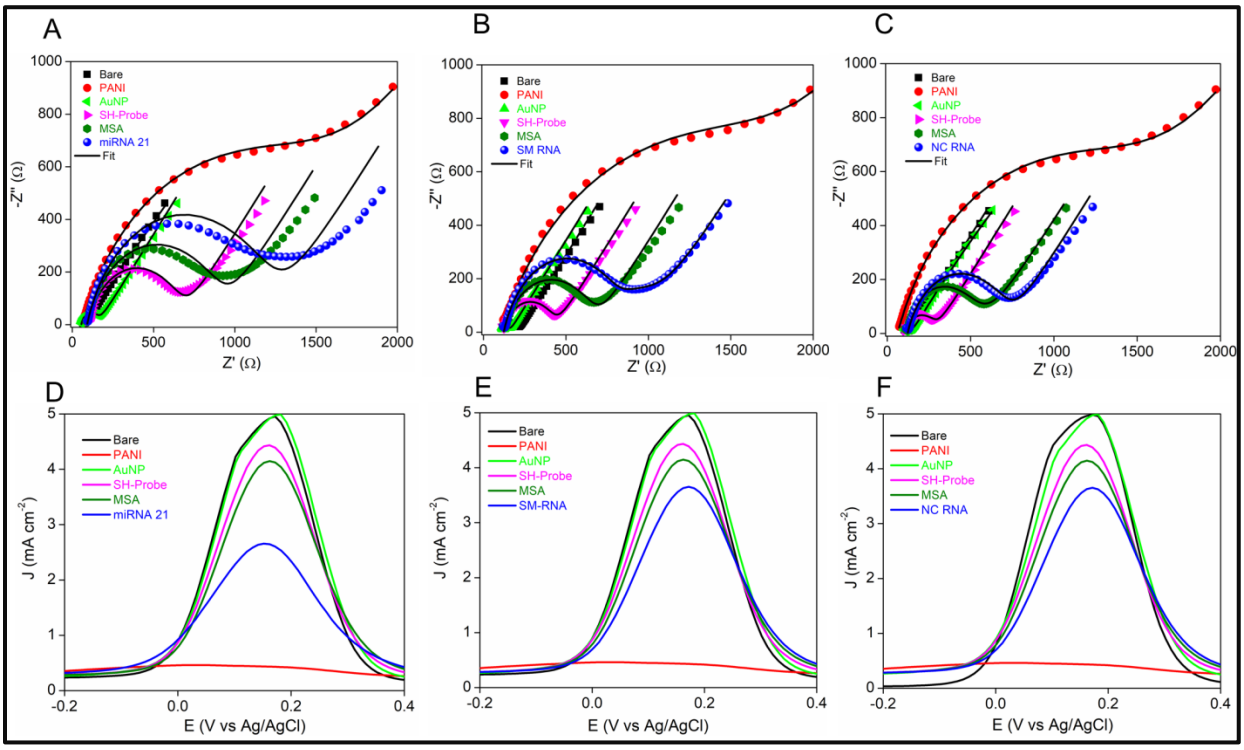
Figure 6.2: Cyclic voltammogram of different modifications of the sensor.



Legends: *A. CV analysis of the sensor for target miRNA-21, B. Single mismatched RNA, C. Non-complementary RNA.*

The chemical species in each modification produced alterations in its electrical features and these electrical properties were identified by using standard redox probe Fe $[(CN)_6]^{3-/4-}$ in PBS (pH 7.4). As shown in the figure 6.2 CV analysis when compared with the bare GCE electrode, the GCE-PANI-AuNP modified with the probe, MSA and miRNA-21 showed decreased cathodic/anodic peak currents and increased potential separation. This confirms that the increased in the charge transfer resistance after modification on the working electrode. Similarly, the corresponding EIS measurements and SWV data were consistent with CV data.

Figure 6.3: EIS and SWV analysis of modified sensor.



Legends: *(A-C: Nyquist plot and D-F SWV of the modified sensor for target miRNA-21, single mismatched RNA, and Non-complementary RNA)*

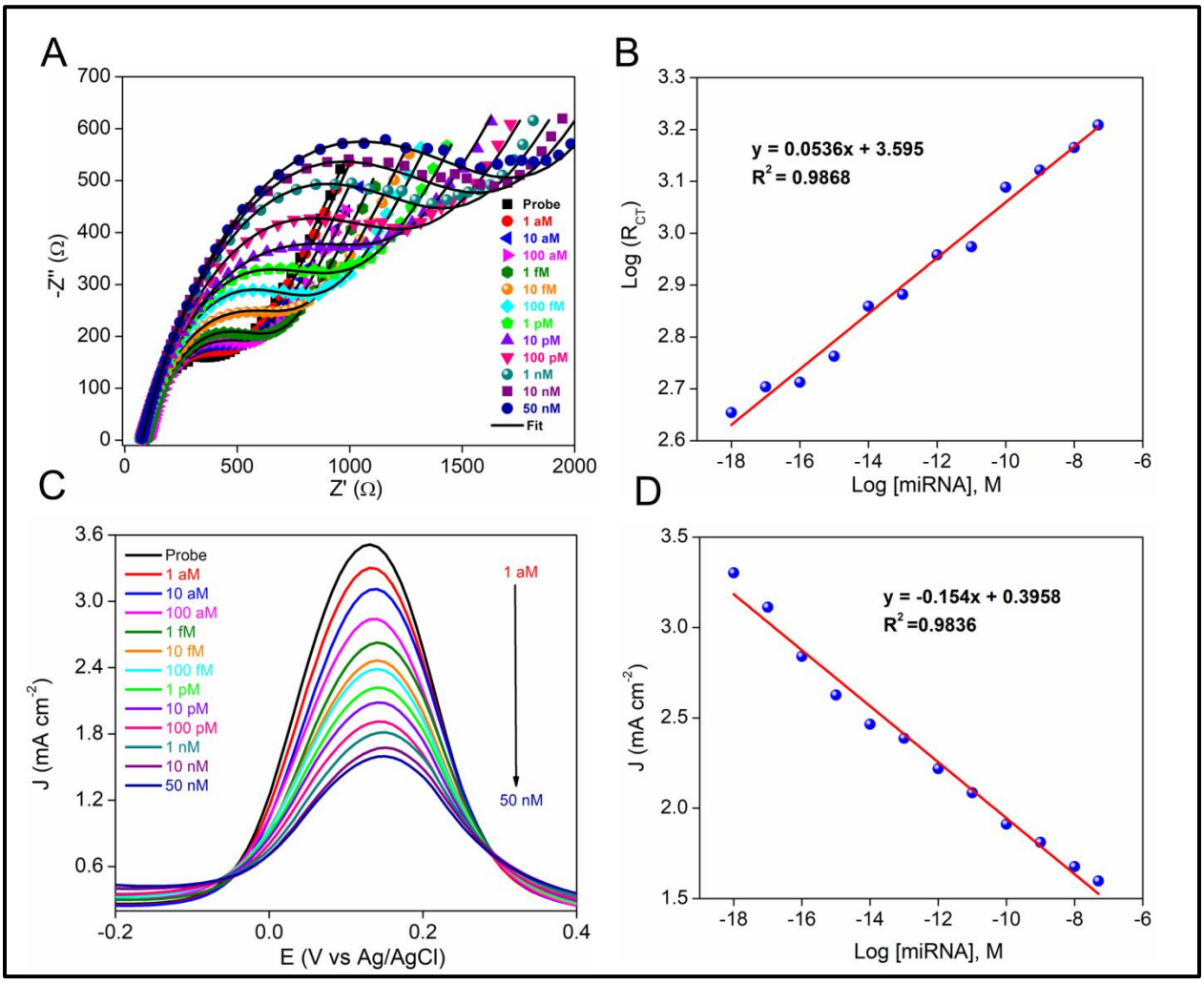
Randle's equivalent circuit was used to fit the physic-chemical process occurring at the working electrode surface. In the Nyquist plot, the semicircle indicates the electron transfer resistance (R_{CT}) and the linear part represents the diffusion limited process. We used Randle's equivalent circuit R(Q(RW)) and modified one in some cases. It provides the magnitude of the electrolyte solution resistance (Rs), the charge transfer resistance (R_{CT}) is inversely proportional to the rate of electron transfer and the Warburg diffusion element (W) at higher frequencies relates the diffusion of ions. The Nyquist plot of each modification was shown in Figure 6.3A-C. After modification on the working electrode the diameter of the semicircle was increased which indicates that the increased electron transfer resistance at the surface.

SWV data were also consistent with EIS and CV analysis Figure 6.3 D-F. SWV is considered more sensitive than CV, the changes in the electrical properties after each addition the decrease in the peak current observed in the voltammograms. This again confirms the immobilization or hybridization of binding elements on the surface. Overall CV, EIS and SWV assays were consistent and confirmed the changes in the electrode surface.

6.3.3 Concentration optimization of the biosensor

The concentration of miRNA was determined by different electrochemical experiments such as EIS and SWV. For this purpose, we selected a concentration range between 1 aM and 50 nM of miRNA, and during each interval, the sensor was incubated for 20 mins. As shown in Figure 6.4A interestingly there was an increase in the R_{CT} upon increasing the concentration, confirming that more charge transfer reactions occur at the surface active area. And the linear fit plot (Figure 6.4B) provides the linear increase in the R_{CT} to the concentration and vice versa. The slope of EIS was found to be 0.0536 and the R^2 was determined as 0.9868. Similarly, the square wave voltammogram consistent with the Nyquist plot, where as in SWV the current density was decreased accordingly as concentration raises (Figure 6.4C), which confirms the electrical property of surface was changed. The slope and R^2 value of SWV was -0.1549 and 0.9836.

Figure 6.4: miRNA concentration optimization



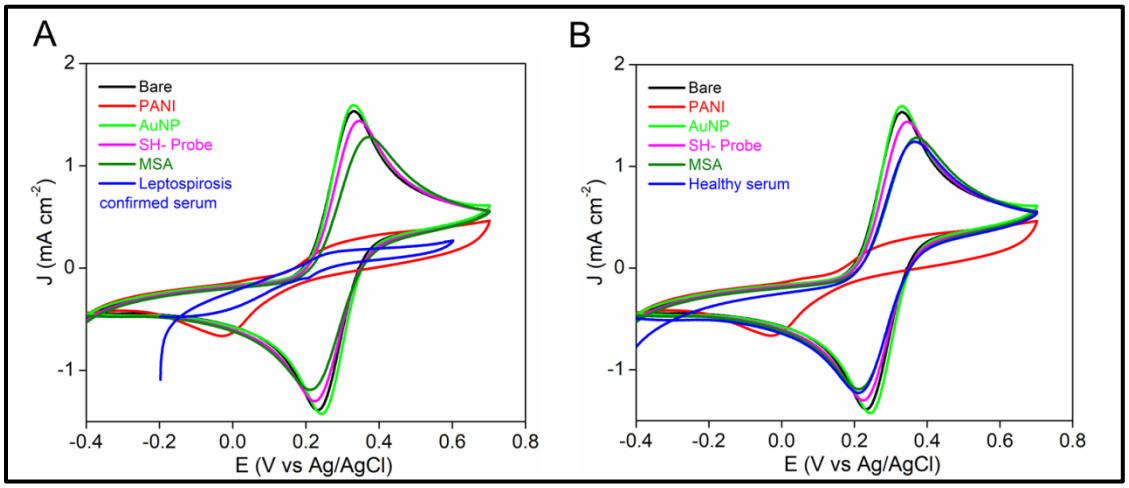
Legends: *(A) Nyquist plot of various concentrations of miRNA (1 aM to 50 nM), (B) Linear plot against the miRNA concentration vs log (R_{CT}), (C) SWV voltammogram and (D) their linear plot against current density vs miRNA concentration.*

6.3.4 Real sample analysis using the proposed sensor

The developed sensor was used for leptospirosis diagnosis using leptospirosis confirmed patients' serum samples and health serum samples. After modification with the thiolated probe to the electroactive surface of the working electrode, the sensor electrode was dipped into the

leptospirosis confirmed patients' serum and incubated for about 20 mins. The figure 6.5 confirms the decrease in the peak current as well slight shift in the peak potential compared with the healthy people serum.

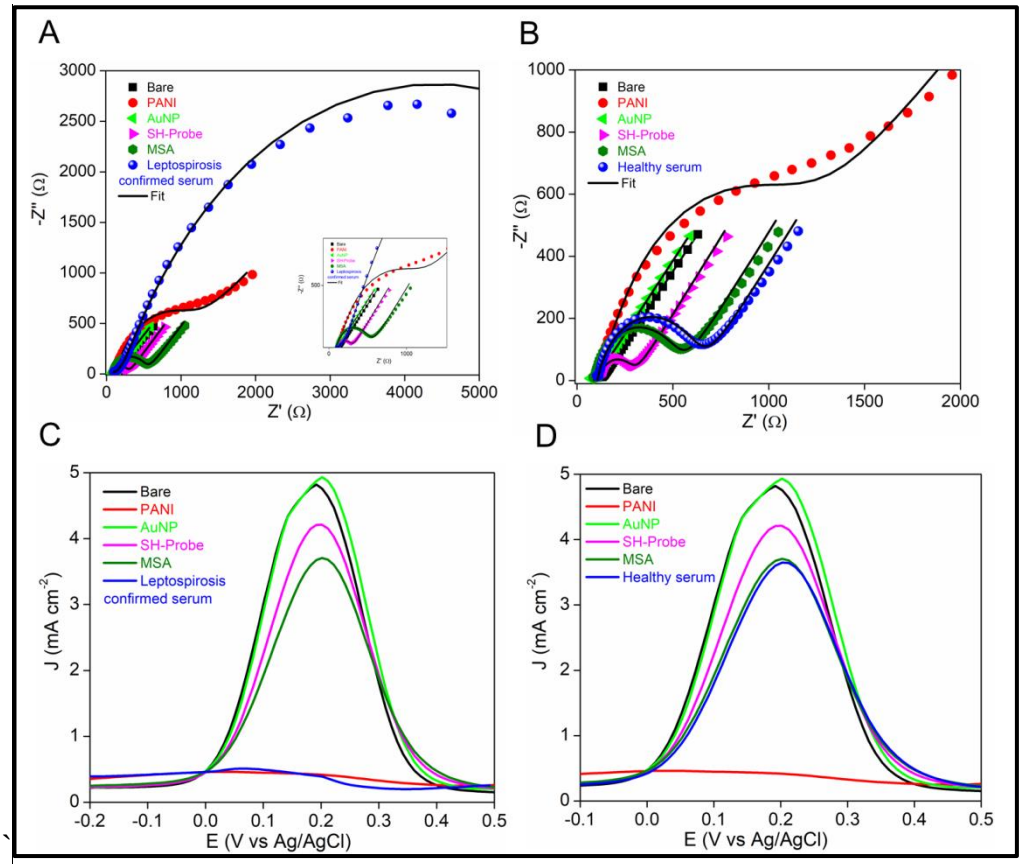
Figure 6.5 Cyclic voltammogram of the sensor selectivity



Legends: *CV of (A) leptospirosis confirmed sera and (B) healthy serum sample analysis of the sensor electrode.*

In EIS spectrum of the figure, 6.6A & B revealed that drastic increase in the R_{CT} value in the leptospirosis serum modified sensor in comparison with the healthy serum sample. This result confirms that there was mass charge transfer occur in the sensor surface area. Also, the SWV also consistent with EIS and CV analysis, as shown in the figure 6.6 C & D the drastic decrease in the current density when compared with healthy serum samples.

Figure 6.6: Nyquist plot and SWV spectrum of the sensor selectivity



Legends: *(A & B) EIS spectrum and (C & D) SWV spectrum of leptospirosis confirmed sera and (B) healthy serum sample analysis of the sensor electrode.*

6.4 Conclusion

This chapter concludes that the developed miRNA based biosensor has higher selectivity towards the target miRNA-21, which is highly upregulated in leptospirosis infected individuals. The data also confirmed that the thiolated probe DNA (anti-miRNA 21) higher affinity towards the target miRNA 21 and leptospirosis positive case serum than the non-complementary sequence as well as healthy serum samples. Overall, the developed sensor had an LOD of 1.5 aM and LOQ of 4.59 aM. This would be the ultralow level miRNA based biosensor for leptospirosis diagnosis.

6.5 References

1. Akino Mercy CS, Suriya Muthukumaran N, Velusamy P, Bothammal P, Sumaiya K, Saranya P, Langford D, Shanmughapriya S, Natarajaseenivasan K. MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis. mSphere. 2020 Jul 15;5(4):e00409-20. doi: 10.1128/mSphere.00409-20. PMID: 32669469; PMCID: PMC7364213.
2. Cardoso AR, Moreira FT, Fernandes R, Sales MG. Novel and simple electrochemical biosensor monitoring attomolar levels of miRNA-155 in breast cancer. Biosensors and Bioelectronics. 2016 Jun 15;80:621-30.
3. Condrat CE, Thompson DC, Barbu MG, Bugnar OL, Boboc A, Cretoiu D, Suciu N, Cretoiu SM, Voinea SC. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. Cells. 2020 Jan 23;9(2):276. doi: 10.3390/cells9020276. PMID: 31979244; PMCID: PMC7072450.
4. Correia CN, Nalpas NC, McLoughlin KE, Browne JA, Gordon SV, MacHugh DE, Shaughnessy RG. Circulating microRNAs as Potential Biomarkers of Infectious Disease. Front Immunol. 2017 Feb 16;8:118. doi: 10.3389/fimmu.2017.00118.
5. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nat Rev Clin Oncol. 2011 Jun 7;8(8):467-77. doi: 10.1038/nrclinonc.2011.76.
6. Cui SY, Wang R, Chen LB. MicroRNA-145: a potent tumour suppressor that regulates multiple cellular pathways. J Cell Mol Med. 2014 Oct;18(10):1913-26. doi: 10.1111/jcmm.12358.

7. Dai X, Nekrassova O, Hyde ME, Compton RG. Anodic stripping voltammetry of arsenic (III) using gold nanoparticle-modified electrodes. Analytical chemistry. 2004 Oct 1;76(19):5924-9.
8. Das Neves S, De Paoli MA. Photoelectrochemistry of polyaniline supported in a microporous cellulose acetate membrane. Synthetic metals. 1998 Jul 15;96(1):49-54.
9. Dinh HN, Ding J, Xia SJ, Birss VI. Multi-technique study of the anodic degradation of polyaniline films. Journal of electroanalytical chemistry. 1998 Nov 23;459(1):45-56.
10. Lee YS, Dutta A. MicroRNAs in cancer. Annu Rev Pathol. 2009;4:199-227. doi: 10.1146/annurev.pathol.4.110807.092222.
11. Li C, Pei F, Zhu X, Duan DD, Zeng C. Circulating microRNAs as novel and sensitive biomarkers of acute myocardial Infarction. Clin Biochem. 2012 Jul;45(10-11):727-32. doi: 10.1016/j.clinbiochem.2012.04.013. PMID: 22713968; PMCID: PMC3965350.
12. Mattiske S, Suetani RJ, Neilsen PM, Callen DF. The oncogenic role of miR-155 in breast cancer. Cancer Epidemiol Biomarkers Prev. 2012 Aug;21(8):1236-43. doi: 10.1158/1055-9965
13. Mohanty US. Electrodeposition: a versatile and inexpensive tool for the synthesis of nanoparticles, nanorods, nanowires, and nanoclusters of metals. Journal of applied electrochemistry. 2011 Mar;41(3):257-70.
14. Tan W, Liu B, Qu S, Liang G, Luo W, Gong C. MicroRNAs and cancer: Key paradigms in molecular therapy. Oncol Lett. 2018 Mar;15(3):2735-2742. doi: 10.3892/ol.2017.7638.
15. Wang Z, Liu E, Zhao X. Glassy carbon electrode modified by conductive polyaniline coating for determination of trace lead and cadmium ions in acetate buffer solution. Thin Solid Films. 2011 May 31;519(15):5285-9.

16. Xu WX, Liu Z, Deng F, Wang DD, Li XW, Tian T, Zhang J, Tang JH. MiR-145: a potential biomarker of cancer migration and invasion. Am J Transl Res. 2019 Nov 15;11(11):6739-6753.
17. Zhao B, Yu Q, Li H, Guo X, He X. Characterization of microRNA expression profiles in patients with intervertebral disc degeneration. Int J Mol Med. 2014 Jan;33(1):43-50. doi: 10.3892/ijmm.2013.1543.

Leptospiral LPS Mediated Pathogenesis in S. cerevisiae

CHAPTER VII

Chapter VII: Analysis of the Leptospiral LPS Mediated Pathogenesis- *Saccharomyces cerevisiae* as a model system

This chapter deals with Hog 1 signaling during induction of leptospiral LPS and the hyperosmotic element NaCl (0.8 M). After the addition of stress elements, the stress activated protein kinase was phosphorylated and led to the expression of various genes responsible for cell survival. In addition, the cytosolic events that occur during the starvation condition are also carried out. The phosphorylation of stress activated kinase during exposure to LPS provides insight and knowledge about the organization of cellular metabolic products and cell survival during stress conditions.

7.1 Background and Motivation

Cells have developed a variety of mechanisms to counteract stress to give a specific and adaptive response. The stress responses rely on the recognition of the specific stress through a cellular sensor followed by a signal transduction pathway that often involves one or more chains of enzymatic activities, leading to several coordinated intracellular actions (Marques *et al.*, 2006). The yeast high osmolarity glycerol protein (*Hog1*) belongs to Mitogen-Activated Protein (MAP) kinases and response to extracellular osmotic stress conditions (de Nadal *et al.,* 2002). This protein is functionally and structurally like the mammalian p38 MAP kinase which is activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), Ultraviolet light and growth factors.

A prototype of the stress activated protein kinases (SAPK) family is Hog1 MAP kinase in yeast, which specifically responds to increased extracellular osmolarity and is essential for cell survival. *Saccharomyces cerevisiae* detects and responds to high extracellular osmolarity through

the high osmolarity glycerol (HOG) MAPK pathway (Albertyn *et al.,* 1994). Phosphorylation of dual specific kinase Pbs2 and *Hog1* can translocate into the nucleus and regulate the gene expression and mediate post-transcriptional and translational activity in the cytoplasm. In the nucleus, *Hog1* associates with stress-responsive promoters via specific transcription factors and stimulates gene expression by recruiting general transcription factors, chromatin-modifying enzymes, and RNA polymerase II (de Nadal and Posas, 2010). Recently, Hog1 was reported to respond to increased extracellular osmolarity and also reported that it may be activated by various stress conditions like temperature, oxidation, LPS, Citric acid, Oleic acid and Palmitic acid (Winkler *et al.*, 2002; Lawrence, 2004). MAP kinase family (p42/44MAP kinase and p38) plays a major role in cellular activation and regulates the translation of cytokines, adhesion, and migration molecules (Han *et al.*, 1994).

7.1.1 HOG Pathway

The *Hog1* was discovered in yeast, followed shortly by the discovery of the p38 and JNK (c-Jun N-terminal kinase) orthologs of this kinase in Mammals. The yeast MAPK *Hog1*-mediated signaling cascades are essential for sensing multiple environmental stress stimuli and for transmitting these signals to the nucleus to control gene expression (O'Rourke and Herskowitz, 2004). The physiological role of the HOG pathway is to orchestrate the adaptation of yeast cells to increased osmolarity of the surrounding medium (Hohmann, 2002). Such increased medium osmolarity leads to water loss and cell shrinking. The cell needs to counteract those effects to maintain shape and turgor and to ensure appropriate water and ion concentration in the cytosol and its organelles for optimal functioning of biochemical reactions. This signal transduction pathway has its role in perceiving environmental changes via phosphorylation and gene activation (Brewster *et al.,* 2014). In addition to its main role in the regulation of

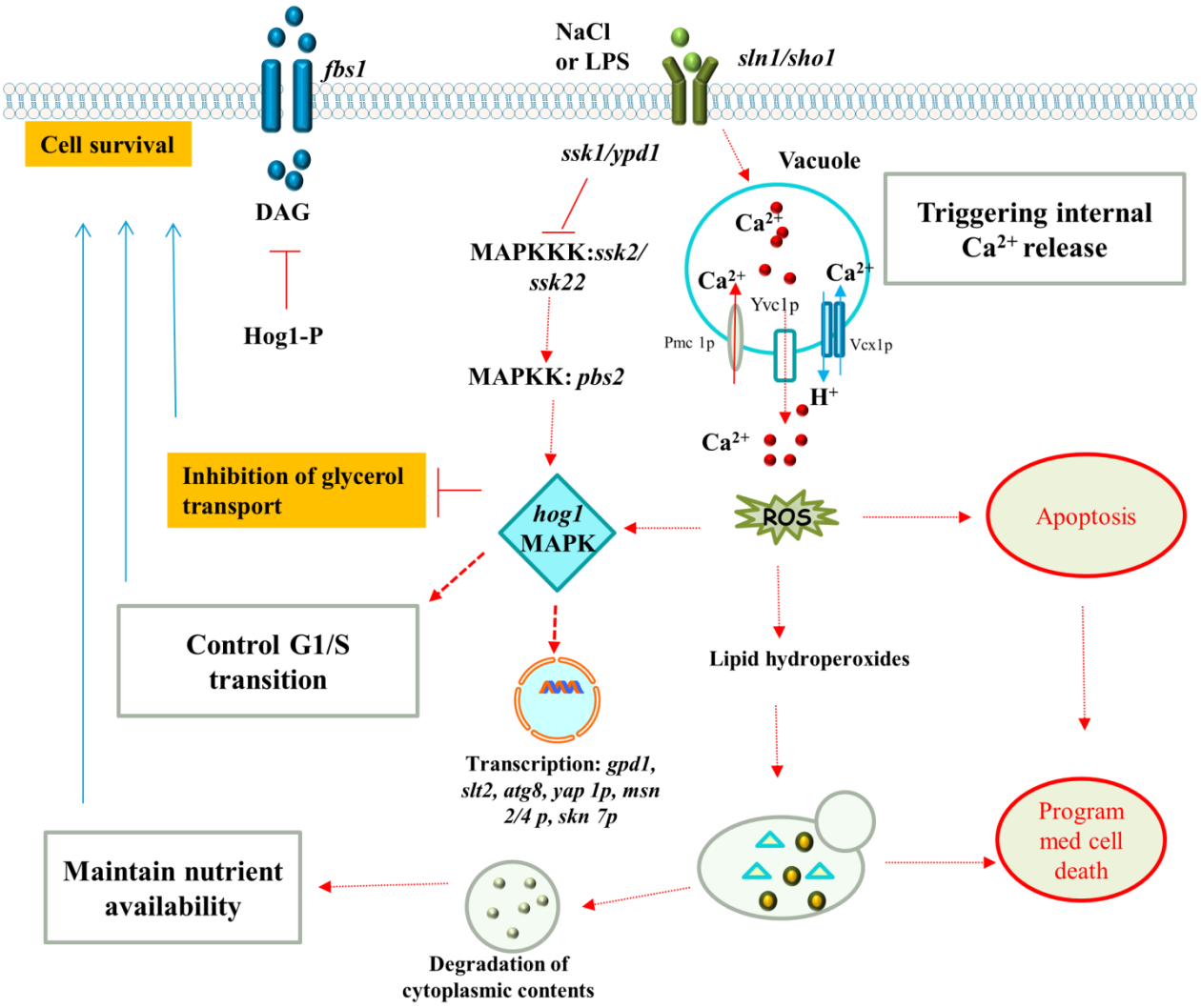
hyperosmotic stress responses, the HOG pathway has also been shown to be activated in response to other stresses, including oxidative stress (Bilsland *et al.,* 2004), acid stress (Lawrence *et al*., 2004), methylglyoxal (Aguilera *et al.,* 2005), temperature downshift (Panadero *et al.,* 2006), arsenite (Thorsen *et al.,* 2006), CsCl (Del Vescovo *et al*., 2008), heat stress (Winkler *et al.,* 2002) and zymolyase (Bermejo *et al*., 2008). The *Hog1* was phosphorylated on both threonine and tyrosine in response to osmotic stress, and this phosphorylation depended on Pbs2 activity. The phosphorylation and hence activity of the *Hog1* MAPK is controlled by two branches, the Sln1 and the Sho1 branch, which converge on the MAP kinase kinase (MAPKK) Pbs2. Phosphorylation of dual specific kinase Pbs2 and *Hog1* can translocate into the nucleus and regulate the gene expression and mediate post-transcriptional and translational activity in the cytoplasm. In the nucleus, *Hog1* associates with stress-responsive promoters via specific transcription factors and stimulates gene expression by recruiting general transcription factors, chromatin-modifying enzymes, and RNA polymerase II. At present, it is not entirely clear why *Hog1* is controlled by two branches, because either branch alone can activate *Hog1* in response to hyperosmotic stress (Maeda *et al*., 1995). The Sln1 branch plays a predominant role over the Sho1 Branch. The Sho1 branch does not seem to be connected to the *Hog1* MAPK cascade in a number of other fungi (Furukawa *et al.,* 2005).

Acute hyperosmotic stress leads to a rapid (sub-minute) increase in the amount of phospho-*Hog1*, the active form of the kinase. Under such conditions, phosphorylation is accompanied (and required for) the import of *Hog1* into the nucleus (Ferrigno *et al.,* 1998). Glycerol is synthesized by reducing de-hydroxy acetone phosphate to glyceroldehyde 3-phosphate which is catalyzed by the NAD-dependent cytosolic G3P dehydrogenase followed by the dephosphorylation of the G3P by a specific phosphatase. Glycerol is produced by yeast cells'

osmoregulation and redox balancing. It is hence a common by-product of yeast fermentations. Hence *Hog1* appears to control glycerol accumulation and maintain cell survival.

With this background, we aimed to analyse whether *Hog 1* is expressed under LPS stress condition and maintain the cell function. This will help to understand the pathogenesis of *Leptospira* in the model organism. LPS mediated Hog 1 signaling was shown in figure 7.1

Figure 7.1: Hog Pathway



7.2 Materials and Methods

7.2.1 Strains and Culture Conditions

7.2.1.1 Yeast Strains

Saccharomyces cerevisiae W303-1A (*Mat α ade 2-1 can 1-100 his 2-11, 15 leu 2-3,112 trp1-1 ura3-1*) and its derivative YSH 444 (*Hog1Δ::TRP1*) mutant were used in this study. Yeast cells were grown in yeast nitrogen base (YNB) medium supplemented with the appropriate selective amino acids and 2% glucose at 30°C for overnight. The culture growth was assessed by measuring the optical density (OD) at 600 nm.

7.2.2 *In silico* characterization

Saccharomyces cerevisiae Hog1 nucleotide (Accession No.: Z73285.1) and protein (Accession No.: CAA97680.1) sequences were retrieved from National Center for Biotechnology Information (NCBI). The complete coding sequences and protein domain were predicted using the CDS tool. The protein sequence was subjected to BLASTp analysis (Altschul *et al.,* 1997) and pairwise sequence alignment using EMBOSS Needle (Needleman & Wunsch 1970) with human *p38* (Accession No.: NP_002736.3) for identification of the similarity and identity.

7.2.3 Characterization of *Hog1* Protein

The molecular weight and the theoretical pI value of the *S. cerevisiae Hog1* protein were predicted using ProtParam and Compute pI tools of UniProt Knowledgebase, respectively. *Hog1* protein is a phosphorylated protein and hence the phosphorylation sites were predicted using NetPhos-3.1 (Blom *et al.,* 2004) and compared with the phosphorylated sites of p38 protein. The three dimensional structure of the *Hog1* protein is not available in PDB and hence it was

modeled using SWISS-MODEL with the template of Mitogen activated kinase 14 (PDB ID: 1WFC).

7.2.4 Cell Viability Assay

7.2.4.1 Spotting assay

The *S. cerevisiae* cells were grown in YNB and supplemented with essential amino acids. The cells were taken at the 0.3-0.4 at OD 640 and induced with stress elements such as Leptospiral LPS (100 ng/mL) and 0.8M sodium chloride (NaCl) was used as a positive control. Then the cells were incubated at 30ºC for 6 h. After incubation, 100 µL aliquots culture were taken and prepared serial dilution and spotted (10^4, 10^5, 10^6, and 10^7 cells/mL) on the YNB agar media and incubated at 30ºC for 3 days.

7.2.4.2 Staining with methylene blue

Yeast cells were suspended in PBS and an aliquot (100 µL) was mixed with 100 µL methylene blue (0.1 mg/mL of 2% dihydrate sodium citrate solution) and incubated at room temperature for 5 min. Viable and dead cells were examined under a microscope (Nikon, Japan).

7.2.5 Protein Extraction and Quantification

Yeast strains were grown to mid-log phase (OD600 0.3-0.4) and then exposed to various stress elements such as leptospiral LPS (100 ng/mL) and 0.8M NaCl used as a positive control. Then the cells were incubated at 30ºC for different time intervals (0 to 12 h). After the appropriate incubation time cells were harvested by centrifugation at 8000 rpm for 5 mins and used for further applications. Protein extracts were obtained by lysing the harvested cells using Tris-$MgCl_2$ buffer (50 mM PMSF). Briefly, the cells were centrifuged at 8000 rpm for 10 min at 4ºC. The pellet was washed with 1 mL of sterile PBS and solubilized using 150 µL of RIPA buffer and incubated on ice for 10 min. Then the

suspension was homogenized using vortex and then sonicated five times at two min time intervals. Then the protein samples were collected by centrifugation as mentioned above and the protein samples were quantified by the Bicinchoninic acid (BCA) method using a kit (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer's instruction.

7.2.6 Western Blot Analysis

Protein samples from various stress-induced and control yeast cells were boiled at 70°C for 10 min and then separated by SDS-PAGE using 8 % acrylamide gel. The proteins were transferred onto nitrocellulose membrane by semi-dry electrotransfer apparatus (Sigma Aldrich, St. Louis, MO, USA) and then blocked at room temperature for 1 h in Tris-buffered saline medium (200 mM Tris and 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) with 5% bovine serum albumin (BSA). Then the membrane was incubated with an anti-rabbit p38 antibody (Cell Signaling Technology, Danvers, MA, USA) at the dilution of 1:250 in TBS-T with 3% BSA at 4°C overnight. After washing the membrane with TBS-T three times, the membrane was incubated with horse-radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Sigma Aldrich, St. Louis, MO, USA, 1:5000) for 60 min at room temperature. Again, the membrane was washed with TBS-T buffer three times, and protein bands were visualized using Luminol/enhancer and peroxide solution (Sigma Aldrich, St. Louis, MO, USA, USA). The membrane was documented using a Chemi-documentation system (Vilber-Laurmat, France).

7.2.7 Determination of cellular by products

7.2.7.1 Nile Red Assay

After induction with stress elements, cells were collected by centrifugation. From the 250 µl of cells add freshly prepared DMSO and PBS (1:1 ration); then 5 µg/ mL of Nile red in acetone was added. Thoroughly mix the cells and incubated 5 mins at room temperature in dark conditions. After centrifugation, the cells were washed twice with PBS (pH: 7.4). Then resuspend the cells

with 100 µL of 10% (v/v) formalin and fix it for about 15 mins under the dark condition at room temperature. Finally, centrifuge the fixed stained cells for 2 min at 492 x g and wash twice with PBS. Take 5 µL of sample and place the cover glass observe under a fluorescence microscope.

7.2.7.2 Dihydroethidium (DHE) staining

To visualize the ROS (Reactive Oxygen Species) upon the addition of stress elements, the cells were satined with DHE. Stress induced cells were collected and washed with PBS. The resulting pellet was resuspended with 500 µL of PBS; add 2.5 µL of DHE (mg/mL stock). Mix well and incubated it for 25 mins at 30ºC under dark conditions. The cells were collected by centrifugation and washed with PBS. PBS resuspended cells were subjected to microscopic observation.

7.2.7.3 Fluo-4 staining

Fluo-4 is analog to Fluo-3 with the two chlorine substituents replaced by fluorines, which cause the excitation at 488 nm. It has been used to analyze the calcium dynamics. Stress induced cells were washed with PBS, after adding 10 µL of Fluo-4 (50µg in (0.1 % DMSO) and incubated for about 60 mins. After washing the cells fluo-4 loaded cells were observed under an inverted fluorescence microscope.

7.3 Results and Discussion

7.3.1 *In silico* Characterization of *Hog1*

The yeast *Hog1* protein is a 1304 bp coding gene, composed of 435 amino acids. The molecular weight and pI of the protein were identified as ~49 kDa and 5.24, respectively. The *Hog1* protein comprises the catalytic domain of the Serine/Threonine Kinases (12-339 residues). The yeast *Hog1* is having 50% identity and 66% of similarity with the mammalian p38 protein (Figure 7.2). The phosphorylation sites on yeast *Hog1* protein were predicted as S18, Y19, and

Y23 (Figure 7.3) and the S18 has a score of 0.942 indicating a very likely phosphorylation site, whereas the other residues showed a score just barely above the threshold (0.500). Protein three dimensional structure was modeled (Figure 7.4) using the template of MAPK14 and the structure was validated using a Ramachandran plot and found more than 90% residues in the allowed regions. This structure will be superimposed on the template and used to calculate the RMSD value of 2.23 and the potential phosphorylation sites were marked on the structure of yeast *Hog1* protein.

Figure 7.2: Pairwise sequence alignment of *Hog1* with *p38* protein

```
HOG1              1 ------MTTNEEFIRTQIFGTVFEITNRYNDLNPVGMGAFGLVCSATDTL     44
                        .....|.:|    |.||::..||.:|:.:|.||:|:||||.|.:
p38               1 MAAAAAAGAGPEMVR----GQVFDVGPRYTNLSYIGEGAYGMVCSAYDNV     46

HOG1             45 TSQPVAIKKIMKPFSTAVLAKRTYRELKLLKHLRHENLICLQDIFLSP--     92
                    ....|||||| .||......:||.||:|:|...||||:|.:.||..:|
p38              47 NKVRVAIKKI-SPFEHQTYCQRTLREIKILLRFRHENIIGINDIIRAPTI     95

HOG1             93 --LEDIYFVTELQGTDLHRLLQTRPLEKQFVQYFLYQILRGLKYVHSAGV    140
                      ::|:|.|.:|..|||::||:|:.|....:.||||||||||||:|||.|
p38              96 EQMKDVYIVQDLMETDLYKLLKTQHLSNDHICYFLYQILRGLKYIHSANV    145

HOG1            141 IHRDLKPSNILINENCDLKICDFGLARIQDPQ------MTGYVSTRYYRA    184
                    :||||||||:|:|..||||||||||||:.||.      :|.||:||:|||
p38             146 LHRDLKPSNLLLNTTCDLKICDFGLARVADPDHDHTGFLTEYVATRWYRA    195

HOG1            185 PEIMLTWQKYDVEVDIWSAGCIFAEMIEGKPLFPGKDHVHQFSIITDLLG    234
                    |||||..:.|...:||||.|||.|||:..:|:||||.:::.|.:.|..:||
p38             196 PEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILG    245

HOG1            235 SPPKDVINTICSENTLKFVTSLPHRDPIPFSERFKTVEPDAVDLLEKMLV    284
                    ||.::.:|.|.:.....::.||||::.:|::..|...:..|:|||:|||.
p38             246 SPSQEDLNCIINLKARNYLLSLPHKNKVPWNRLFPNADSKALDLLDKMLT    295

HOG1            285 FDPKKRITAADALAHPYSAPYHDPTDEPVADAKFDWHFNDADLPVDTWRV    334
                    |:|.|||....||||||...|:||:|||:|:|.|.:.....|||.:..:.
p38             296 FNPHKRIEVEQALAHPYLEQYYDPSDEPIAEAPFKFDMELDDLPKEKLKE    345

HOG1            335 MMYSEILDFHKIGGSDGQIDISATFDDQVAAATAAAAQAQAQAQAQVQLN    384
                    :::.|...|.....|
p38             346 LIFEETARFQPGYRS----------------------------------    360

HOG1            385 MAAHSHNGAGTTGNDHSDIAGGNKVSDHVAANDTITDYGNQAIQYANEFQ    434

p38             361 -------------------------------------------------    360

HOG1            435 Q    435

p38             361 -    360
```

Figure 7.3: Prediction of phosphorylation sites of *Hog1* protein

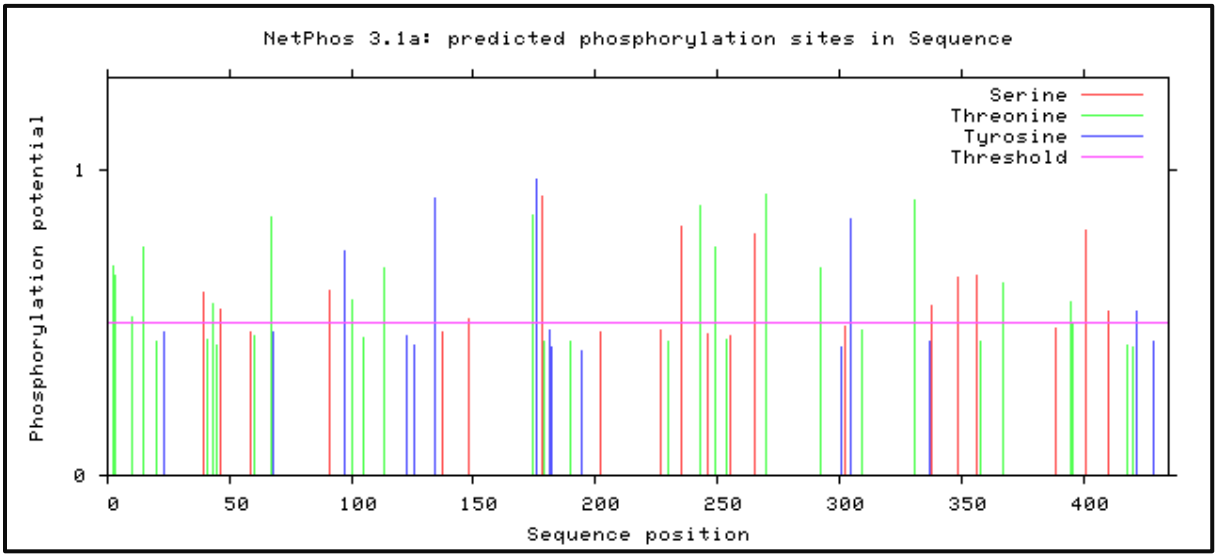
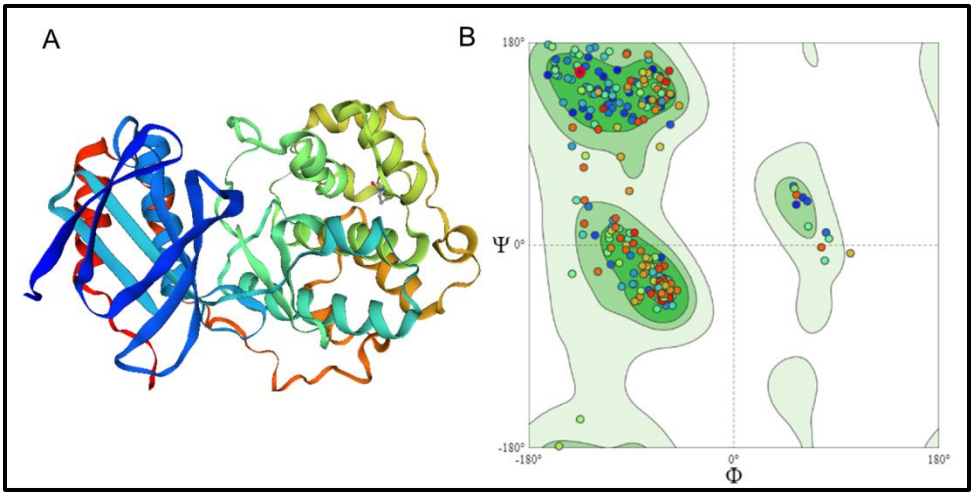


Figure 7.4: 3D structure (homology model) of *Hog1* protein and structural validation (Ramachandran plot).



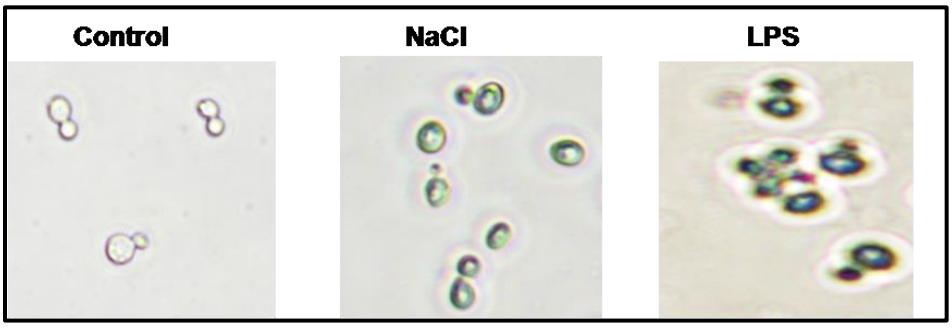
Note: Ramachandran favoured 95.63%

7.3.2 Yeast Phenotypic Characterization

Morphological changes of yeast cells in stress induced and un-induced state was examined under the microscope (Figure 7.5). Yeast cells are known to produce budding, and it was identified in the control whereas the condensation and loss of efficient budding were

observed in the NaCl (positive control) treated cells. The leptospiral LPS-induced cells showed cell shrinkage as well as condensation of cellular parts. These results clearly suggest that osmotic stress caused morphological changes in the cells.

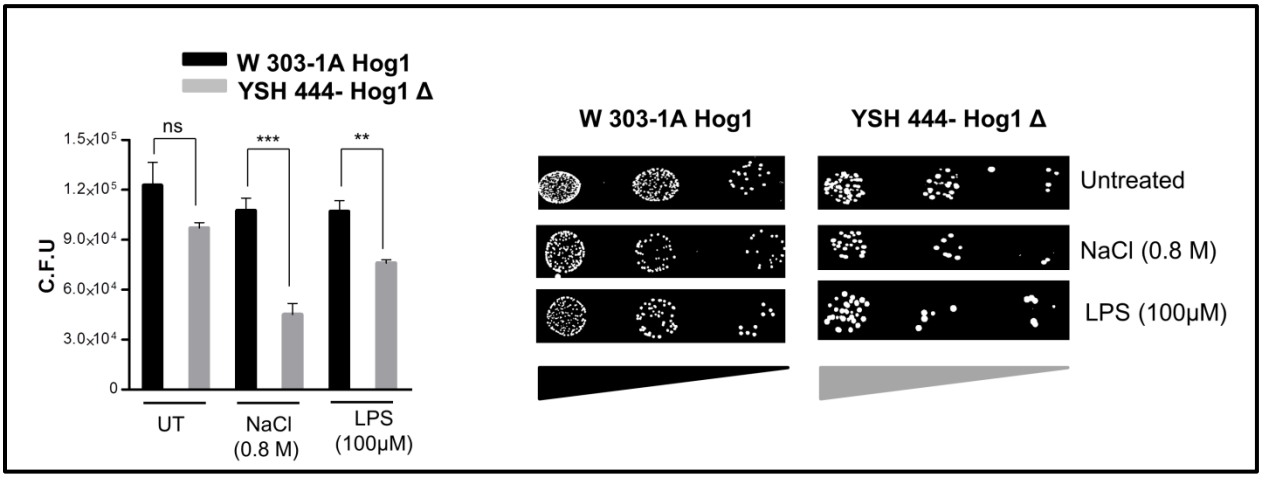
Figure 7.5: Phenotypic characterization of the *S. cerevisiae* under stress conditions



7.3.3 Yeast Cell Viability Assay

The osmotic stress was induced in the wild and *Hog1* mutant yeast strains with different stress elements and compared with the control. The effect of stress elements on the survival of yeast cells was examined by spotting the serially diluted cultures on YNB agar plates (Fig7.6).

Figure 7.6: Cell survivability of yeast strains under various stress conditions.



Legends: *Untreated (UT), LPS: Autumnalis LPS*

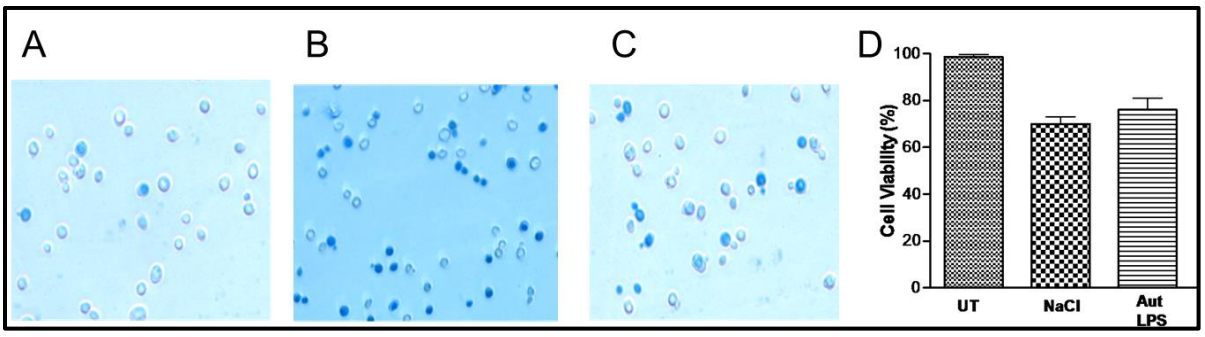
Stress elements are identified to reduce the cells in the *Hog1* mutant strain whereas the wild type yeast strain survived under stress conditions due to combat of *Hog1* protein. The cells were

quantified as CFU, and the results reflect that CFU is not affected by stress in wild type whereas the CFU was reduced in the *Hog1* mutant yeast strain.

7.3.4 Staining with methylene blue

The cell viability was also measured based on methylene blue staining as colorimetric or fluorescent dyes are much more efficient and give more sensitive results. Methylene blue dye was used for the analysis of individual yeast cells. The dye penetrates into every cell and the viable cells reduce the dye to a colourless product and become unstained. Whereas dead cells are stained in blue and observed by microscopy (Figure 7.7). When compared to control, more dead cells were observed under the stress of NaCl and found a reduction of ~30% cells.

Figure 7.7: Measurement of cell survival by methylene blue staining method



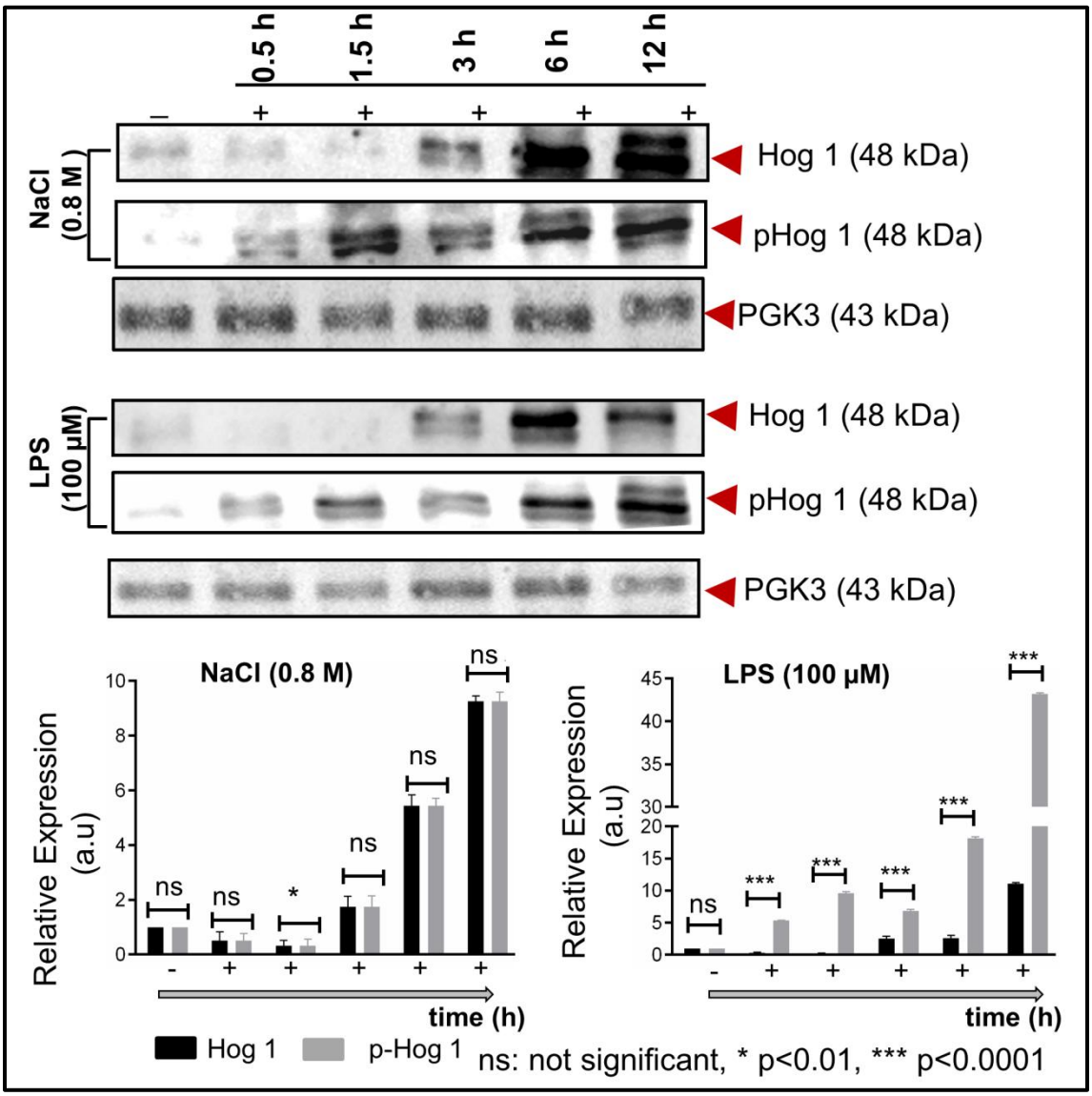
Legends: *Untreated (A), NaCl (B), and Autumnalis LPS (C).*

7.3.5 Optimization of Stress Elements on *Hog1* Phosphorylation

The optimal concentration of leptospiral LPS to activate the *Hog1* protein was determined as 100 µM and the time dependent phosphorylation was studied by analysis of *Hog1* phosphorylation by Western blot for up to 12 h of exposure. The time taken for observable phosphorylation was identified as 30 min and it was gradually increased to 1.5 fold for 3 hours of induction. Then found about two fold rise in phosphorylation of *Hog1* protein (Figure 7.8). The ratio of relative density of phosphorylated *Hog1* and total *Hog1* was calculated for the cells exposed to NaCl and LPS to adequately compare the level of phosphorylation irrespective of

changes in the total *Hog1* levels. Regardless of the phosphorylation kinetics, taken together, these data clearly showed that LPS exposure induces *Hog1* phosphorylation.

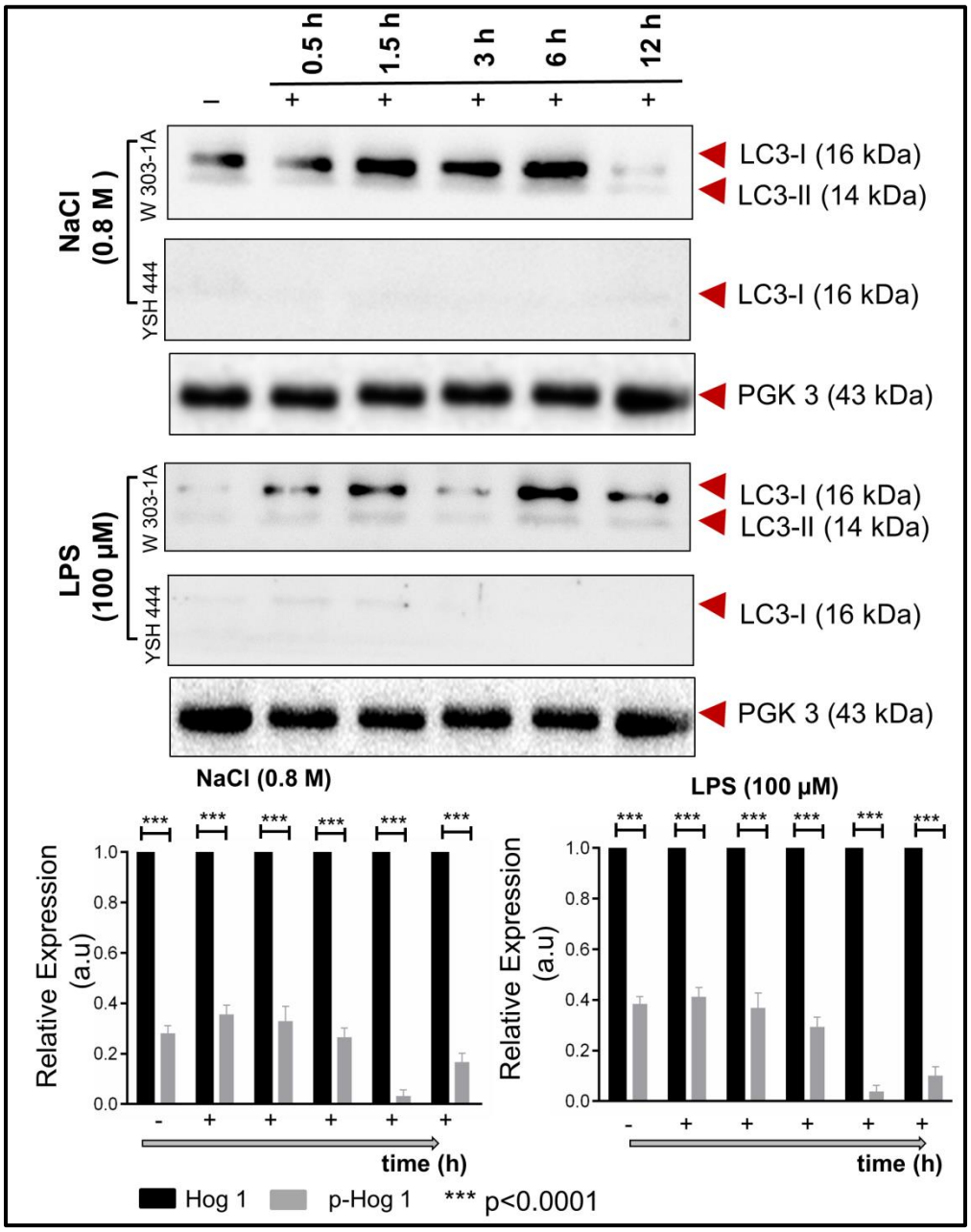
Figure 7.8: Phosphorylation of Hog 1upon exposure to stress elements.



7.3.6 Induction of Autophagy related genes

Autophagy is an important physiological process for survival during starvation as well as under normal conditions. Studies reported that Hog1 helps to maintain autophagy during osmotic conditions and reported that loss of Hog1 leads to osmosensitivity (Prick et al., 2006)

Figure7.9: Autophagy gene phosphorylation during stress conditions

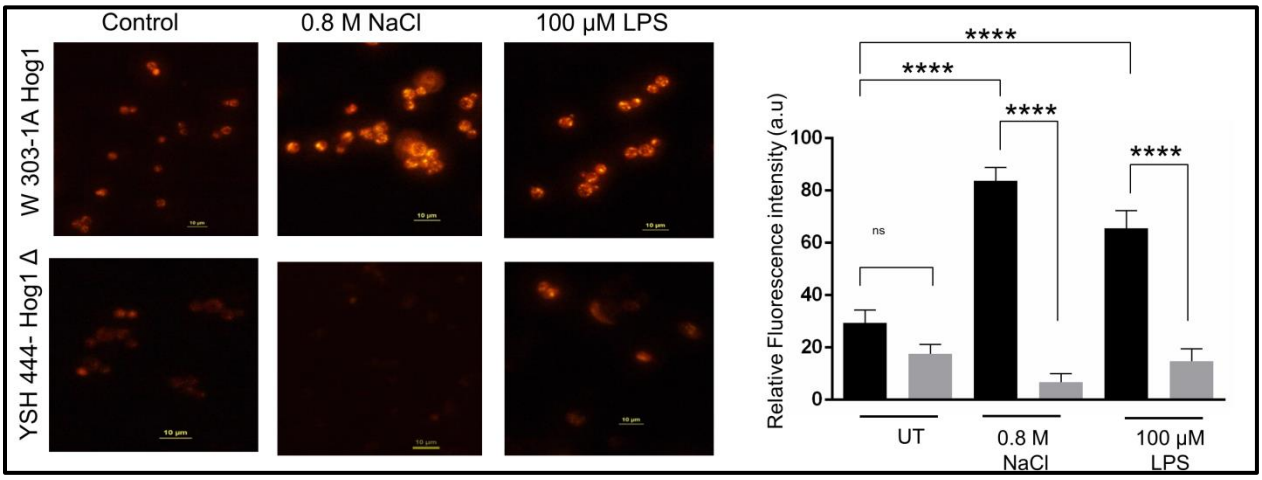


In yeast the autophagy is regulated by Apg8p, homolog to mammalian LC3. Two forms of LC3 are present, LC3-I is cytosolic, and LC3-II is membrane bound (Kabeya et al., 2000). LC3, which is the marker to identify autophagy. As shown in figure 7.9 we observed that in wild type cells the Apg8p was phosphorylated upon exposure to LPS and NaCl. However, hog1 mutant

cells lack the ability to phosphorylate the autophagy related genes. These results confirmed that Hog1 is essential to maintain cell survival through autophagy during stress conditions.

7.3.7 Lipid Droplet accumulation

Figure 7.10: Accumulation of lipid droplets during starvation conditions

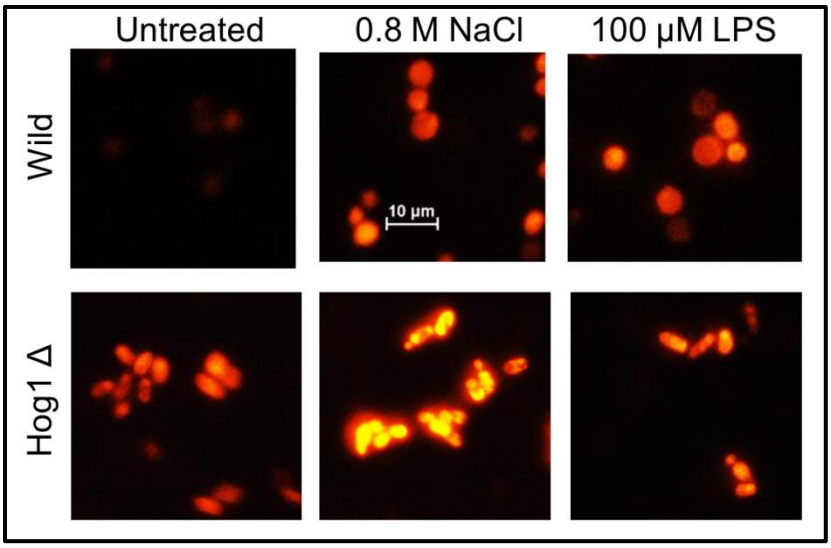


Hog1 is required for intracellular lipid accumulation to maintain cell viability during stress conditions. The loss of viability or an alteration of lipids in the membrane can increase cell permeability. During osmotic conditions, it is observed that the loss of viability in hog1 mutant cells. We quantified the lipid content using the lipophilic dye Nile Red, which is used to localize and quantify lipids in the biological systems. Membrane lipids play important role in MAP kinase signaling. Accumulation of membrane lipids during starvation conditions is a compensatory mechanism to recruit signaling elements and then activate Hog1 (Figure 7.10). Once Hog 1 is activated it controls the lipid metabolism and maintains cell viability. However, in the case of Hog 1 mutant, fails to activate hog1 signaling and there would be no repression of lipid synthesis and increase in the membrane lipids.

7.3.8 ROS Production

Programmed cell death (PCD) is a fundamental cellular process. In *Saccharomyces cerevisiae,* the apoptotic cell death was first described in a temperature-sensitive cdc48 mutant. Evidence suggested that hyperosmotic stress induces time dependent cell viability.

Figure 7.11: ROS production analysis by DHE staining



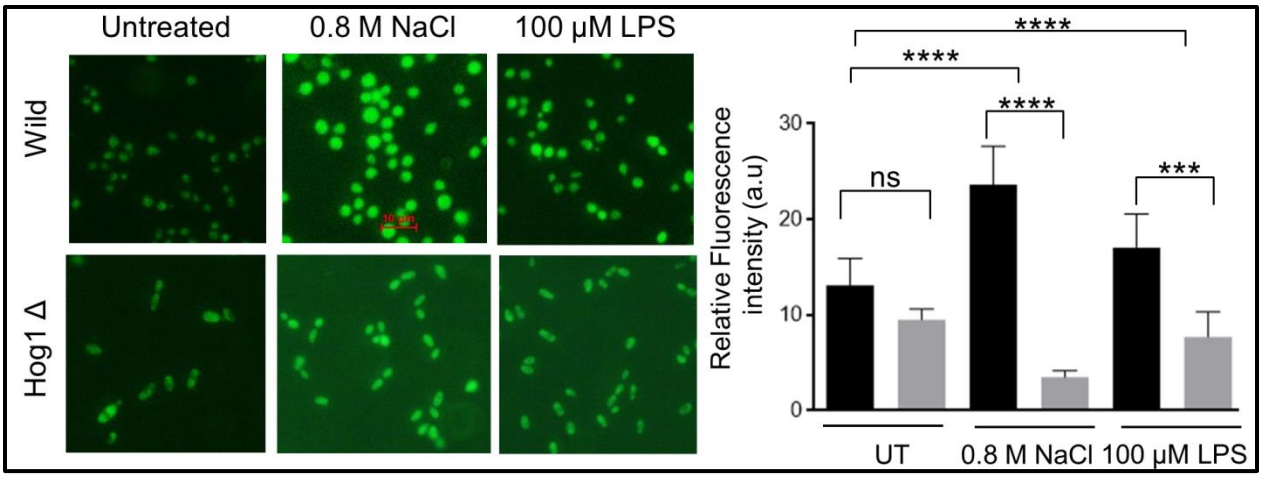
We observed that under starvation conditions the mutant cells accumulate more ROS production (Figure 7.11) Similarly under LPS treatment the mutant cells have more ROS than the wild type cells. Accumulation of more ROS leads to PCD, thereby cells fail to survive under starvation conditions.

7.3.9 Cytosol Calcium (Ca^{2+}) Increase

In yeast, increasing cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration by the response to any stress elements like the mating pheromone α factor (Iida et al., 1990), the addition of glucose to starving cells, and hypotonic shock. Studies suggested that vacuole is involved to increase the internal calcium and mediates the calcium homeostasis. It is evidenced that vacuolar membrane

protein Yvc1 p (homology to transient receptor potential (TRP)) mediates the hyperosmolarity induced Ca^{2+} (Denis and Cyert, 2002). In our study, we observed that Hog1 mediates the increase of the ($[Ca^{2+}]_{cyt}$ upon addition of leptospiral lipopolysaccharide in comparison with NaCl (0.8 M). (Figure 7.12)

Figure 7.12: Cytosolic calcium dynamic measurement



Overall Hog 1 wild-type cells had maintained the cell survival through several intracellular events such as lipid droplet accumulation, controlling the ROS species generation, and maintaining the intracellular calcium dynamics. However, loss of Hog 1 leads to dysfunction of lipid accumulation and calcium signaling during the starvation condition. Moreover, mutant cells exhibit high ROS production leads to cell death.

7.4 Conclusion

This chapter confirms that the leptospiral LPS induces the hog1 signaling and maintain the cell survival during LPS stress by maintaining the cellular events such as production of lipid droplets. So that cells maintain the energy state during the starvation conditions. Moreover, the stressed cells increase the cytosolic calcium concentration and induce the transcription of genes

responsible for cell survival under stress conditions. All the results were also consistent with using hog 1 deletion strain.

7.5 References

1. Aguilera J, Rodríguez-Vargas S, Prieto JA. The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in Saccharomyces cerevisiae. Mol Microbiol. 2005 Apr;56(1):228-39. doi: 10.1111/j.1365-2958.2005.04533.x.
2. Albertyn J, Hohmann S, Prior BA. Characterization of the osmotic-stress response in Saccharomyces cerevisiae: osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently. Curr Genet. 1994 Jan;25(1):12-8. doi: 10.1007/BF00712960.
3. Bermejo C, Rodríguez E, García R, Rodríguez-Peña JM, Rodríguez de la Concepción ML, Rivas C, Arias P, Nombela C, Posas F, Arroyo J. The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. Mol Biol Cell. 2008 Mar;19(3):1113-24. doi: 10.1091/mbc.e07-08-0742.
4. Bilsland E, Molin C, Swaminathan S, Ramne A, Sunnerhagen P. Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. Mol Microbiol. 2004 Sep;53(6):1743-56. doi: 10.1111/j.1365-2958.2004.04238.x
5. Brewster JL, Gustin MC. Hog1: 20 years of discovery and impact. Sci Signal. 2014 Sep 16;7(343):re7. doi: 10.1126/scisignal.2005458.
6. de Nadal E, Alepuz PM, Posas F. Dealing with osmostress through MAP kinase activation. EMBO Rep. 2002 Aug;3(8):735-40. doi: 10.1093/embo-reports/kvf158.

7. de Nadal E, Posas F. Multilayered control of gene expression by stress-activated protein kinases. EMBO J. 2010 Jan 6;29(1):4-13. doi: 10.1038/emboj.2009.346.
8. Del Vescovo V, Casagrande V, Bianchi MM, Piccinni E, Frontali L, Militti C, Fardeau V, Devaux F, Di Sanza C, Presutti C, Negri R. Role of Hog1 and Yaf9 in the transcriptional response of Saccharomyces cerevisiae to cesium chloride. Physiol Genomics. 2008 Mar 14;33(1):110-20. doi: 10.1152/physiolgenomics.00251.2007.
9. Denis V, Cyert MS. Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. J Cell Biol. 2002 Jan 7;156(1):29-34. doi: 10.1083/jcb.200111004.
10. Ferrigno P, Posas F, Koepp D, Saito H, Silver PA. Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. EMBO J. 1998 Oct 1;17(19):5606-14. doi: 10.1093/emboj/17.19.5606.
11. Furukawa K, Hoshi Y, Maeda T, Nakajima T, Abe K. Aspergillus nidulans HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. Mol Microbiol. 2005 Jun;56(5):1246-61. doi: 10.1111/j.1365-2958.2005.04605.x.
12. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science. 1994 Aug 5;265(5173):808-11. doi: 10.1126/science.7914033.
13. Hohmann S. Osmotic adaptation in yeast--control of the yeast osmolyte system. Int Rev Cytol. 2002;215:149-87. doi: 10.1016/s0074-7696(02)15008-x.
14. Iida H, Yagawa Y, Anraku Y. Essential role for induced Ca2+ influx followed by [Ca2+]i rise in maintaining viability of yeast cells late in the mating pheromone response

pathway. A study of $[Ca2+]_{in}$ single Saccharomyces cerevisiae cells with imaging of fura-2. J Biol Chem. 1990 Aug 5;265(22):13391-9.

15. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000 Nov 1;19(21):5720-8. doi: 10.1093/emboj/19.21.5720.
16. Lawrence CL, Botting CH, Antrobus R, Coote PJ. Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. Mol Cell Biol. 2004 Apr;24(8):3307-23. doi: 10.1128/MCB.24.8.3307-3323.2004
17. Lawrence CL, Botting CH, Antrobus R, Coote PJ. Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. Mol Cell Biol. 2004 Apr;24(8):3307-23. doi: 10.1128/MCB.24.8.3307-3323.2004.
18. Maeda T, Takekawa M, Saito H. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science. 1995 Jul 28;269(5223):554-8. doi: 10.1126/science.7624781.
19. Marques JM, Rodrigues RJ, de Magalhães-Sant'ana AC, Gonçalves T. Saccharomyces cerevisiae Hog1 protein phosphorylation upon exposure to bacterial endotoxin. J Biol Chem. 2006 Aug 25;281(34):24687-94. doi: 10.1074/jbc.M603753200.
20. O'Rourke SM, Herskowitz I. Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. Mol Biol Cell. 2004 Feb;15(2):532-42. doi: 10.1091/mbc.e03-07-0521.

21. Panadero J, Pallotti C, Rodríguez-Vargas S, Randez-Gil F, Prieto JA. A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in Saccharomyces cerevisiae. J Biol Chem. 2006 Feb 24;281(8):4638-45. doi: 10.1074/jbc.M512736200.
22. Prick T, Thumm M, Köhrer K, Häussinger D, Vom Dahl S. In yeast, loss of Hog1 leads to osmosensitivity of autophagy. Biochem J. 2006 Feb 15;394(Pt 1):153-61. doi: 10.1042/BJ20051243.
23. Thorsen M, Di Y, Tängemo C, Morillas M, Ahmadpour D, Van der Does C, Wagner A, Johansson E, Boman J, Posas F, Wysocki R, Tamás MJ. The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol Biol Cell. 2006 Oct;17(10):4400-10. doi: 10.1091/mbc.e06-04-0315.
24. Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. Eukaryot Cell. 2002 Apr;1(2):163-73. doi: 10.1128/EC.1.2.163-173.2002.
25. Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. Eukaryot Cell. 2002 Apr;1(2):163-73. doi: 10.1128/EC.1.2.163-173.2002

Summary and Conclusion

CHAPTER VIII

Chapter VIII: Summary and Conclusion

This chapter describes the overall summary and conclusion of the thesis.

8.1 Summary of chapter I

This chapter describes the importance of the diagnosis of leptospirosis and the detection methods available for leptospirosis diagnosis. Also deals with the biosensor principle and applications in biomedical diagnosis.

8.2 Summary of chapter II

This chapter describes a brief explanation of the basic principle of methods used in the thesis. Mainly it describes (i) Serological methods like MAT, ELISA, and Dot Blot Assay. (ii) Structural characterization methods such as Atomic Force Microscopy (AFM), X-ray Photoelectron Spectroscopy (XPS), Zeta potential, and Contact angle measurement analysis. (iii) Electrochemical techniques including Cyclic Voltammetry (CV), Electrochemical impedance spectroscopy (EIS), and Square Wave Voltammetry (SWV).

8.3 Summary of chapter III

This chapter summarizes that the seroprevalence of leptospirosis among the human population is 56.4 % and the bovine population is 33.9%. It is noted that exposure to infected cattle urine and their associated activities was responsible for the leptospiral infection. It is also evidenced that the leptospiral serovar Grippotyphosa was prevalent among each population, which suggested that cattle act as a carrier for leptospirosis in this geographical location (Salem, Tamil Nadu).

8.4 Summary of chapter IV

This chapter summarizes that the leptospiral LPS is a potent biomarker for leptospirosis diagnosis. The molecular weight of extracted LPS lies between 18 and 25 kDa. The optimal concentration of target analyte (LPS) was determined as 1 µg/mL for serological assays such as ELISA and dot blot. The diagnostic accuracy of serodiagnosis of human leptospirosis has more than 90 % sensitivity, about 70 % specificity, and around 90 % PPV and NPV. Likewise, the diagnostic accuracy of bovine leptospirosis was found to be about 90% of statistics. Together dot blot has more specificity than the ELISA. The AUC values obtained from the ROC curve analyses conducted on the ELISA data were all relatively high (>0.9), indicating that this assay is very accurate.

8.5 Summary of chapter V

This chapter summarizes that the developed LPS-based electrochemical biosensor detects leptospirosis at a minimal concentration of 100 nM LPS. Using thiol modified gold electrode allows hydrophobic interaction with leptospiral LPS and strongly binds to the sensor surface. Finally, the LPS imprinted biosensor able to detect serovar specific antibodies present in the serum sample, the sensor has higher selectivity towards the homologous sera than the heterologous sera. Similarly, the sensor specificity achieved by other infectious diseases such as dengue and typhoid, again confirms that the developed sensor has highly specific to the leptospirosis and also serovar specific.

8.6 Summary of chapter VI

This chapter, summarizes the detection of leptospirosis using microRNA. According to the literature, miRNA was increased during the leptospiral infection in particular miRNA 21. Using

a complementary probe to the miRNA 21 the PANI and AuNP modified sensor allows ultralow level detection of miRNA. The limit of detection was found to be 1.5 aM and the limit of quantification is 4.5 aM. The sensor selectivity is achieved by hybridization with the target miRNA-21 single mismatched RNA and the non-complementary RNA. As we expected the sensor has higher R_{CT} towards the target miRNA-21 than the non-complementary RNA sequence. Further, the sensor allowed to detect of real sample analysis using leptospirosis confirmed sera, the sensor achieved drastic increase in the R_{CT} values and decrease in the current density than the healthy sera. So that the developed miRNA sensor used to rapidly detect acute leptospirosis.

8.7 Summary of chapter VII

This chapter summarizes the leptospiral LPS involves the phosphorylation of Hog 1 (homolog to mammalian p38) and leads to the activation of Hog 1 signaling, which helps to transcribes genes or other events responsible for yeast cells under stress conditions. We found that LPS mediated hog 1 phosphorylation leads to activation autophagy related gene phosphorylation, thereby cells encounter and digest the metabolic waste or organelles for their energy during starvation. And also, the cells accumulate lipid droplets, to maintain cell survival. Loss of Hog1 leads to shrinkage in the cell wall and condensation of cytoplasmic part, high level ROS production. This led to the hog 1 mutant cell death under LPS or stress condition.

IX. List of Publications

9.1 First Author Publications

1. **Bothammal P**, Michelraj S, Ganesh V, Verma A, Natarajaseenivasan K. Electrochemical Biosensor for Serogroup Specific Diagnosis of Leptospirosis. Bioelectrochemistry. (IF: 5.373)
2. **Bothammal P**, Ganesh M, Vigneshwaran V, Anbarasu K, Ponmurugan K, Al-Dhabi Naif , Natarajaseenivasan K. Construction of Genomic Library and Screening of Edwardsiella tarda Immunogenic Proteins for Their Protective Efficacy Against Edwardsiellosis. Frontiers in immunology. 2021Nov 16; 12:4504 (IF: 7.561)
3. **Bothammal P**, Ganesh V, Natarajaseenivasan K. A ultra-low level detection of miRNA based biosensor for leptospirosis detection. (Manuscript under preparation)
4. **Bothammal P**, Saranya P, Michelraj S, Sumathi K, Verma A, Ganesh V, Kalimuthusamy Natarajaseenivasan K. Biosensor for diagnosis of bovine leptospirosis: A simple, sensitive, and serovar electrochemical detection strategy. (Manuscript under preparation)
5. **Bothammal P**, Natarajaseenivasan K. Review on the incidence and prevalence of human leptospirosis in five continents of the world. (Manuscript submitted to Critical reviews in Microbiology)

9.2 Co-Author Publications

6. Saranya P, Goswami C, Sumathi K, Balasundareshwaran AH, **Bothammal P**, Dutta LJ, Muralitharan G, Bora DP, Natarajaseenivasan K. Prevalence of leptospirosis among animal herds of north eastern provinces of India. Comparative Immunology, Microbiology and Infectious Diseases. 2021 Dec 1;79:101698. (IF: 2.268)
7. Vanithamani S, Akino Mercy CS, Kanagavel M, Sumaiya K, **Bothammal P**, Saranya P, Prasad M, Ponmurugan K, Muralitharan G, Al-Dhabi NA, Verma A, Vijayachari P,

Natarajaseenivasan K. Biochemical analysis of leptospiral LPS explained the difference between pathogenic and non-pathogenic serogroups. Microb Pathog. 2021 Mar;152:104738 (IF:3.378)

8. Akino Mercy CS, Suriya Muthukumaran N, Velusamy P, **Bothammal P**, Sumaiya K, Saranya P, Langford D, Shanmughapriya S, Natarajaseenivasan K. MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis. mSphere. 2020 Jul 15;5(4):e00409-20. (IF:3.68)
9. Prasad M, **Bothammal P**, Akino Mercy CS, Sumaiya K, Saranya P, Muralitharan G, Natarajaseenivasan K. Leptospiral protein LIC11334 display an immunogenic peptide KNSMP01. Microb Pathog. 2020 Dec;149:104407. (IF: 3.378)
10. Raja V, Prasad M, **Bothammal P**, Saranya P, Sumaiya K, Akino Mercy CS, Natarajaseenivasan K. Silver enhanced nano-gold dot blot immunoassay for leptospirosis. J Microbiol Methods. 2019 Jan;156:20-22. (IF:2.363)

Bioelectrochemistry 144 (2022) 108005

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Electrochemical biosensor for serogroup specific diagnosis of leptospirosis

Palanisamy Bothammal [a], Singarayan Michelraj [b,c], Ganesh Venkatachalam [b,c,*], Ashutosh Verma [d,*], Kalimuthusamy Natarajaseenivasan [a,e,*]

[a] Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India
[b] Electrodics and Electrocatalysis (EEC) Division, CSIR – Central Electrochemical Research Institute (CSIR – CECRI), Karaikudi – 630003, Tamil Nadu, India
[c] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India
[d] Lincoln Memorial University, College of Veterinary Medicine, Harrogate, TN 37752, USA
[e] Department of Neural Sciences, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA

ARTICLE INFO

Article history:
Received 5 April 2021
Received in revised form 18 November 2021
Accepted 21 November 2021
Available online 25 November 2021

Keywords:
Leptospirosis
LPS
Electrochemical biosensor
Serogroup
Impedimetry

ABSTRACT

A problem with the current leptospirosis diagnostic methods is the low sensitivity and specificity during the acute phase of illness. Rapid point-of-care (POC) assays with minimal sample utilization and low cost are desired in clinical practice. Here, we report for the first time lipopolysaccharide (LPS) based electrochemical biosensor that offers a rapid, highly sensitive, serogroup specific diagnosis of leptospirosis during the acute stage of infection and also to distinguish from other flu like infections. The proposed sensor is fabricated by the immobilization of LPS onto dodecanethiol (DT) modified gold electrode. Monolayer of DT is attached through covalent bond (Au-S) interaction onto the gold electrode. Thus, leptospiral antibodies from the human serum samples bind to the LPS present on self-assembled monolayer (SAM) of DT and showed a higher R_{CT} value compared to SAM. The detection limit of the developed LPS sensor is estimated to be 100 nM. This biosensor is the first electrochemical sensing platform used for detection of LPS from *Leptospira* spp. This method is completely a solution-based diagnostic method and therefore it is rapid, simple, and sensitive; thus establishing a key technology towards a useful POC diagnostic strategy in serogroup level and hence an alternative to MAT.

© 2021 Elsevier B.V. All rights reserved.

1. Introduction

Leptospira is a member of order Spirochaetales and belongs to the family Leptospiraceae, which can be either pathogenic or saprophytic [1]. Pathogenic leptospires are present in the renal tubules of infected or carrier animals [2]. Rodents are the major reservoir of leptospirosis, which is an important emerging global public health problem distributed worldwide. The diagnostic tests incurred costs, between 15.3 and 17.2 USD per patient [3]. Usually the infection rate is higher in the tropical regions than in the temperate region [4]. The clinical manifestations vary from mild flu like illness to multi-organs failure and in serious conditions it may even lead to death of the infected host [5]. However, during the early stage of illness the clinical symptoms of leptospirosis are non-specific and challenging to distinguish from dengue, malaria, influenza, and many other febrile diseases [6]. Hence, laboratory based tests are needed to confirm the diagnosis to initiate proper treatment and that is vital for controlling the morbidity and mortality levels [7]. The incidence of leptospirosis and its associated mortality rates with an increased outbreak identified leptospirosis as a fatal disease worldwide [8].

Abbreviations: **LPS**, **Lipopolysaccharide**; **DT**, **Dodecanethiol**; **MAT**, **Microscopic Agglutination Test**; **ELISA**, **Enzyme-linked immuno-sorbent assay**; **ICG-LFB**, **Immuno-chromatography Lateral Flow Assay**; **MCDA-LFB**, **multiple cross displacement amplification-lateral flow biosensor**; **ET**, **Electron Transfer**; **EIS**, **Electrochemical Impedance Spectroscopy**; **EMJH**, **Ellinghausen-McCullough-Johnson-Harris Medium**; **IEC**, **Institutional Ethics Committee**; **PBS**, **Phosphate Buffer Saline**; **SDS-PAGE**, **Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis**; **kDa**, **Kilo Dalton**; **PPV**, **Positive Predictive Value**; **NPV**, **Negative Predictive Value**; **XPS**, **X-ray Photoelectron Spectroscopy**; **CV**, **Cyclic Voltammetry**; **$K[Fe(CN)_6]^{3-/4-}$**, **Potassium hexacyanoferrate (III & IV)**; **SCE**, **Saturated Calomel Electrode**; **NaF**, **Sodium Fluoride**; BSA, Bovine Serum Albumin; AFM, Atomic **Force** Microscopy; WDCA, Water- Drop Contact Angle; MCAT, Micro Capsule Agglutination Test; Au electrode, Gold Electrode; E/V vs. SCE, Potential with respect to the saturated calomel electrode; Z' (Ω cm2), Real part of impedance; - Z'' (Ω cm2), Imaginary part of impedance; J (A/cm2), Current density; RCT, Charge transfer resistance; RS, Solution Resistance.

* Corresponding authors at: Electrodics and Electrocatalysis (EEC) Division, CSIR – Central Electrochemical Research Institute (CSIR – CECRI), Karaikudi – 630003, Tamil Nadu, India (G. Venkatachalam), Lincoln Memorial University, College of Veterinary Medicine, Harrogate, TN – 37752, USA (A. Verma), and Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India (K. Natarajaseenivasan).

E-mail addresses: vganesh@cecri.res.in (G. Venkatachalam), ashutosh.verma@lmunet.edu (A. Verma), natarajaseenivasan@gmail.com (K. Natarajaseenivasan).

https://doi.org/10.1016/j.bioelechem.2021.108005
1567-5394/© 2021 Elsevier B.V. All rights reserved.

Even though numerous techniques are used for the diagnosis of leptospirosis, performing microscopic agglutination test (MAT) to demonstrate four-fold raise in antibody titre and isolation of leptospires from the infected specimen (blood, urine and/or relevant fluids are employed as target samples) are the direct confirmatory evidences of leptospirosis [9]. Though MAT is deemed as a "gold standard" reference serovar specific test for diagnosis of leptospirosis, it is difficult to perform. Also it involves a large panel of live leptospiral culture as antigen and moreover it needs professional experience and skilled manpower for interpretation of results [10]. As leptospirosis is a critical human health problem in developing or under-developed tropical countries, thus, a precise diagnostic format that can be used for point-of-care analysis is vital. Enzyme-linked immuno-sorbent assay (ELISA), dipstick assay, Immuno-chromatography Lateral Flow Assay (ICG-LFA), multiple cross displacement amplification-lateral flow biosensor (MCDA-LFB) method, macroscopic agglutination test, and microcapsule agglutination are the other diagnostic formats developed for the diagnosis [11,12]. Among these methods, ELISA offers acceptable sensitivity and the option of handling many samples at a time and has been established and evaluated with number of variations starting with the use of crude antigens to conserved peptides etc. [13,14]. A Recent study established the role of circulating stable microRNAs (miRNAs) (miR-21-5p, miR-144-3p, and miRlet-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs were able to discriminate the acute leptospiral infection from other febrile diseases [15]. However, the above mentioned diagnostic techniques are primarily based on the conserved protein antigens/miRNAs and cannot be employed for serogroup level identification. Till date there is no serogroup specific diagnostic formats to replace the reference, MAT.

Lipopolysaccharide (LPS), a major component present in the outer membrane of *Leptospira* is one of the major essential immuno-dominant antigens. The antigenic nature of LPS initiates the immuno-modulatory activity during pathogenesis and structural variation between serogroup helps in the serogroup level identification of *Leptospira* species [16,17]. Concerning, validation of such serogroup specific antigens in diagnostics can serve as an alternative to MAT. Electrochemical detection is a desirable unconventional technique for application in primary health care diagnosis due to its considerable advantages for a point-of-care testing facility. Electrochemical immunosensor based on gold-labeled monoclonal anti-LipL32, Loa22 gene based DNA biosensor for the diagnosis of leptospirosis was developed and being a genus-specific technique unable to find the infecting serovar [18–20].

In this context, here, for the first time, we attempt to develop an electrochemical biosensor for serogroup level diagnosis of leptospirosis. Electrochemical sensors offer rapid response, higher sensitivity, simplicity and ease to use [21]. Particularly the sensing layer for LPS detection includes a SAM of dodecanethiol (DT), antibodies and functionalized LPS that generates the change in the electronic properties upon binding of LPS to specific antibodies. Binding events are then probed by measuring the electron transfer (ET) reaction associated with the redox-active species in a solution using electrochemical impedance spectroscopy (EIS) [22]. The charge transfer resistance, R_{CT} is determined by fitting the experimental data to a semicircle and determining its intercept on the x-axis based on the modified Randle's equivalent circuit. Later, this parameter is correlated with the binding of target analyte onto the modified electrode surface.

In order to develop a serogroup specific diagnosis, we target to build up leptospiral LPS based electrochemical immunosensor using a gold electrode modified with SAM of DT. The binding events were measured using EIS, and our results reveal that the sensor has higher selectivity and sensitivity to the leptospiral specific homologous sera compared to heterologous and sera from other febrile cases. The analytical performances were evaluated systematically for the developed immuno-sensors for their applicability for a serogroup specific diagnosis of leptospirosis. Proposed LPS based detection strategy allows serogroup specific detection due to the unique structural difference exists between the serogroups. Until now, MAT is considered as gold standard technique for serogroups level identification of leptospirosis, but it is laborious and difficult to maintain live leptospiral cultures all the time for while performing the experiments and inter-laboratory variations may also occur. Hence the proposed LPS based strategy allows one to overcome the drawbacks of MAT and is an ideal for alternative to MAT.

2. Experimental procedures and characterization

2.1. Leptospiral strains and media

A panel of pathogenic leptospiral strains: *Leptospira interrogans* serovar, Australis strain Ballico, *Leptospira interrogans* serovar, Autumnalis strain N2, *Leptospira borgpetersenii* serovar Ballum strain Mus 127, *Leptospira interrogans* serovar Bataviae strain Swart, *Leptospira interrogans* serovar Canicola strain Hond Utrecht IV, *Leptospira kirschneri* serovar Grippotyphosa strain Moskva V, *Leptospira interrogans* serovar Hebdomadis strain Hebdomadis, *Leptospira borgpetersenii* serovar Javanica strain Poi, *Leptospira interrogans* serovar Pomona strain Pomona, *Leptospira interrogans* serovar Sejroe strain Hardjoprajitno, *Leptospira interrogans* serovar Pyrogenes strain Salinem, and the non-pathogenic *Leptospira biflexa* serovar Andamana CH11 were obtained from World Health Organization (WHO) Reference Centre for Leptospirosis, Regional Medical Research Centre, Indian Council of Medical Research (ICMR), Port Blair, India and used for the present study. Strains were maintained by regular sub-culturing in Ellinghausen-McCul lough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) in a shaking incubator (100 rpm) at 30 °C.

2.2. Patients and case definition

In total, 252 serum samples collected during the early phase of illness (0 to 10 days after the onset of disease) were selected from a bank of 370 laboratory-confirmed cases of leptospirosis (a positive IgM ELISA or MAT titer of $\geq$ 1:160 or isolation of leptospires from the blood). A total of 56 seronegative healthy controls matched with respect to age (±5 years) and sex, and 39 patients who were hospitalized with a clinical suspicion of leptospirosis and subsequently diagnosed as having other illness including dengue (18), malaria (17) and typhoid (04) based on laboratory evidences were also included in the study. Informed written consent was obtained from both the cases and controls before sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (BDU/IEC/2020/02; DM/2014/101/49), as well as permitted by the Directorate of Health Services (5796/TV1/07), Tamil Nadu with due approval.

2.3. Extraction of leptospiral LPS, western blotting and ELISA

Leptospires were collected from mid-log cultures by centrifugation at 10,000xg for 30 min and then washed three times with sterile 1X Dulbecco's phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA) before LPS extraction. LPS was extracted following the standard hot phenol-water extraction as described previously [16]. LPS was quantified by the phenol/sulfuric acid method using sucrose as a standard [23]. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) profiling

of extracted was performed on a 12% polyacrylamide gel using a discontinuous buffer system. The extracted LPSs were mixed with 2X SDS-PAGE sample loading buffer (BioRad, Hercules, CA, USA) and boiled for 5 min before loading. Electrophoresis was carried out in a vertical electrophoretic mini-cell unit (Bio-Rad, Hercules, CA, USA) for 2 h at 100 V using Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) [24]. A modified silver staining procedure was performed to detect the LPS bands on SDS-PAGE [16,25] and documented using a gel documentation system (Bio-Rad, Hercules, CA, USA). The apparent molecular mass of the extracted LPSs lies between 14 and 28 kDa (**Fig. S1A**). The separated LPSs were transferred to nitrocellulose membranes (0.2 μm; Bio-Rad, USA) [24] using V20 semi-dry blotter (Scie-Plas, UK), and probed with serum (1:200) from confirmed cases for leptospirosis, followed by incubation with anti-human IgG antibody conjugated with *horseradish peroxidase* (Sigma-Aldrich, St. Louis, MO, USA) and the bands were visualized by using 4-chloro-α-naphthol (Sigma-Aldrich, St. Louis, MO, USA) (**Fig. S1B**) and it showed the reactivity to respective pathogenic leptospiral LPS. There was no reactivity was observed in non-pathogenic serovar Andamana. Serogroups reactivity was observed for all leptospiral LPSs against their homologous and heterologous sera confirming the serogroups specificity and reactivity. Moreover, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of homologous sera values obtained by ELISA were represented in Table 1.

2.4. Electrochemical studies

The binding events occur during the sensing of these LPSs for the diagnosis of leptospirosis were monitored using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). All these electrochemical studies were performed with an Autolab PGSTAT 302 instrument (Metrohm, Autolab-Potentiostat/ Galvanostat Instruments (PGSTAT302N), Netherlands). The electrochemical properties of the monolayer towards electron transfer process were studied using $K[Fe(CN)_6]^{3-/4-}$ redox couple as a probe. The electrolyte solutions used for electrochemical measurements were prepared using Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany) having a resistivity of 18.2 MΩ cm. CV and EIS studies were conducted in a three-electrode system at 25 °C consisting of a platinum rod/ wire, a saturated calomel electrode (SCE) and modified gold electrode as a counter, reference, and working electrodes respectively. Cyclic voltammetry was performed in 1 mM potassium ferro/ferricyanide aqueous solution containing 0.1 M NaF as a supporting electrolyte. Impedance measurements were carried out at the formal redox potential ($E_{1/2}$ = 0.16 V vs. SCE, as determined from cyclic voltammetry) in an aqueous solution containing equal concentrations of both the oxidized and reduced forms of $[Fe(CN)_6]^{3-/4-}$ (1 mM each) with 0.1 M NaF as a supporting electrolyte [26]. The frequency ranging from 100 KHz to 0.01 Hz with an ac amplitude of 5 mV was used for the impedance analysis.

2.5. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra were recorded with a MulitiLAB system (Multilab 2000, Twin anode X-ray source, Thermo scientific, UK). Gold-coated plates were used to prepare the samples that were mounted with a spring clip during the measurement. A monolayer of DT was prepared by immersing the cleaned gold strips into neat DT for 1 h. After this, the SAM modified gold surface was thoroughly rinsed with ethanol and Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany). Then, DT monolayer-modified gold electrode was dipped into an aqueous solution of LPS having a concentration ranging from 20 nM to 100 μM for about 1 h. After this LPS-DT modified gold electrode was rinsed with Milli-Q water. Further, the LPS coated DT monolayer-modified gold electrodes were dipped into an aqueous solution of 100 μM bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for about 1 h each, after which they were thoroughly rinsed with Milli-Q water. After blocking with BSA, the LPS coated electrodes were dipped into an aqueous solution of either the homologous or the heterologous patient's sera (contain the specific antibodies against the leptospiral LPS) and sera of healthy controls. In a typical experiment, a few survey scans in the binding energy ranging from −10 eV to 1350 eV were collected at a resolution of 1.0 eV. Then, detailed scans of 10 eV – 30 eV over a single feature/ element were collected at a resolution of 0.1 eV. XPS is primarily used to identify the presence of elements along with their corresponding oxidation states and the confirmation of LPS binding onto the modified electrode surfaces.

2.6. Contact angle measurements and Atomic Force Microscopy (AFM) analysis

Water-drop contact angle (WDCA) measurements were also performed at room temperature by employing the sessile drop technique using a commercial VCA Optima instrument. All the contact angle values were determined from the average of at least three independent measurements performed under the same conditions at different locations on the sample surface. Similarly, the surface topography of bare gold electrode, DT monolayer modified Au, leptospiral LPS immobilized DT electrode and the anti-leptospiral antibody functionalized Au electrode was analyzed using Atomic Force Microscopy [AFM] (5500 scanning probe microscope, Agilent Tech., USA) in a non-contact mode. The surface morphological features and thickness profiles were analyzed using the software provided by them.

3. Results

3.1. Construction and characterization of the sensing electrode

Here in, we demonstrated a simple and efficient way to detect *Leptospira* specific antibodies using chemically modified gold elec-

Table 1
Sensitivity, specificity, PPV and NPV of homologous sera.

LPS (Serogroup)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	kappa value
Australis	93.75	99.16	83.30	99.71	0.876
Autumnalis	91.67	100	100	99.43	0.953
Bataviae	100	99.72	92.85	100	0.962
Canicola	96.43	99.68	98.18	99.37	0.968
Grippotyphosa	93.40	99.03	98.80	99.51	0.984
Javanica	92.86	100	100	99.72	0.962
Pomona	93.30	99.71	96.55	99.42	0.945
Pyrogenes	100	99.71	96.00	100	0.978

trodes. The overall construction process is depicted in Scheme 1A-E. Evaluation of surface modification steps and probing of the binding events towards the development of electrochemical biosensor for the detection of leptospirosis is carried out using electrochemical techniques namely cyclic voltammetry and impedance measurements using potassium ferro/ferricyanide as a redox probe. Impedance spectroscopy data were analyzed using an equivalent circuit fitting procedure based on Randle's equivalent circuit model **(Fig. S2A and S2B)** (sometimes modified one) to determine the charge transfer resistance (R_{CT}), a parameter used to quantify the rate of electron transfer across the interface in turn the binding events. **Fig. S2C and S2D** show the CVs and Nyquist plots of bare Au electrode before modification and after different modification steps used for the fabrication of sensor along with the corresponding equivalent circuits used for fitting the measured impedance data and for the determination of parameters like R_{CT}. Here Z' represents the real part and Z" denotes the imaginary part of the impedance at the frequency range between 100 kHz and 0.01 Hz with an alternative current amplitude of 5 mV.

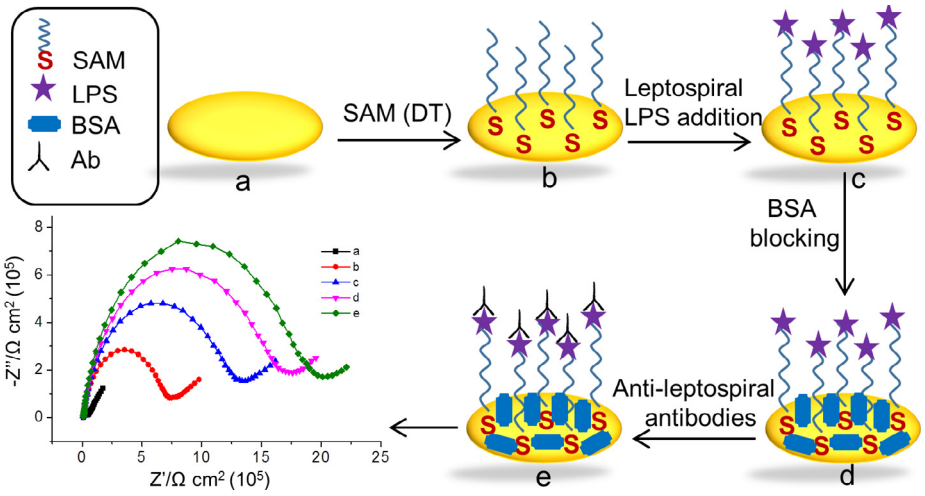
Several factors contribute the electronic or ionic properties of the surface electrode, such as the solution resistance (RS), the double layer capacitance (C) associated with Warburg formed by the ions at the vicinity of the electrode, the charge transfer resistance (RCT) that represents the current flow due to redox reactions at the electrode electrolyte interface upon addition of different element such as LPS, BSA and anti-leptospiral antibodies and a constant phase element Table 2.

To illustrate the surface binding after each modification step, WDCA measurements were performed and the results are shown in **Fig. S2E**. WDCA analysis is used to determine the wettability of the functionalized surfaces. Initially bare Au surface without any modification exhibits a contact angle of 83.20°, while the contact angle of SAM modified surface was measured to be 99.30°, revealing the formation of monolayer of DT leading to hydrophobicity of the resultant modified surface. Further the addition of leptospiral LPS onto the SAM modified surface increases the contact angle to 102.40° indicating the enhancement in the hydrophobic nature of the surface due to the presence of long alkyl chain from the LPS moiety. In contrast, after attachment of the anti-leptospiral antibody to LPS anchored DT-modified surface through the antigen–antibody interaction a decrease in the contact angle of 90.00° is noted; revealing its switch over to a slight hydrophilic nature due to the presence of hydrophilic amino acids in the antibody. Thus these results provide evidence for the modification steps used in our study for the fabrication of sensor.

Fig. 1 displays the representative AFM images of gold electrodes before and after each modification step employed for the fabrication of biosensor. As seen from Fig. 1, the bare gold surface showed a smooth surface with an average height of 3.45 nm (Fig. 1A). After the formation of monolayer of DT, the surface height increased to 9 nm (Fig. 1B), further upon immobilization of LPS, the surface height increased to12.8 nm (Fig. 1C). Finally, after the functionalization with antibody, the surface height increased further up to 16 nm (Fig. 1D). The increasing surface height along with the roughness factor is mainly attributed to the adsorption of molecules in each and every modification step. These results clearly confirm the functionalization of Au electrodes with the target molecules and reaffirm the formation of different layers on Au surface during the modification.

3.2. Impedimetric response of the chemically modified gold electrodes

Before modification, electrochemical response of the bare electrode was recorded. Bare Au electrode shows a clear redox peak observed at 0.276 V and 0.201 V in cyclic voltammograms due to the redox reaction occurs on the surface of gold electrode. Initially, a self-assembled monolayer of DT is formed on the gold electrode, which leads to the formation of a highly organized hydrophobic monolayer that blocks the electron transfer process. A drastic decrease (2-fold in comparison to bare Au) in the redox current is noted in CV after the addition of DT, which indicates that the electron transfer reaction is inhibited (Fig. 2A). The Nyquist plot shows, an obvious semicircle up to low frequency with the R_{CT} value of 1.28×10^4 Ω cm^2, reveals the charge transfer controlled process for the redox probe on SAM modified electrode (Fig. 2B). In Fig. 2B inset shows the Nyquist plot of bare gold electrode shows a small semicircle with a straight line in the Nyquist plot of impedance spectroscopy and the corresponding R_{CT} value is measured to be 3850 Ω cm^2 which defines the characteristic of a diffusion-controlled process. This EIS data indicates the successful formation of DT monolayer on the gold electrode and hindering the electron transfer reaction on the surface which correlates well with the CV results. This is due to the inability of the redox species to penetrate the monolayers onto the conductive electrode surface. To examine the surface structure of DT monolayer modified electrode XPS studies were performed. **Fig. S3A** depicts the XPS survey spectrum of DT monolayer modified Au electrode and **Fig. S3B** reveals the presence of sulphur peaks (S2p) at 162.3 eV corresponding to the sulfhydryl group. DT can interact with Au electrode through



Scheme.1. Schematic representation of the biosensor construction process. (a) bare gold electrode, (b) DT monolayer modification, (c) DT + LPS modification, (d) DT + LPS + BSA addition and (e) DT + LPS + BSA + Anti-leptospiral antibody modification.

Table 2
Elements present in the equivalent circuit used for fitting the impedance data of proposed sensor (Grippotyphosa).

Monolayer	$R_S u$	CPE (x 10^{-7})	Freq	R_{CTCT}	Warburg(x 10^{-5})	Chsq
Bare	342	0.104	0.914	3964	7.793	7.43 x10^{-3}
SAM	444.7	3.649	0.861	1.669 x10^{5}	4.786	1.59 x10^{-2}
LPS	409.7	2.785	0.828	4.19 x10^{5}	2.279	1.48 x10^{-2}
BSA	622.7	6.01	0.835	6.86 x10^{5}	2.241	9.09 x10^{-3}
HS	357.8	3.149	0.818	3.24 x10^{6}	1.179	2.21 x10^{-2}
HES	353.9	6.572	0.814	2.218 x10^{6}	1.097	3.14 x10^{-2}
HC	376.2	3.67	0.807	1.757 x10^{6}	1.586	2.95 x10^{-2}

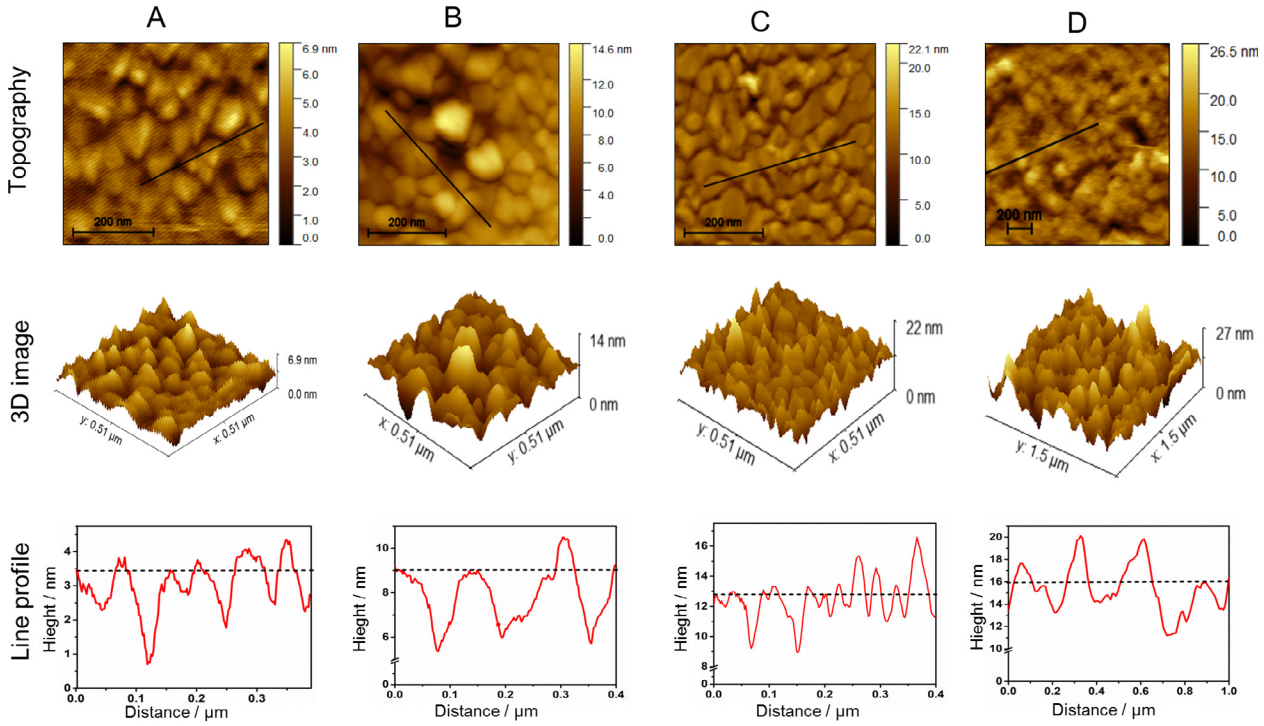


Fig. 1. Representative AFM images displaying surface topography, 3D-images and line profiles of various modified electrodes used in this study. Panel A- bare gold electrode, Panel B- DT-monolayer, Panel C- leptospiral LPS modified and Panel D- anti-leptospiral antibody functionalized electrodes respectively.

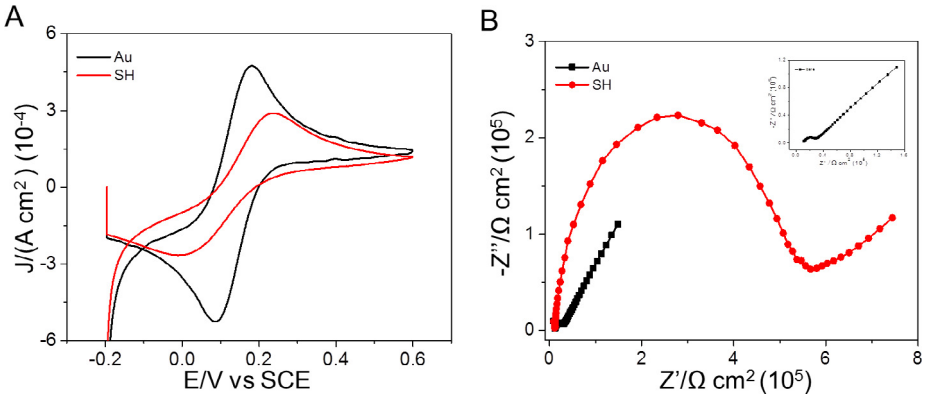


Fig. 2. Electrochemistry characterization of the sensing electrode. (A) Cyclic voltammograms, and (B) Impedance (Nyquist) plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for bare gold electrode and DT monolayer modified electrode compared to bare Au electrode. (Inset. Nyquist plot of bare Au electrode).

sulfhydryl groups forming Au-S interaction leading to the formation of organized SAM.

3.3. Functionalization of SAM coated sensing electrode

In order to achieve the serogroup detection of leptospirosis, the SAM coated electrode was functionalized with the addition of leptospiral LPS. Such chemical modification is carried out by anchoring the leptospiral LPS onto the DT monolayer films through hydrophobic interactions between the long aliphatic chains from LPS with the alkyl chains of the monolayer. To detect the optimal concentration of leptospiral LPS for the detection of leptospirosis the impedance responses were recorded for the addition of different concentrations of leptospiral LPS to the SAM coated surface in

the range from 10 pM, 100 pM, 500 pM, 100 nM, 500 nM to 1 μM and their corresponding R_{CT} values were measured to be 7.6×10^5 Ω cm^2, 9.74×10^5 Ω cm^2, 10.09×10^5 Ω cm^2, 11.76×10^5 Ω cm^2, 11.79×10^5 Ω cm^2 and 12.44×10^5 Ω cm^2 respectively (Fig. 3A). The Binding of LPS is identified to be remarkably effective in the concentration range of 100 nM (Fig. 3B). The increase in R_{CT} is probably related to both an increase in negative charges and thickness of the molecular layer on the electrode surface. However, in some serogroups the effect of mass transfer persists at the lower frequency range.

To further probe into the structure of modified surfaces, XPS analysis of SAM coated gold electrode was carried out. XPS spectra of these modified electrodes display peaks due to nitrogen and phosphorous that appear at 399.1 eV and 132.8 eV due to the presence of LPS apart from the carbon and sulphur peaks noted at 285 eV and 162.9 eV arising from the DT monolayer respectively **(Fig. S4)**. These results clearly suggest the anchoring of LPS onto a monolayer modified gold electrode. In order to saturate the electrode surface, BSA was immobilized and used as a surface covering agent. The addition of BSA, which blocks the surface other than to LPS has the R_{CT} value of 4.8×10^4 Ω cm^2 and allows the binding of antibodies specific to the target analyte.

3.4. Development of serovar specific biosensor

To develop the serovar-specific biosensor and to eliminate the possible cross reactive binding of other serogroups, independent experiments were conducted using LPS of different leptospiral serogroups. Moreover to improve the specific binding and to decrease the nonspecific binding from other sera, homologous sera confirmed by MAT and ELISA analyses were used for the study. The LPS modified electrode was immobilized with the anti-leptospiral antibodies, for that the laboratory-confirmed leptospirosis patient's serum samples were used. EIS response of the sensor is drastically increased further, which indicates strong affinity between the LPS and anti-leptospiral antibodies. In order to avoid hook effect of the antigen antibody interaction we design the experiments at different dilutions of the patient's serum containing the circulating anti-leptospiral antibodies against the leptospiral LPS. The representative R_{CT} values for the dilution of 1:100 (5.682×10^7 Ω cm^2), 1:200 (5.475×10^7 Ω cm^2), 1:500 (5.089×10^7 Ω cm^2) and 1:1000 (4.77×10^7 Ω cm^2) respectively were determined (**Fig. S5A and S5B**). These results indicate the decreasing R_{CT} value while increasing the dilution of the sera. Upon increasing the dilution of antibody further the R_{CT} values are found to be decreased, confirming the decreasing concentrations of antibody. For all further experiments, a dilution of 1:1000 was used. At the same time, upon addition of healthy control serum samples there was no significant increase in R_{CT} is noted indicating the selectivity of the biosensor against only leptospiral specific antibodies.

Since the focus of our study is the serogroup specific detection of leptospirosis, we analyzed the interaction between Grippotyphosa LPS molecule to the MAT and ELISA confirmed patients' sera (anti-leptospiral antibodies). Hence the sensing electrode consisting of Grippotyphosa LPS was subjected into various MAT confirmed leptospiral cases which have specific reactivity to Australis, Autumnalis, Ballum, Canicola, Pomona, Hebdomadis, Pyrogenes, Sejroe and Grippotyphosa. Their corresponding impedance responses were shown in Fig. 4A from which their R_{CT} values were determined and shown in Fig. 4B. Together the sensing electrode comprising of Grippotyphosa LPS has a higher affinity to the Hebdomadis ($3.6x\ 10^8$ Ω cm^2) and Ballum ($2.46x\ 10^8$ Ω cm^2) compared to the Grippotyphosa positive case (7.3×10^8 Ω cm^2). From these results, it is clear that the proposed biosensor possesses higher specificity to the homologous sera than the heterologous sera.

A limited number of clinical samples (n = 12) were used for the evaluation to assess the ability of biosensor in analyzing the 'real world' samples. As mentioned before the clinical samples were analyzed with serological assays such as MAT and ELISA to confirm the presence of anti-leptospiral antibodies prior to the biosensor experiments. The results summarized in Table 3 reveal that the sensor could able to differentiate the healthy individual sera. The representative histogram (Fig. 4C and 4D) demonstrates the specificity associated with each sensor. From these results, it is clear that the sensor has higher affinity to LPSs of Hebdomadis (8.694×10^7 Ω cm^2), Pyrogenes (2.071×10^7 Ω cm^2), Sejroe (1.331×10^7 Ω cm^2), Ballum (1.033×10^7 Ω cm^2) followed by Australis (0.979×10^7 Ω cm^2), Canicola (0.707×10^7 Ω cm^2), Autumnalis (0.683×10^7 Ω cm^2), Bataviae (0.547×10^7 Ω cm^2), Pomona (0.165×10^7 Ω cm^2) and Javanica (0.142×10^7 Ω cm^2) **[Fig. S6A-J]**. Then there is a slight increase in R_{CT} value upon addition of BSA, which indicates the prevention of nonspecific interactions on the LPS functionalized electrode. Further addition of specific anti-leptospiral antibody (leptospirosis confirmed (MAT) patients samples), a sharp increase in the R_{CT} was observed. The representative R_{CT} values for Hebdomadis, Pyrogenes, Sejroe, Ballum, Australis, Canicola, Autumnalis, Bataviae, Pomona and Javanica were 15.69, 4.252, 3.117, 2.502, 2.926, 1.343, 1.172, 2.402, 0.225 and 0.188×10^7 Ω cm^2 respectively. These findings reveal that the antigen –antibody complex was formed on the surface of sensing elec-

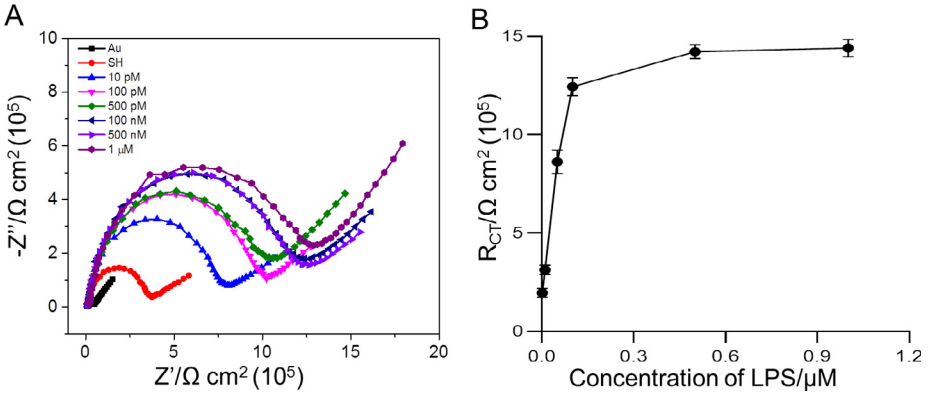
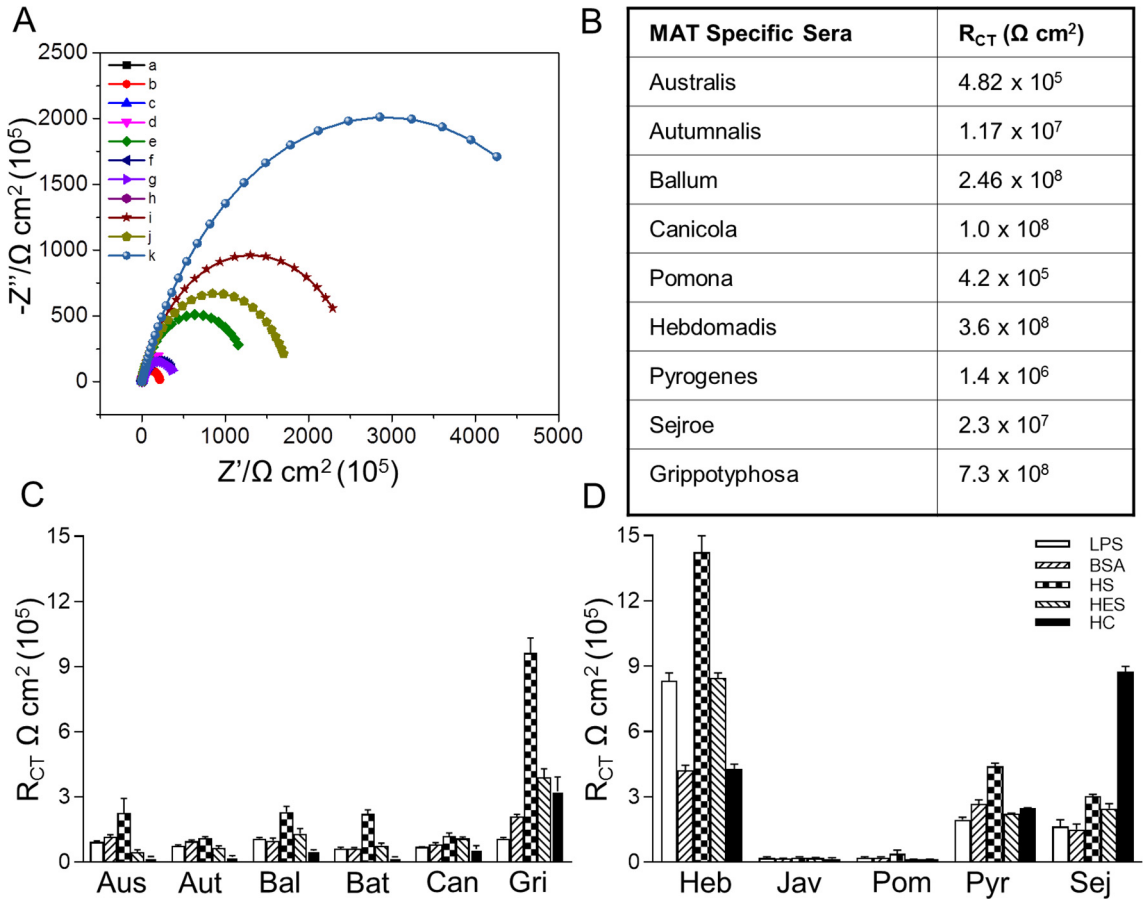


Fig. 3. Optimization of leptospiral LPS concentration. (A) Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for different concentrations of Grippotyphosa lipopolysaccharide (Au- Bare Au, SH- DT-monolayer, 10 nM – 1 μM- different concentrations of LPS). The lines show the fitting of measured impedance data points using Randle's equivalent circuit. (B) Plot of the ratio, R_{CT}/R^0_{CT}, (where R^0_{CT} is the resistance to the electron transfer reaction before LPS addition and R_{CT} is the measured values for each addition of LPS) vs. concentration of Grippotyphosa LPS.



MAT Specific Sera	R_{CT} (Ω cm^2)
Australis	4.82 x 10^5
Autumnalis	1.17 x 10^7
Ballum	2.46 x 10^8
Canicola	1.0 x 10^8
Pomona	4.2 x 10^5
Hebdomadis	3.6 x 10^8
Pyrogenes	1.4 x 10^6
Sejroe	2.3 x 10^7
Grippotyphosa	7.3 x 10^8

Fig. 4. Serovar specific analysis. (A and B) Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for the Grippotyphosa LPS against homologous and heterologous sera (a- bare Au, b- DT monolayer, c- DT + GrippoLPS, d- DT + Grippo LPS + BSA, e-j (DT + Grippo LPS + BSA + Heterologous sera, k- DT + GrippoLPS + BSA + homologous sera), and their R_{CT} values. (C and D) Histrogram of the R_{CT} values for different serovars namely Australis, Autumnalis, Ballum, Bataviae, Canicola, Hebdomadis, Javanica, Pomona, Pyrogenes and Sejroe respectively.

Table 3
R_{CT} values of the sensing electrode consisting of various leptospiral lipopolysaccharide with respect to the homologous and heterologous sera.

Serovar	R_{CT} values of different modified electrodes (x 10^7 Ω cm^2)				
	LPS	BSA	Homologous sera	Heterologous sera	Healthy sera
Australis	0.979	1.092	2.926	0.574	0.193
Autumnalis	0.683	0.893	1.172	0.745	0.156
Ballum	1.033	1.103	2.051	1.538	0.475
Bataviae	0.547	0.666	2.402	0.648	0.169
Canicola	0.707	0.710	1.343	1.158	0.442
Grippotyphosa	1.134	2.199	10.32	4.290	3.920
Hebdomadis	8.694	4.459	15.69	8.694	4.093
Javanica	0.142	0.151	0.188	0.169	0.109
Pomona	0.165	0.177	0.225	0.109	0.128
Pyrogenes	2.071	2.874	4.252	2.230	2.452
Sejroe	1.331	1.251	3.117	2.698	8.991

trode. Further the binding of anti-leptospiral antibody to the specific leptospiral LPS to form the antigen–antibody complex occur on the gold electrode as confirmed by XPS, which shows S 2p peak at 162.5 eV, and N 1 s peak at 400.1 eV. Moreover, their corresponding intensity values were increased when compared to SAM and LPS modified electrodes confirming the presence of antibody (**Fig. S7**) and reconfirms the binding.

3.5. Selectivity of the developed biosensor

In order to investigate the selectivity of this particular biosensor, the modified electrode has been tested with sera samples of dengue and typhoid patients, where the R_{CT} values were measured to be 2.3 × 10^6 Ω cm^2 and 1.4 × 10^6 Ω cm^2 and compared the R_{CT} value of *Leptospira* specific sera of 7.3 × 10^8 Ω cm^2, which was significantly higher. As shown in the Nyquist plot (Fig. 5A and 5B), the biosensor responses to the other samples are significantly lower due to the nonspecific interaction with the antibody, which makes the sensor electrode very specific to the target analyte of leptospiral LPS. Further, we also performed the electrochemical response for non-pathogenic leptospiral LPS to the anti- leptospiral antibody (patient's serum) **Fig. S8A**, where the R_{CT} value was found to be lower (2.8 × 10^6 Ω cm^2) compared to the pathogenic LPS. Also the healthy control sera showed decreased R_{CT} (2.0 × 10^6 Ω cm^2) **Fig. S8B**, thus confirming the higher specificity of the proposed biosensor.

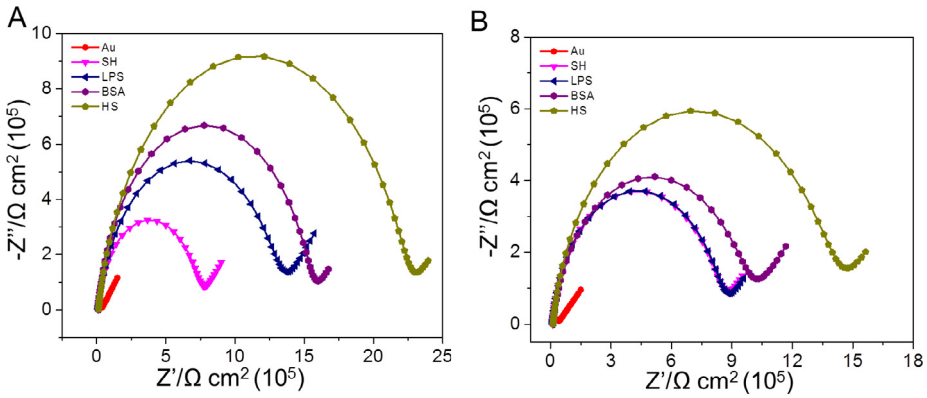


Fig. 5. Selectivity of the proposed sensor. Nyquist plots for the leptospiral LPS reactivity against the dengue (A) and typhoid (B) patients sera. Legends: (Au- bare Au, SH- DT - monolayer, LPS- DT + LPS, BSA- DT + LPS + BSA complex, HS- DT + LPS + BSA + Patient's sera (Dengue or Typhiod).

We also compared our sensor performance with that of other methods or functionalized materials used for LPS detection (Table 4). Though previous detection methods mostly used *E.coli* LPS [27,28] and this is the first attempt on leptospiral LPS based detection for real-world sample diagnosis with a low limit of detection range consistent with other LPS detection methods.

4. Discussion

In the outer membrane of leptospires, LPS is the major antigenic component present and it is thought as the best immunogenic molecule because they are serogroup specific [29]. Since the laboratory diagnosis for leptospirosis mainly depends upon the serological techniques, and the serogroup-specific MAT is the most widely used reference method these days. Generally, MAT detects both anti-leptospiral, IgM and IgG and most of the agglutinating antibodies confronted in MAT are IgM that is produced against the LPS in the acute phase of illness [30,31]. Even though MAT is considered as the gold standard test for the diagnosis of leptospirosis, but it is complex to control, perform, and interpret. Mostly it is performed with locally circulating predominant leptospiral serovars as live antigens and MAT forms the bases for serological diagnosis and for the classification of leptospires. Especially during outbreak condition, investigation of such a large number of serum samples by a complicated technique like MAT may compromise the quality of results. Apart from these drawbacks, MAT also suffers from other major issues like cross-contamination of the antigen cultures, interference of the culture medium to affect the MAT titers, and the maintenance of a large number of strains are hazardous for laboratory workers [32].

Under critical circumstances, the use of an alternate rapid serological diagnostic test is highly warranted. Serological tests like the microcapsule agglutination test (MCAT), Lepto Dipstick, Lepto Lateral Flow, and Lepto Dri Dot have been evaluated as rapid tests for leptospirosis as alternative techniques. But the majority of them are genus-specific and they mainly suffer from low sensitivity and specificity during the acute stage of the disease [33–36]. An attempt was made to develop a serogroup specific diagnosis of leptospirosis by ICG-LFA, but in this array the Ballum LPS showed cross-reactivity with other serovars [16].

Further to improvise the rapidity of the technique and make it applicable in outbreak situations, a point of care serogroup specific diagnosis, LPS based electrochemical biosensor is proposed and demonstrated in this work for the first time and evaluated for the diagnosis of leptospirosis. This particular sensor is fabricated by immobilizing LPS onto the DT modified gold electrode. This test could potentially be assessed and employed as a point of care alternative test for MAT to detect the locally prevalent infecting serogroups in endemic regions and offers many advantages.

5. Conclusions

In conclusion, our results clearly demonstrate the rapid diagnostic method for the specific diagnosis of acute leptospirosis using electrochemical impedance measurements by employing LPS based serovar detection strategy. The proposed biosensor could sense the wider concentration of leptospiral LPS ranging from 10 pM to 1 μM. EIS measurements of LPS based sensor offers a simple and promising method to develop extremely effective and sensitive serogroup specific diagnosis of acute leptospirosis. This method has several significant advantages towards the prevention of leptospirosis diseases, whereby this specific sensor could be directly employed to identify the serogroup specific leptospiral agglutinins. Further, the proposed sensor could also help for the timely initiation of antibiotics that may be critical for the treatment of leptospirosis.

Table 4
Comparison of selected methods for detection of LPS.

Recognition material	Detection Method	Detection range	Sensitivity	Reference
293/hTLR4-MD2-CD14-pGL4.26-m.cherry-NF-kB cells	Fluorescent	0.01 – 100 ng mL^{-1}	0.01 ng mL^{-1}	[37]
Aptamer	Electrochemical Impedance Spectroscopy	0.001 – 1.0 ng mL^{-1}	0.001 ng mL^{-1}	[38]
Polymyxin B	Electrochemical Impedance Spectroscopy	0.2 – 0.8 ng mL^{-1}	0.2 ng mL^{-1}	[39]
Au NPs/ CramoLLlectin	Electrochemical Impedance Spectroscopy	Not reported	200 μg mL^{-1}	[40]
Aptamer	Electrochemical Impedance Spectroscopy	0.01 aM – 1.0 pM mL^{-1}	7.94×10^{-21} M mL^{-1}	[41]
rhTLR4/MD-2complex	Differential Pulse Voltammetry	0.0005 – 5 EU mL^{-1}	0.0002 EU mL^{-1}	[42]
Dodecanethiol- modified Au electrode	Cyclic Voltammetry and Electrochemical Impedance Spectroscopy	10 pM – 1 μM mL^{-1}	10 pM mL^{-1}	This study

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

PB is thankful to Indian Council of Medical Research for financial support through ICMR-SRF (5/3/8/39/ITR-F/2020). We greatly thank Dr. P. Vijayachari, Director, Regional Medical Research Centre, Indian Council of Medical Research, Port Blair, Andaman Islands for the leptospiral strains. We also acknowledge Vice-Chancellor, Bharathidasan University for the support and facilities to carry out this work.

Funding

This work was supported by Indian Council of Medical Research, Government of India, for the research and development grant (ICMR-Leptos/33/2013-ECD-I; ICMR-Leptos/34/2013-ECD-I) to KNS and the Lincoln Memorial University (LMU), College of Veterinary Medicine, United States of America (USA) for the research and development grant (19873/P3/2018 dated 12.07.2018) to AV and KNS.

CECRI Manuscript Communication Number: CECRI/PESVC/Pubs./2021–017.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.108005.

References

[1] A.R. Bharti, J.E. Nally, J.N. Ricaldi, M.A. Matthias, M.M. Diaz, M.A. Lovett, P.N. Levett, R.H. Gilman, M.R. Willig, E. Gotuzzo, J.M. Vinetz, Leptospirosis: A zoonotic disease of global importance, Lancet Infect. Dis. 3 (12) (2003) 757–771, https://doi.org/10.1016/S1473-3099(03)00830-2.

[2] S. Faine, B. Adler, C. Bolin, P. Perolat, Leptospira and leptospirosis, Melbourne, MediSci, Australia, 1999, p. 259.

[3] J.M. Sanhueza, M.G. Baker, J. Benschop, J.M. Collins Emerson, P.R. Wilson, C. Heuer, Estimation of the burden of leptospirosis in New Zealand, Zoonoses Public Health. 67(2) (2020) 167–176, doi: 10.1111/zph.12668

[4] D.A. Haake, P.N. Levett, Leptospirosis in humans, Leptospira Leptospirosis, Springer 387 (2015) 65–97, https://doi.org/10.1007/978-3-662-45059-8_5.

[5] M.G.A. Goris, J.F.P. Wagenaar, R.A. Hartskeerl, E.C.M. van Gorp, S. Schuller, A.M. Monahan, J.E. Nally, T. van der Poll, C. van 't Veer, S. Costa Oliveira, Potent innate immune response to pathogenic leptospira in human whole blood, PLoS One. 6 (3) (2011) e18279, https://doi.org/10.1371/journal.pone.0018279.

[6] A.J.A. McBride, D.A. Athanazio, M.G. Reis, A.I. Ko, Leptospirosis, Curr. Opin. Infect. Dis. 18 (5) (2005) 376–386, https://doi.org/10.1097/01.qco.0000178824.05715.2c.

[7] S.V. Budihal, K. Perwez, Leptospirosis diagnosis: competancy of various laboratory tests, J. Clin. Diagnostic Res. JCDR. 8 (1) (2014) 199–202, https://doi.org/10.7860/JCDR/2014/6593.3950.

[8] F. Merien, D. Portnoi, P. Bourhy, F. Charavay, A. Berlioz-Arthaud, G. Baranton, A rapid and quantitative method for the detection of Leptospira species in human leptospirosis, FEMS Microbiol. Lett. 249 (1) (2005) 139–147, https://doi.org/10.1016/j.femsle.2005.06.011.

[9] C. Hull-Jackson, M.B. Glass, M.D. Ari, S.L. Bragg, S.L. Branch, C.U. Whittington, C. N. Edwards, P.N. Levett, Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis, J. Clin. Microbiol. 44 (5) (2006) 1853–1855, https://doi.org/10.1128/JCM.44.5.1853-1855.2006.

[10] W.E. Winslow, D.J. Merry, M.L. Pirc, P.L. Devine, Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection, J. Clin. Microbiol. 35 (8) (1997) 1938–1942, https://doi.org/10.1128/jcm.35.8.1938-1942.1997.

[11] D. Widiyanti, N. Koizumi, T. Fukui, L.T. Muslich, T. Segawa, S.Y.A.M. Villanueva, M. Saito, T. Masuzawa, N.G. Gloriani, S.I. Yoshida, Development of immunochromatography-based methods for detection of leptospiral lipopolysaccharide antigen in urine, Clin. Vaccine Immunol. 20 (5) (2013) 683–690, https://doi.org/10.1128/CVI.00756-12.

[12] S. Li, Y. Liu, X. Chen, M. Wang, W. Hu, J. Yan, Visual and rapid detection of Leptospira interrogans using multiple cross-displacement amplification coupled with nanoparticle-based lateral flow biosensor, Vector-Borne Zoonotic Dis. 19 (8) (2019) 604–612, https://doi.org/10.1089/vbz.2018.2395.

[13] W.J. Terpstra, G.S. Ligthart, G.J. Schoone, ELISA for the detection of specific IgM and IgG in human leptospirosis, J. Gen. Microbiol. 131 (2) (1985) 377–385, https://doi.org/10.1099/00221287-131-2-377.

[14] M. Kanagavel, S. Shanmughapriya, K. Anbarasu, K. Natarajaseenivasan, V.M. Litwin, B-cell-specific peptides of Leptospira interrogans LigA for diagnosis of patients with acute leptospirosis, Clin. Vaccine Immunol. 21 (3) (2014) 354–359, https://doi.org/10.1128/CVI.00456-13.

[15] C.S. Akino Mercy, N. Suriya Muthukumaran, P. Velusamy, P. Bothammal, K. Sumaiya, P. Saranya, D. Langford, S. Shanmughapriya, K. Natarajaseenivasan, MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis, MSphere. 5 (4) (2020) e00409-20, doi: 10.1128/mSphere.00409-20.

[16] S. Vanithamani, S. Shanmughapriya, R. Narayanan, V. Raja, M. Kanagavel, K. Sivasankari, K. Natarajaseenivasan, A. Calderaro, Lipopolysaccharide specific immunochromatography based lateral flow assay for serogroup specific diagnosis of leptospirosis in India, PLoS One. 10 (9) (2015) e0137130, https://doi.org/10.1371/journal.pone.0137130.

[17] L.R. Stromberg, H.M. Mendez, H. Mukundan, Detection methods for lipopolysaccharides: past and present, Escherichia Coli. Recent Adv. Physiol. Pathog. Biotechnol. Appl., Amidou Samie, IntechOpen, (2017) 141–168, DOI: 10.5772/intechopen.68311.

[18] S. Jampasa, P. Lae-ngee, K. Patarakul, N. Ngamrojanavanich, O. Chailapakul, N. Rodthongkum, Electrochemical immunosensor based on gold-labeled monoclonal anti-LipL32 for leptospirosis diagnosis, Biosens. Bioelectron. 142 (2019) 111539, https://doi.org/10.1016/j.bios.2019.111539.

[19] V. Verma, D. Kala, S. Gupta, H. Kumar, A. Kaushal, K. Kuča, N. Cruz-Martins, D. Kumar, Leptospira interrogans outer membrane protein-based nanohybrid sensor for the diagnosis of leptospirosis, Sensors. 21 (7) (2021) 2552, https://doi.org/10.3390/s21072552.

[20] R. Nagraik, A. Kaushal, S. Gupta, P. Dhar, S. Sethi, D. Kumar, Optimized DNA-based bioassay for Leptospira interrogans detection: a novel platform for leptospirosis diagnosis, 3 Biotech. 9 (7) (2019) 284, doi: 10.1007/s13205-019-1815-4.

[21] J.L. Hammond, N. Formisano, P. Estrela, S. Carrara, J. Tkac, Electrochemical biosensors and nanobiosensors, Essays Biochem. 60 (1) (2016) 69-80, doi: 10.1042/EBC20150008.

[22] E. Boubour, R.B. Lennox, Insulating properties of self-assembled monolayers monitored by impedance spectroscopy, Langmuir. 16 (9) (2000) 4222–4228, https://doi.org/10.1021/la991328c.

[23] M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, A colorimetric method for the determination of sugars, Nature. 168 (4265) (1951) 167, doi: 10.1038/168167a0.

[24] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (9) (1979) 4350–4354, https://doi.org/10.1073/pnas.76.9.4350.

[25] C.M. Tsai, C.E. Frasch, A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels, Anal. Biochem. 119 (1) (1982) 115–119, https://doi.org/10.1016/0003-2697(82)90673-x.

[26] A.D. Chowdhury, K. Takemura, T.C. Li, T. Suzuki, E.Y. Park, Electrical pulse-induced electrochemical biosensor for hepatitis E virus detection, Nat. Commun. 10 (1) (2019) 3737, https://doi.org/10.1038/s41467-019-11644-5.

[27] P. Ertl, S.R. Mikkelsen, Electrochemical biosensor array for the identification of microorganisms based on lectin– lipopolysaccharide recognition, Anal. Chem. 73 (17) (2001) 4241–4248, https://doi.org/10.1021/ac010324l.

[28] P. Sondhi, M.H.U. Maruf, K.J. Stine, Nanomaterials for biosensing lipopolysaccharide, Biosensors. 10 (1) (2020) 2, https://doi.org/10.3390/bios10010002.

[29] G.A. Trueba, C.A. Bolin, C.O. Thoen, Evaluation of an enzyme immunoassay for diagnosis of bovine leptospirosis caused by Leptospira interrogans serovar hardjo type hardjo-bovis, J. Vet. Diagnostic Investig. 2 (4) (1990) 323–329, https://doi.org/10.1177/104063879000200413.

[30] C. Gowri Priya, K. Bhavani, S.R. Rathinam, V.R. Muthukkaruppan, Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis, J. Med. Microbiol. 52(Pt 8) (2003) 667-673, doi: 10.1099/jmm.0.05120-0.

[31] T. Toyokawa, M. Ohnishi, N. Koizumi, Diagnosis of acute leptospirosis, Expert Rev. Anti. Infect. Ther. 9 (1) (2011) 111–121, https://doi.org/10.1586/eri.10.151.

[32] P. Ramadass, B.D.W. Jarvis, R.J. Corner, D. Penny, R.B. Marshall, Genetic characterization of pathogenic Leptospira species by DNA hybridization, Int. J. Syst. Bacteriol. 42 (2) (1992) 215–219, https://doi.org/10.1099/00207713-42-2-215.

[33] S.C. Sehgal, P. Vijayachari, V. Subramaniam, Evaluation of leptospira micro capsule agglutination test (MCAT) for serodiagnosis of leptospirosis, Indian J. Med. Res. 106 (1997) 504–507.

[34] S.C. Sehgal, P. Vijayachari, A.P. Sugunan, T. Umapathi, Field application of Lepto lateral flow for rapid diagnosis of leptospirosis, J. Med. Microbiol. 52 (10) (2003) 897–901, https://doi.org/10.1099/jmm.0.05064-0.

[35] S.C. Sehgal, P. Vijayachari, S. Sharma, A.P. Sugunan, LEPTO Dipstick: A rapid and simple method for serodiagnosis of acute leptospirosis, Trans. R. Soc. Trop. Med. Hyg. 93 (2) (1999) 161–164, https://doi.org/10.1016/s0035-9203(99)90293-6.

[36] P. Vijayachari, A.P. Sugunan, S.C. Sehgal, Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis, Epidemiol. Infect. 129 (3) (2002) 617–621, https://doi.org/10.1017/s0950268802007537.

[37] H. Jiang, D. Jiang, J. Shao, X. Sun, J. Wang, High-throughput living cell-based optical biosensor for detection of bacterial lipopolysaccharide (LPS) using a red

fluorescent protein reporter system, Sci. Rep. 6 (2016) 36987, https://doi.org/10.1038/srep36987.
[38] W. Su, M. Lin, H. Lee, M.S. Cho, W.S. Choe, Y. Lee, Determination of endotoxin through an aptamer-based impedance biosensor, Biosens. Bioelectron. 32 (1) (2012) 32–36, https://doi.org/10.1016/j.bios.2011.11.009.
[39] S.J. Ding, B.W. Chang, C.C. Wu, C.J. Chen, H.C. Chang, A new method for detection of endotoxin on polymyxin B-immobilized gold electrodes, Electrochem. Commun. 9 (5) (2007) 1206–1211, https://doi.org/10.1016/j.elecom.2006.12.029.
[40] M.D.L. Oliveira, C.A.S. Andrade, M.T.S. Correia, L.C.B.B. Coelho, P.R. Singh, X. Zeng, Impedimetric biosensor based on self-assembled hybrid cystein-gold nanoparticles and CramoLL lectin for bacterial lipopolysaccharide recognition, J. Colloid Interface Sci. 362 (1) (2011) 194–201, https://doi.org/10.1016/j.jcis.2011.06.042.
[41] B. Posha, S.R. Nambiar, N. Sandhyarani, Gold atomic cluster mediated electrochemical aptasensor for the detection of lipopolysaccharide, Biosens. Bioelectron. 101 (2018) 199–205, https://doi.org/10.1016/j.bios.2017.10.030.
[42] T.Y. Yeo, J.S. Choi, B.K. Lee, B.S. Kim, H.I. Yoon, H.Y. Lee, Y.W. Cho, Electrochemical endotoxin sensors based on TLR4/MD-2 complexes immobilized on gold electrodes, Biosens. Bioelectron. 28 (1) (2011) 139–145, https://doi.org/10.1016/j.bios.2011.07.010.

frontiers in Immunology

ORIGINAL RESEARCH
published: 16 November 2021
doi: 10.3389/fimmu.2021.764662

Construction of Genomic Library and Screening of *Edwardsiella tarda* Immunogenic Proteins for Their Protective Efficacy Against Edwardsiellosis

Palanisamy Bothammal[1†], Mohan Ganesh[1†], Vellaisamy Vigneshwaran[1], Kumarasamy Anbarasu[2], Karuppiah Ponmurugan[3], Naif Abdullah Al-Dhabi[3] and Kalimuthusamy Natarajaseenivasan[1]*

[1] Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli, India, [2] Microbial Biotechnology Laboratory, Department of Marine Biotechnology, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, India, [3] Department of Botany & Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia

OPEN ACCESS

Edited by:
Minjun Yang,
Lund University, Sweden

Reviewed by:
Lihua Song,
Beijing University of Chemical Technology, China
Jieh-Juen Yu,
University of Texas at San Antonio, United States

***Correspondence:**
Kalimuthusamy Natarajaseenivasan
natarajaseenivasan@gmail.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: 25 August 2021
Accepted: 11 October 2021
Published: 16 November 2021

Citation:
Bothammal P, Ganesh M, Vigneshwaran V, Anbarasu K, Ponmurugan K, Al-Dhabi NA and Natarajaseenivasan K (2021) Construction of Genomic Library and Screening of Edwardsiella tarda Immunogenic Proteins for Their Protective Efficacy Against Edwardsiellosis. Front. Immunol. 12:764662. doi: 10.3389/fimmu.2021.764662

Edwardsiella tarda is a severe aquaculture pathogen that can infect many hosts including humans, animals, and fish. Timely diagnosis and treatment are crucial for the control of edwardsiellosis in the aqua industry. By using rabbit polyclonal antibody, an expression gene library of virulent *Edwardsiella tarda* strain ED-BDU 1 isolated in south India was constructed and screened. The identified immune expressive proteins were characterized, and the corresponding coding sequences were cloned, expressed, and the purified recombinant proteins were used as antigens. The identified immunoreactive proteins namely HflC, HflK, and YhcI were studied for their immune protective potential *in vivo* by challenge experiments. The protective efficacy of HflC, HflK, and YhcI showed that the clearance of *Edwardsiella* from the host with ~ 60% survivability. Further, the immunoreactive proteins induce a strong immune response upon infection and elicit the significant production of IL-10, IFN-γ, Th1, and Th2 mediated mRNA expression and were therefore effective in vaccine production for edwardsiellosis.

Keywords: ***Edwardsiella tarda*, edwardsiellosis, immunoreactive protein, vaccine, IL-10, IFN-γ**

INTRODUCTION

Aquaculture is a fast-growing fisheries sector globally, and India accounts second in culture fisheries production with an annual growth rate of over 7%. Aquaculture not only supplies dietary essentials for human consumption but provides opportunities for employment and income, especially in less economically developed rural areas. Sixty million people are directly engaged, part-time or full-time, in the primary production of fish, either by fishing or in aquaculture, supporting the livelihoods of 10-12% of the world population. It currently accounts for over 50% of global fish consumption. In India, carp varieties are highly cultivated, namely catla (*Catla catla*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*) (1).

The aquaculture industry has become increasingly vulnerable to exotic, endemic, and emerging disease epizootics. It is estimated that as much as 10% of all aquatic aquaculture animals die of infectious diseases, representing losses of more than USD 10 billion per year globally. The most prevalent bacterial pathogens in Indian aquaculture belong to the genera *Aeromonas, Edwardsiella, Vibrio*, and *Flavobacterium*, infecting the top farmed fish species (2–4). The genus *Edwardsiella* belongs to the family *Enterobacteriaceae* and consists of three known species, namely *E. tarda, E. ictaluri, E. hoshinae* (5). The Gram-negative *E. tarda*, ubiquitous in the microbial biosphere and has the broadest host range, infecting both freshwater and marine life. Fish infected by *E. tarda* develop edwardsiellosis, generalized septicemia with symptoms including distended abdomen, prolapsed rectum, and gross lesions of internal organs (2). Clinical presentation of edwardsiellosis such as extensive skin lesions that progress into necrotic abscesses, distended abdomen, and swollen anus due to the accumulation of ascetic fluid, pigment loss, enlarged kidney, and abscesses on internal organs.

Virulence factors of *Edwardsiella* help entry to host fish through the gastrointestinal tract, the gills, and the body surface and can resist the host immune system mediated by host complements and phagocytes. *E. tarda* uses various secretion systems as virulence factors likely type III (T3SS) and type VI (T6SS) secretion systems, which are vital to invasion and intracellular replication of microorganisms in the host (6–9). Apart from that, the production of enzymes such as hemolysins and chondroitinase helps the bacterium to invade inside the host (10, 11). *In vivo* induced antigen technology (IVIAT) used examined the biological properties and function of a putative adhesin, Eta1, which is drastically enhanced during infection of host cells (12). Thus, studies of *in vivo* expressed proteins contribute to the understanding of host-pathogen interactions and are helpful in the design of novel diagnostics and vaccines.

The outbreak of edwardsiellosis causes major economic losses in cultured fish. Currently, the control of edwardsiellosis relies chiefly on the use of antibiotics. The use of antibiotics poses the risk of selection of drug resistance in pathogens, making the treatment ineffective, and risking the spread of resistance determinants to other bacteria (13). Pathogenic *E. tarda* is found to be an intracellular pathogen and is naturally resistant to benzylpenicillin, oxacillin, macrolide, lincosamides, streptogramins, and glycopeptides which increases the difficulty of antibiotic-based treatment and their application in aquaculture has become more and more restricted (14, 15). It is, therefore, urgent to develop new prevention and treatment methods.

Routinely using antibiotics in aquaculture leads to the emergence of multi-drug resistant pathogens in aquaculture, in order to avoid such multi drug resistance and spreading of infection vaccination would be best the eco-friendly practical approach. Several vaccines are reported so far against edwardsiellosis. Immunization using FKC or LPS showed protective effects after challenge with a virulent strain of *E. tarda* in Japanese eel (16). Kawai et al (17), reported ghost cells and bacterin of *E. tarda*, showing that this was most effective (relative percent of survival rate above 90%). Using purified recombinant subunit vaccine as antigen is more immunoprotective. Recombinant vaccines such as OmpA and Omp48 showed high levels of immunogenicity against edwardsiellosis (18). The main advantage of recombinant vaccines is that they are safer because they only contain the antigenic protein and not the entire pathogens, and these genetically engineered vaccines help remove undesired harmful antigens.

In the present study, we have identified *in vivo*-expressed proteins of virulent *Edwardsiella tarda* ED-BDU 1 through an expression gene library screening. The library was screened with *Edwardsiella tarda* specific rabbit polyclonal sera, and the identified proteins were evaluated for their protective efficacy for edwardsiellosis.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Ethics

E. coli BL21, BL21 (DE3), and DH5α were purchased from Novagen (Novagen Inc., Madison, Wisconsin). *E. tarda* BDU-1 was isolated from the kidneys of diseased *Labeo rohita* and is naturally resistant to rifampin. All strains and isolates were grown in Luria-Bertani broth (LB) (28) at 37°C (for *E. coli*) or 28°C (for *E. tarda* ED-BDU1).

Animal experiments described in this study were carried out in strict accordance with the recommendations approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), and Bharathidasan University Ethics Committee in Animal Experimentation (Approval number: BDU/IAEC/2011/29).

Study Area and Collection of Diseased Fish

The present study was conducted in and around Tiruchirappalli district, Tamil Nadu. A total of 41 farms were included in the study. Of the 41 fish farms, 10 were reported to have a high mortality rate among farmed fishes. The infected fishes were collected and transported on ice to the Medical Microbiology Laboratory on the same day of collection. Information data sheets comprising the clinical sign and mortality rate of fish were collected with duly signed by the fish farm proprietor.

Antibiotic Susceptibility Test

All the isolates were subjected to the antibiotic susceptibility test by Kirby-Bauer disk diffusion method by using Muller-Hinton Agar (Merck, Germany). Briefly, the sample of *E. tarda* culture in Tryptic soy broth was swabbed onto Muller Hinton agar uniformly for a lawn of bacterial growth. Antibiotic discs were gently placed on the surface of the agar using sterile forceps and were kept in the incubator for 24h at 30°C. Interpretation of the resulted inhibition zones, namely sensitivity and resistance, was done according to the standard measurement in millimeter (mm) following National Committee for Clinical Laboratory Standards.

Phenotypic and Genotypic Characterization

The *E. tarda* isolates from diseased fishes were characterized by morphological and biochemical tests. The surface of the fish was washed, and visceral organs were removed aseptically. The organs were homogenized, serially diluted in PBS, and plated on Rimler-Shotts medium. Isolates identified as *E. tarda* by biochemical test and further confirmed by molecular characterization of the 16S rRNA sequencing using specific 16S-F 5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3' and 16S-R 5'- AAGGAGGTGATCCAG -3' primers. The temperature profile for amplification was initial denaturation at 95°C for 4 min, denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, for 30 cycles, followed by a final extension at 72°C for 5 min. The PCR products were purified, sequenced, and analyzed for phylogenetic relatedness to strains from type culture collections.

Enzyme-Linked Immunosorbent Assay

Two young healthy rabbits weighing 3kg were selected and their sera were pretested for *E. tarda* antibodies prior to inoculation. Live antigens were prepared from 24 hours old *E. tarda* ED-BDU1 culture (1-2 X 10^8/ml). The cultures were centrifuged at 10,000 x g for 10 mins and dissolved in Phosphate buffered saline (PBS) and the cells count was determined by using a Neubauer counting chamber. Briefly, 1-2 x 10^8 cells were injected subcutaneously into the rabbits on day 1, followed by booster inoculums on days 14 and 28. The level of antigen-specific circulating antibodies was determined by titrating the serum samples in ELISA. For performing serum dilution in ELISA, wells were coated with 100µl carbonate coating buffer (pH 7.2) containing 1µg of sonicated antigen and stored at 4^0C overnight. Then the plate was proceeding to wash with phosphate-buffered saline with Tween 20 (PBST) (8mM Na_2HPO_4, 150mM NaCl, 2mM KH_2PO_4, 3mM KCl, 0.05% Tween® 20, pH 7.4) and blocked with 200µl/well of 4% non-fat milk in PBST for 1 h at 37°C. Sera were diluted from starting dilution of 1:25 to 1:51200 in triplicate and incubated for 1 h at 37°C. Bound IgG was detected using peroxidase-conjugated Protein A (Sigma, St. Louis, MO), followed by development using 100µl of ortho phenylenediamine (OPD). Optical densities (OD's) were read at 490nm using an ELISA reader (Bio-Rad, Hercules, CA, USA).

Genomic DNA Library Construction and Screening

Among all the isolates *E. tarda* ED-BDU1 was found to be highly resistant, so we constructed a genomic DNA expression library for our local isolate. *E. tarda* strain ED-BDU1 was constructed using λZAPII library (Strategene, San Diego, CA). Briefly 5 x 10^4 pfu of library construct was used for amplification. Validation of the entire library was carried out by PCR. A λZAPII library consisting of 3- to 5-kb random fragments of *E. tarda* ED–BDU1 was screened to identify phage that expressed gene products reactive with rabbit polyclonal antibodies specific for *E. tarda*. The secondary antibody was peroxidase-conjugated Protein A (Sigma, St. Louis, MO) diluted 1:2000. After primary and secondary antibody treatment, the NC membrane was developed with the chromogenic substrate 4-chloro-1-napthol (Sigma, St. Louis, MO).

In Vivo Excision of Immunoreactive Clones and DNA Sequencing

Excision of *in vivo* expressed clones was performed by the previously reported method with slight modifications (19). Suspected plaques on agar plugs were transferred to 500 µl of SM buffer and allowed to elute overnight. Insert-carrying plasmids were rescued from selective reactive phages by using ExAssist helper phage and *E. coli* SOLR according to the manufacturer's instructions. Plasmid DNA was isolated from reactive clones using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and sequenced in a commercial sequencing facility (Macrogen, South Korea) using T3, T7, and custom-designed primers. Sequences were edited with Chromas 1.61 (Technelysium Pty. Ltd., Queensland, Australia) and aligned and connected with DNASIS (Hitachi Software Engineering Co., Ltd., San Francisco, CA). DNA sequences thus obtained were compared with the whole-genome sequences of *E. tarda* strain FL95-01 and *E. tarda* strain ET-1 using the National Centre for Biotechnology Information server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and highly identical sequences were retrieved.

Cloning

The specific primers used in this study were listed in **Table 1**. The sequence of respective genes was PCR amplified from genomic DNA of ED-BDU1, which was denatured initially at 94°C for 5 mins followed by 30 cycles of 94°C for 1 min, 57°C for 45 sec and 72°C for 1 min and 72°C for 7 mins. Following PCR amplification, the amplicons were cloned into predigested pET28a (Novagen, Madison, WIS) (*hflC, hflK*, and *yhcI*). Recombinant plasmids were transformed into *E. coli* BL21 (DE3). Colony PCR, restriction endonuclease analysis (REA), and DNA sequencing were performed to confirm the presence of the correct insert.

Colony PCR

The construct was extracted from the transformed cells by the boiling method. Briefly, each single colony was picked separately and dissolved in MilliQ water. The content was boiled for 8 mins in a water bath and immediately kept in ice for 2 min. The vial was centrifuged at 9000 rpm for 10 min and the supernatant was directly used as a template for the colony PCR. Amplification was performed in a thermal cycler (Eppendorf, Germany) at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 69°C for 45 s, and 72°C for 1 min, with a final single extension of 72°C for 7 min.

TABLE 1 | List of primers used for amplification of hflC, hflK, and yhcI genes.

Gene name	Sequence
hflC- F	5'- CCGGAATTCATGCGTAAGTCTTTGTTAGTGATTC-3'
hflC- R	5'- CCCAAGCTTTTACTTTGCGCTTTTGCCG-3'
hflK- F	5'-CGGAATTCATGGCGTGGAATCAGCCC-3'
hflK- R	5'-CCCAAGCTTTTATTGCTCTTGTCATCCACCAG-3'
yhcI- F	5'- CGCGGATCCATGAATACGCTGGCCATT -3'
yhcI- R	5'-GTGCTCGAGTCATGCCGTTTCTCTCC -3'

The presence and absence of amplicons in the corresponding colonies were confirmed by agarose gel electrophoresis.

Restriction Endonuclease Analysis

The positive colony was picked and streaked into the fresh LB agar plate with kanamycin as a resistant marker. Once the desired growth was attained, a single colony was inoculated into the 5 ml of LB broth containing appropriate antibiotic and incubated 37°C for 12-16 hrs. The construct was extracted by using a mini plasmid extraction procedure (Qiagen mini preparation kit) as described above. The restriction analysis was carried out as follows, 5 ul of universal fast digest buffer (33mm Tris Acetate, 10 mM Magnesium Acetate, 66mM Potassium Acetate and 0.1 mg/ml BSA), 1 µl of Hind III and 1 µl of EcoRI, 1µl of BamHI, 1 µl of XhoI, 10 µl of construct (hflc + pET-28a, hflk + pET-28a, yhc1 + pET-28a) and 33 µl of Milli-Q. The digestion was carried out at 37°C for 1 hour. Agarose gel electrophoresis was carried out with empty pEt-28a, construct single and double digest and finally amplified product of *hflc*, *hflk*, and *yhc1* in ascending order with molecular markers on both sides of the gel.

Expression and Purification

Recombinant plasmid pET28a+hflC, pET28a+hflK, and pET28a +yhcI were transformed into *E. coli* BL21 (DE3). Briefly, the recombinant cells were cultured in 500 mL of LB broth containing antibiotic Kanamycin 3 mg/ml with proper agitation. When cultures reached an optical density of 0.6 at 600 nm 1mM IPTG was added to the culture to induce the expression of polyhistidine-tagged recombinant proteins (HflC, HflK, and YhcI) and cells were harvested after 3h. Cultures measuring 500mL were pelleted by centrifugation at 10,000 rpm for 10 mins at 4°C in a falcon tube and the pellets were resuspended with 1:10 (w/v) of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 2.5mM imidazole, 8M urea, 200 µg/ml of lysozyme, and 2 mM phenylmethylsulfonyl fluoride [PMSF] pH 8.0). Cells were lysed by sonication five times at setting 3 for 10 seconds each time, with cooling on ice for 1 minute between each sonication. Cell extract was centrifuged at 10,000 x g for 30 minutes at 4°C in a high-speed cooling centrifuge. Recombinant His-tagged proteins (HflC, HflK, and YhcI) were purified by using IMAC Ni2+ resin (Bio-Rad, USA) in a buffer containing 8M urea. Then 500 µl cell lysate supernatant was added onto a 5 mL nickel column equilibrated with 0.5 ml binding buffer (20 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, and 8M urea, pH 8.0). It was incubated in ice for 5 minutes and centrifuged at 1000 x g for 1 minute at 4°C in a high-speed cooling centrifuge. The column was washed with the addition of 0.5 ml of wash buffer (20 Mm sodium phosphate, 300 mM NaCl, 10 mM imidazole, and 8M urea, pH 8.0). The elution was performed for the target by adding a five-column volume of elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM Imidazole, and 8M urea pH 8.0), was added onto the column, mixed well, and incubated for 5 mins to elute the His-tagged protein. The concentration of purified recombinant protein was determined using a Bicinchoninic acid assay kit and measured at 562 nm in the biophotometer.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Proteins were separated on 10% acrylamide gels by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. After blocking, membranes were incubated with rabbit polyclonal antibodies followed by incubation with anti-human or anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO). Membranes were developed and visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, Massachusetts, United States). All incubations were performed at room temperature and TBST washing was done thrice each for 10 min after every incubation. The NC membranes were documented with a Fusion Solo 6S (VilberLourmat, Collegien, France).

Edwardsiella Bacterins and Recombinant Proteins

Edwardsiella bacterins, whole-cell lysate (WCL) was prepared from *E. tarda* strain ED-BDU1. For vaccine preparation and immunization, ED-BDU1 cells with an OD of 1.0 were harvested by centrifugation at 4,000 g for 15 min and washed three times with saline solution. Washed cells were suspended in sterile saline solution as a live *E. tarda* vaccine or then incubated at 100°C to produce the inactivated vaccines. Plate counting assay was performed to examine bacterial sterility in the inactivated vaccines. HflC, HflK, and YhcI recombinant proteins were over-expressed and purified using the *E. coli* expression system as described above. The recombinant proteins were dialyzed, and the protein concentration was determined using a Bicinchoninic acid assay (BCA) kit (Sigma, St. Louis, MO). Then 100 µg of protein in Alhydrogel (Sigma, St. Louis, MO) was used as a vaccine formulation for the first booster on day 0. The second booster of 100 µg protein was administered by subcutaneous injections on the 21st day.

Immunization and Challenge Experiments

Healthy Indian common carp (*L. rohita*, weighing 80-90 g) were purchased from the SDS Fish Farm, Tanjore District, Tamil Nadu, India. The fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were maintained in aerated water and fed daily with commercial dry pellets. Before each of the immunization experiments, the fish were anesthetized with tricaine methanesulfonate (TMS, Sigma) prior to immunization experiments.

Grouping of mice: Laboratory mice were divided randomly into the following six groups, each group containing five mice (i) First group, positive control, were injected with heat-killed whole cell lysate; ii) Second group, negative control, were injected with PBS alone; iii) Third group, internal control, were injected with adjuvant (Alhydrogel (Sigma, St. Louis, MO)) alone; iv) Fourth group mice were injected with HflC recombinant protein mixed with adjuvant; v) Fifth group mice were injected with HflK recombinant protein mixed with adjuvant; and vi) the Sixth group of mice were injected with YhcI recombinant protein mixed with adjuvant.

Grouping of fishes: *L. rohita* were divided randomly into the following six groups, each group containing ten fish. (i) Positive control fish were injected with heat-killed whole cell lysate, ii) Negative control fish were injected with PBS alone, iii) Internal control fish were injected with adjuvant [Alhydrogel (Sigma, St. Louis, MO)] alone, iv) group four fish were injected HflC recombinant protein mixed with adjuvant, v) group five fish were injected HflK recombinant protein mixed with adjuvant, vi) and group six fish were injected YhcI recombinant protein mixed with adjuvant.

In brief, the recombinant proteins were mixed with an equal volume of complete adjuvant and injected into the animals on day 1, and followed by 2 booster doses which were injected with recombinant protein mixed with an equal volume of incomplete adjuvant at days 21 and 35. For each dose 10μg of recombinant proteins were injected into the respective group. The positive control was injected with heat-killed whole cell *Edwardsiella*, as a negative control animals received PBS.

The animals were challenged intraperitoneally with 5x median lethal dose (LD50) of *E. tarda* ED-BDU1 (4 x 10^7 CFU) 42 days after the first immunization. Animals were monitored daily and those who survived were euthanized on day 70 after the challenge. Similarly, pathogen-free 4-6 weeks old female BALB/c mice with a weight of 20–35 g were used for immunization and challenge experiments. Animals were bled through the tail vein before each immunization on day 0 (pre-vaccination), 14th (after 1st immunization), 28th (after booster), 42nd (after booster), 49th and 70th day (after challenge), and serum was collected and kept at -80°C until use.

Humoral Immune Response

Humoral immune response was determined using ELISA as mentioned before. In Brief, 0.2 μg/well of rHflC, rHflK, and rYhcI were coated in polystyrene 96 well microtiter plates (Nunc; Thermo Scientifics, USA) using carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. The plate was blocked with 4% non-fat milk in PBST. Mice sera (1:200) dilution in PBST were added and incubated for 1h at 37°C. Anti-mouse IgG peroxidase conjugate (Sigma Aldrich, St. Louis, MO) (1:4000) dilution in PBST was added and incubated at 37°C

The reaction was developed with o-phenylenediaminedi] hydrochloride (Sigma-Aldrich, St. Louis, MO). After 5 mins dark incubation the reaction was stopped by the addition of 0.1M sulphuric acid (Merk, Germany). Optical density (OD) values were measured at 490 nm (Bio-Rad, Hercules, CA, USA).

Cell-Mediated Immune Response

Cell-mediated immune responses were analyzed by cytokine profiling of immunized mice spleen cells. Briefly, total RNA was isolated from the immunized mice spleen cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Using the iScript cDNA synthesis kit the cDNA was synthesized (Bio-Rad, Hercules, CA, USA). A 25 μL reaction mixture containing 50 ng cDNA, 12.5μL Master Mix (SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA)), 0.5μM of each primer) was used, the amplification process performed in CFX96 TouchTM Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). All the primers used in this experiment are given in **Table 2**. The amplification process consisted of 95°C for 10 min for initial denaturation, followed by for 45 cycles of 95°C for 5 s, 60°C for 30 s, and a variable extension time at 72°C. After amplification, the melting curves were analyzed at a linear temperature transition rate of 0.1°C/s from 55 to 95°C, with continuous fluorescence acquisition.

Statistical Analysis

All data were normally distributed and presented as mean values ± SEM. In the case of single mean comparisons, data were analyzed by Student's t-test or, when not normally distributed a nonparametric Mann-Whitney U test. Differences in means among multiple data sets were analyzed using 1-way ANOVA/ 2-way ANOVA with Tukey test. P values less than 0.05 were found to be significant. All the data were computed with GraphPad Prism 8 or SigmaPlot 11.0 Software.

RESULTS

Identification and Characterization of *E. tarda*

In 10 different high mortality fish farms (**Table S1**), 27 infected Indian common carp (*Labeo rohita*) exhibited clinical manifestations such as gross lesions on the skin, pale gills, and tumefaction of the eye. A total of 54 isolates were obtained from 27 infected fishes with an isolation frequency of two isolates per infected fish. The isolates thus obtained were sub-cultured until they reached clonality. The morphological and biochemical studies identified 13 isolates (24.1%) as *E. tarda* (**Table S2**), 11 (20.4%) as *Salmonella* sp., and 10 (18.5%) as *Shigella* sp. All the isolates were found to be resistant to penicillin (piperacillin, oxacillin, ampicillin), aminoglycoside (neomycin), sulfonamides (sulfamethoxazole), peptide (polymyxin), macrolide (oleandomycin, erythromycin), and lincosamide (lincomycin) groups of antibiotics. Intermediate resistance was observed for tetracyclines (tetracycline; 100%), aminoglycosides (gentamycin, 75% and streptomycin, 95%), and other (chloramphenicol, 90%) groups of antibiotics (**Figure 1A**). The strain *E. tarda* ED–BDU1 showed excellent

TABLE 2 | List of cytokine primers used for qPCR analysis.

Gene Name	Direction	Sequence (5'- 3')
TNFα	Forward	GGACTAGCCAGGAGGGAGAA
	Reverse	CGCGGATCATGCTTTCTGTG
IL 10	Forward	GCCCTTTGCTATGGTGTCCT
	Reverse	TTTTCAGGGATGAAGCGGCT
IL 4	Forward	CAAACGTCCTCACAGCAACG
	Reverse	AAGCCCGAAAGAGTCTCTGC
IL 12p40	Forward	GGAAGCACGGCAGCAGAATA
	Reverse	AACTTGAGGGAGAAGTAGGAATGG
IFNγ	Forward	ATTCAGAGCTGCAGTGACCC
	Reverse	GGAAGCACCAGGTGTCAAGT
GAPDH	Forward	AACGACCCCTTCATTGAC
	Reverse	TCCACGACATACTCAGCAC

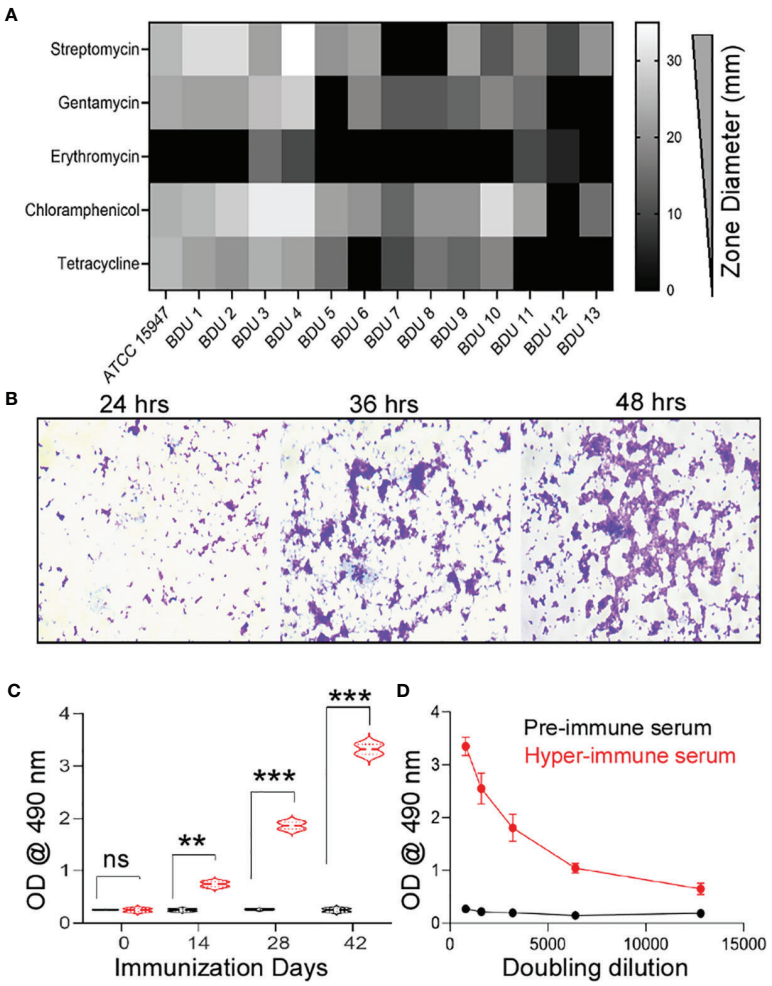


FIGURE 1 | **(A)** Antimicrobial Resistance of the *E. tarda* ATCC 15947 and the clinical isolates. x-axis: Antimicrobial zone in millimeter, y-axis: different antimicrobial agents. **(B)** Biofilm forming ability of the strain *E. tarda* ED-BDU1 at different time intervals (400X). **(C)** Immune response in rabbits against the live antigen of ED-BDU1 at different days of immunization. X-axis- Immunization days, Y-axis- OD @ 490 nm, ns, no significance, **P < 0.01, ***P < 0.001. **(D)** Doubling dilution of the polyclonal rabbit antisera raised against the live antigen of ED-BDU1, X-axis- Doubling dilution of the sera taken after 42 days of immunization, Y-axis- OD @ 490 nm.

biofilm-forming ability apart from antibiotic resistance (**Figure 1B**). **Figure S1A** represents the genomic DNA of the *E. tarda* isolates on a 0.8% agarose gel stained with ethidium bromide. Further, the size of the 16S rRNA product of *E. tarda* was found to be 1537 bp (**Figure S1B**). Then the isolates shared 93-96% homology to known *E. tarda* isolates. Three sequences were deposited in NCBI GenBank (Accession no: KT001505, KT001506, KT001507).

Rabbit Polyclonal Antibody Against *E. tarda*

The immunized sera from rabbits presented significant levels of circulating antibodies at 14, 28, and 42 days ($P < 0.05$) compared to pre-immune rabbit sera ($p > 0.05$) (**Figure 1C**). The antibody titer was found to be higher and of no or less reactivity for pre-immune rabbit serum. The graph was plotted for the obtained OD values against different dilutions from 1:800 to 1:12800 (**Figure 1D**).

Immunoscreening of the *E. tarda* ED–BDU1 Gene Expression Library

A λ ZAPII library containing 2-5 kb random fragments of *E. tarda* was screened to identify phage that expressed gene products reactive with polyclonal rabbit sera. Primary screening was done with a titer of 5 X 10^4 pfu and 600 µl of XL1-MRF cells at OD 600 of 0.5. Primary screening of approximately 10^5 plaques of the lambda library against pooled rabbit hyperimmune serum revealed around 20 reactive plaques, which were further preceded for secondary screening (**Figure 2A, B**). Secondary screening shows 7 clones as hyper-reactive and others as less immunogen. Therefore the 7 clones that showed as positive were subjected to tertiary screening to

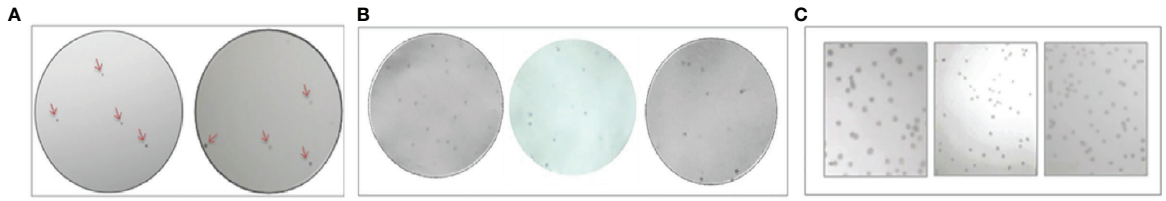


FIGURE 2 | DNA library screening against rabbit polyclonal antiserum. **(A)** Primary screening in duplicates, arrow mark indicates the prominent spots developed. The positive plaques appeared as "doughnuts" with clear centers, **(B)** Secondary screening and **(C)** Tertiary screening (ED-BDU1 λ 1-1 III, ED-BDU1 λ 3-1 III, and ED-BDU1 λ 5-1 III).

identify the plaques showing 100% immunoreactivity frequency in the NC membrane. The tertiary screening identified 3 highly expressive immunoreactive clones namely BDU 1 λ 1-3 III, BDU 1 λ 3-1 III, and BDU 1 λ 5-1 III (**Figure 2C**). The ExAssist helper phage with SOLR strain (Strategene, San Diego, CA) was used to allow efficient excision of the pBluescript phagemid from the Lambda ZAP II vector.

Obtaining of the Sequence of Antigenic Protein Gene Encoders for *E. tarda*

Plasmids were excised from the highly immunoreactive ED-BDU1+ λ recombinant phages (ED-BDU1 λ 1-3 III, ED-BDU1 λ 3-1 III, and ED-BDU1 λ 5-1 III) with the aid of ExAssist helper phage and *E.coli* SOLR. Insert DNAs were sequenced by using standard T7 and T3 primers. Sequence results found that the ED-BDU1 λ 1-3 III has two open reading frames, the first encodes a protein *hflK*, and the second encodes a protein *hflC*. Whereas the second clone (ED-BDU1 λ 3-1 III) encodes *crcA*. The third clone (ED-BDU1 λ 5-1 III) encodes the *yhcI*. The genetic sequencing analysis was performed by comparing the positive clones of *E. tarda* with the GenBank (**Table 3**).

Cloning, Expression, and Immunoreactivity

Figure 3A shows that the PCR amplified product of the *hflC, hflK*, and *yhcI* from the genomic DNA of ED-BDU1 and were found to be1000 bp, 1100 bp, and 850 bp respectively. **Figure 3B** shows the REA and colony PCR analysis of *hflC, hflK*, and *yhcI* which reveals that the presence of the right insert. The expression of recombinant proteins (HflC, HflK, and YhcI) was confirmed by SDS- PAGE, and the molecular weight was found to be 36kDa, 40kDa, and 31kDa respectively (**Figure 3C**). The SDS profiling of purified proteins (**Figure 3D**) and the purified proteins were immunoblotted with rabbit's sera and the immunoreactivity of the purified proteins was found to be specific (**Figure 3E**).

Humoral Immune Response

The specific reactivity of the serum samples against the recombinant HflK, HflC, and YhcI proteins was analyzed by IgG ELISA (**Figures 4A–C**). After the 21st and 42nd day of immunization, the significant induction of antibody titer was observed against the respective recombinant proteins in comparison with the negative control. There were significant levels of circulating anti-HflK, HflC, and YhcI were detected ($P<0.0001$). The developed sera were highly selective to the recombinant proteins ruling out the possibility of immunization. Similarly, the vaccinated groups show significant IgG response ($P<0.0001$), but lower compared to immunized alone.

Cytokine Expression Profile

mRNA abundance of both Th1 and Th2 type cytokines was evaluated by qRT-PCR analysis. Our results clearly show that our recombinant protein-based vaccines could induce significant levels of both Th1 and Th2 cytokines ($p<0.05$) (**Figure 5**). TNF- α (**Figure 5A**) mRNA expression levels were found to be upregulated in immunized groups like rHflC group (ratio = 4.05), rYhcI group (ratio = 1.56), and rHflK group (ratio = 2.26). IL10 (**Figure 5B**) mRNA expression was found to be significantly upregulated in HflC group (ratio = 3.35), YhcI group (ratio = 2.8) and HflK group (ratio = 3.34). IL-4 (**Figure 5C**) mRNA expression levels were found to be upregulated in the immunized HflK group (ratio =2.63) and YhcI group (ratio = 1.91) whereas others has less significant upregulation in the HflC group (ratio = 1.41). IL-12p40 (**Figure 5D**) mRNA expression levels were found to downregulated in YhcI group (ratio = 0.805) and the remaining groups HflC group (ratio = 1.655) and HflK

TABLE 3 | Genetic sequencing analysis by comparing the positive clones of *E. tarda* with the Gen Bank.

Clones	Genes	E-value[a]	Identity (%)	Region of the genome (Total gene)
ED-BDU1 λ 1-3 III	*hflK*,	0	100	399,238-400,527
	hflC	0	100	400,527-401,531
ED-BDU1 λ 3-1 III	*crcA*	0	100	2,823,483-2,823,956
ED-BDU 1 λ 5-1 III	*yhcI*	1e-07	97%	1,493,555-1,494,424

[a]*The closer to zero the E-value, the lower the probability of the alignment of the gene sequences occurring at random.*

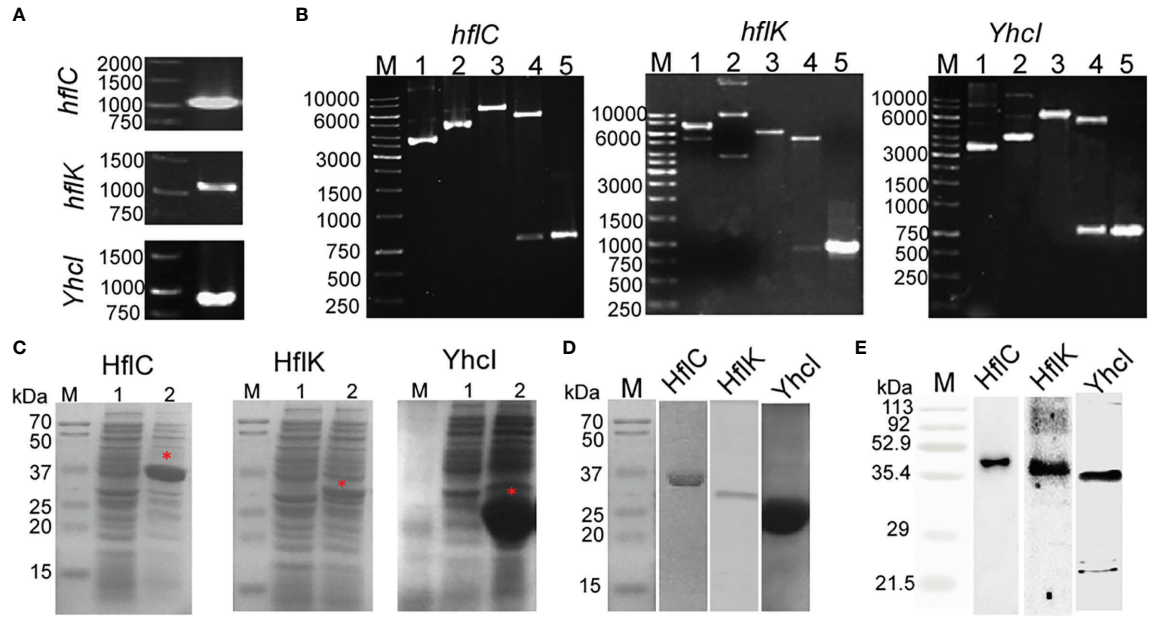


FIGURE 3 | **(A)** PCR amplified product of hflC (a), hflK (b), and YhcI (c) from the genomic DNA of strain *E. tarda* ED-BDU1, **(B)** Restriction analysis and colony PCR analysis of hflC (a), hflK (b), and YhcI (c) from the *E. coli* BL21 transformants. (M- 1kb marker, 1- Vector pET28a, 2- construct pET28a+amplified gene, 3- single digestion of construct pET 28a+ amplified gene using EcoRI for hflC and hflK genes, BamHI for YhcI gene, 4-Double digestion of construct pET28a+ amplified gene using EcoRI and HindIII for hflC and hflK, BamHI genes and XhoI for YhcI gene and 5- Colony PCR product of the respective gene). **(C)** Expression of the hflC (a), hflK (b), and YhcI (c) proteins as confirmed by SDS-PAGE profiling. (Lane M: Marker, Lane 1: Uninduced whole cell lysate, Lane 2: IPTG induced whole cell lysate). **(D)** SDS- PAGE profiling of purified recombinant proteins (HflC, HflK, and YhcI). **(E)** Immunoblot analysis of purified recombinant proteins (HflC, HflK, and YhcI) probed with specific rabbit polyclonal antiserum.

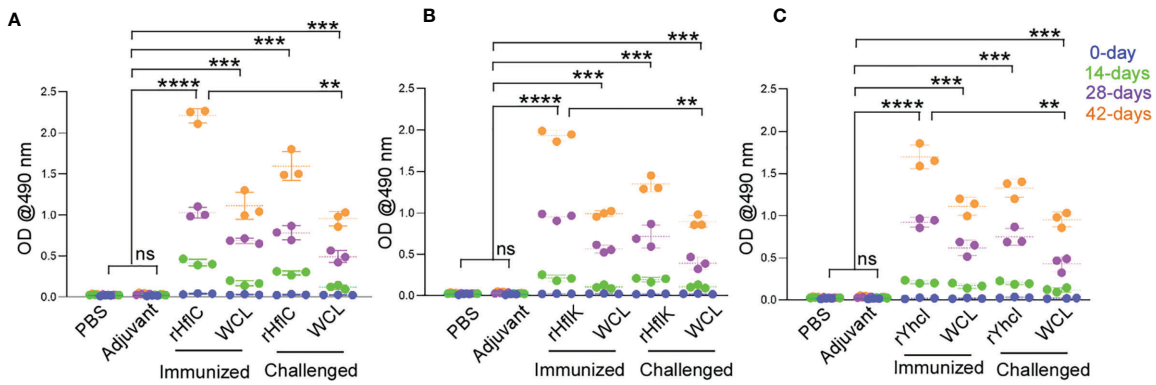


FIGURE 4 | Evaluation of humoral immune response in control and immunized mice groups by ELISA. **(A)** rHflC antigen used, **(B)** rHflK antigen used and **(C)** rYhcI antigen used. Graphs represent the mean + SD of the optical density sera obtained at different day intervals (0, 14, 28, 42 days before challenge) and after challenge. ns, no significant, **P < 0.05, ***P < 0.01, ****P < 0.0001. P values were obtained by comparison with control (PBS and Adjuvant) using Tukey's multiple comparisons test by 2-way ANOVA. (WCL- Whole cell lysate of *E. tarda*).

group (ratio = 1.64) have significant levels of expression. IFN-γ (**Figure 5E**) mRNA expression levels were found to be upregulated in the HflC group (ratio = 3.57), YhcI group (ratio = 2.75) and HflK group (ratio = 2.22). Overall significant changes in mRNA expression levels were found to be present only in the HflC vaccine group for both the Th1 and Th2 type cytokines. Particularly, HflC based recombinant protein vaccine shows a good level of cytokine induction.

Protective Efficacy of Vaccines

The LD50 dose was in the range of 1.6×10^5 c.f.u. The protective efficacy of HflC, HflK, and YhcI was evaluated in terms of

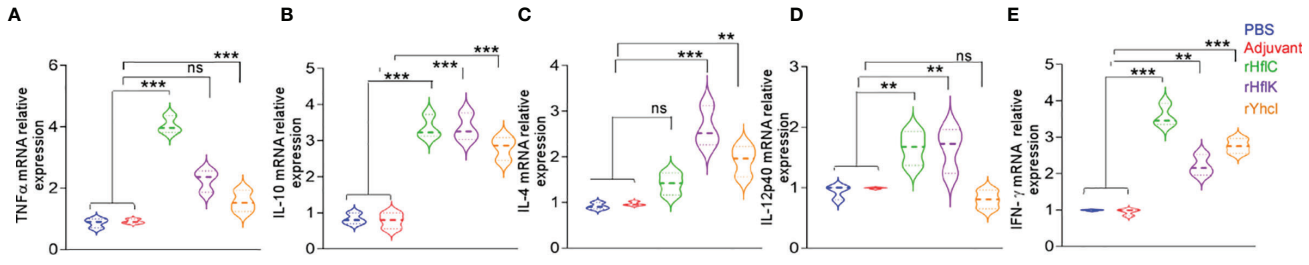


FIGURE 5 | Cytokine profiling of the control and immunized mice. **(A)** TNFα, **(B)** IL-10, **(C)** IL-4, **(D)** IL-12p40, **(E)** IFN-γ. The relative CT (ΔΔ CT) method was used to quantify cytokine gene expression: CTs were normalized against the GAPDH gene CT (ΔCT) and then compared to the same normalized gene in the PBS immunized mice group. The control groups were set to 1. ns, no significance, **P < 0.01, ***P < 0.001. x-axis: Study groups, y-axis- Relative gene expression (fold).

survival and reisolation of *Edwardsiella*. The survival data on the 30 days post-infection showed (60-100%) survival of animals in the vaccinated group (P <0.05), while none of the animals survived in the non-vaccinated group either PBS or Adjuvant (P <0.05). The rate of death of animals in PBS or adjuvant control (median survival time, 5 days) occurred immediately after the challenge confirming the virulence of the low passaged strain used for challenge experiments (**Figures 6A, B**).

DISCUSSION

The advantage of building an immune expressive library is that prior knowledge of target antibodies is not required, eliminating the need to design primer oligonucleotides, and allowing the identification of new antigens. Moreover, with a large part of the bacterium's genome used in the library, it is possible to screen for the expression of several genes in the same experiment (20). In this study, immunoscreening of the gene expression library was efficient at finding genes that encode antigenic proteins of *E. tarda*.

The genes of *hflK* and *hflC* are adjacent to each other in the same operon and both having a single transmembrane segment located at the N-terminal region, form a complex (HflKC), remaining large C-terminal domain exposed on the periplasmic side. Also, evidence suggests that HflKC was inhibitory against the Sec-Y degrading function of FtsH both *in vivo* and *in vitro*. HflC (~45.5 kDa) and HflK (~38 kDa) help govern the stability of phage lambda cII protein, and thereby control the lysogenization frequency of phage lambda (21–24). Akiyama et al. proposed that HflC contains a serine–protease-like sequence motif. HflKC also forms a complex with FtsH, and represses the proteolytic activity of FtsH (25). HflKC, having an evolutionarily conserved domain (protein homology domain), may have protein/lipid-binding properties (26). HflK and HflC form a large complex with FtsH protease, that contributes to both membrane protein quality control and regulation of the cellular response to environmental stress in many bacteria. Both activities are crucial to the Lyme disease pathogen *Borrelia burgdorferi*, which depends on membrane functions, such as motility, protein transport, and cell signaling, to respond to

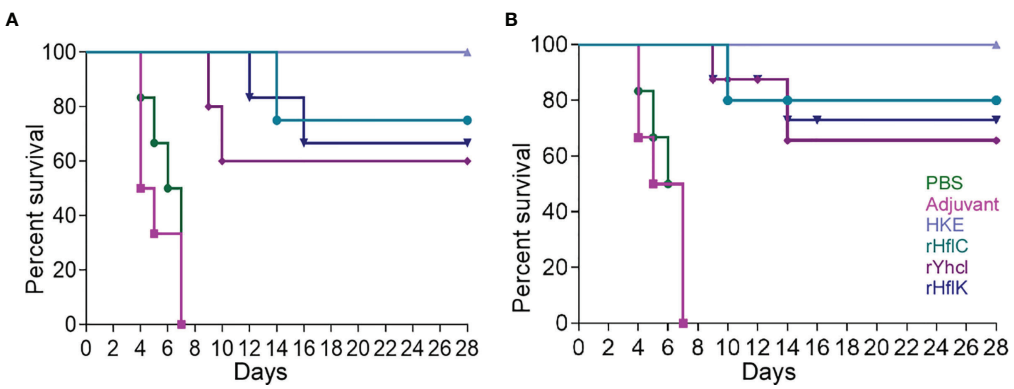


FIGURE 6 | Survivability of the immunized mice **(A)** and fish **(B)** (*Labeo rohita*) against virulent *E. tarda* strain ED-BDU1 and the survival curves were compared using log-rank analysis. The number of survived animals in different day intervals (0–28) after the challenge was estimated as Mean ± SD to plot the graphs. Two-tailed P value was determined by Fisher exact test in comparison to the result of different groups observed as follows: PBS, Adjuvant, Heat Killed *Edwardsiella* (HKE), rHflC, rHflK, rYhcI. x-axis: Immunization days, y-axis: percent survival.

rapid changes in its environment (27). In *Pseudomonas aeruginosa*, insertions inactivation of two FtsH protease accessory factors (HflK and HflC) and a cytoplasmic protease (HslUV) increased tobramycin sensitivity (28). YhcI encodes N-acetylmannosamine kinase [ManNac kinase (nanK)] which catalyzes the phosphorylation ManNAc to ManNAc-6-P liberated from N-acetylneuraminic acid (NANA) by NanA protein. Titgemeyer et al. (29) reported that YhcI is homologous to NagC, Mlc, and proteins of the ROK (repressor, ORF, and kinase) family. YhcI is also homologous to the bifunctional UDp-GlcNAc 2-epimerase/ManNAc kinase from the mammalian liver (30, 31).

Subunit vaccines are found to be safe and have negligible adverse effects when introduced in the form of purified recombinant proteins and formulated with certain adjuvants, which can induce the production of serum antibodies and produce highly protective effects (32, 33). In the present work, the recombinant proteins (rHflC, rHflK, and rYhcI) with 100 μg concentrations were injected into mice along with adjuvant (Alhydrogel). During the 4-week observation period, no mortality was found in the heat-killed *Edwardsiella* group when compared to rHflC (median survival: 14 days), rHflK (median survival: 16 days), rYhcI (median survival: 10 days), PBS (median survival: 6 days) and adjuvant alone group (median survival: 5 days). Nevertheless, the concentration used in the present study might not be the most optimal concentration, so further research is needed to optimize the concentration dose for challenges. A significant level of humoral immune response was detected in immunized animals with respective recombinant proteins. Moreover, the vaccinated animals showed significantly lower antibody titer than animals that underwent only immunization. Meanwhile, significant expression levels of Th1 and Th2 type cytokines were observed in the rHflC group compared to other groups. These findings relate that the rHflC could induce a strong humoral immune response.

In conclusion HflC, HflK and YhcI were identified as *in-vivo* expressed immunogenic proteins, which could induce a strong immune response upon infection and their recombinant proteins evoke a highly protective effect against *E. tarda* challenge. Together recombinant HflC elicits significant IL-10, IFN-γ, Th1, and Th2 mediated mRNA expression. This indicates that rHflC protein plays as a promising vaccine candidate against edwardsiellosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Bharathidasan University Ethics Committee in Animal Experimentation.

AUTHOR CONTRIBUTIONS

PB: Conceptualization, methodology, software, and writing – original draft preparation. MG: Conceptualization, data curation, and methodology, Writing – original draft preparation. VV: Data curation and methodology. KA: Visualization and nvestigation. KP: Software, data curation, and methodology. NA-D: Supervision, editing, and funding acquisition. KN: Conceptualization, validation, funding acquisition, project administration, writing, and reviewing and editing. All authors contributed to the article and approved the submitted version.

FUNDING

We acknowledge the Department of Biotechnology (DBT), the Government of India for the research and development grant (BT/PR12133/AAQ/3/707/2014 dated 02-09-2016) and the Researchers Supporting Project (RSP-2021/20) of King Saud University, Riyadh, Saudi Arabia.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.764662/full#supplementary-material

REFERENCES

1. Swain P, Nayak SK. Comparative Sensitivity of Different Serological Tests for Seromonitoring and Surveillance of Edwardsiella Tarda Infection of Indian Major Carps. *Fish Shellfish Immunol* (2003) 15:333–40. doi: 10.1016/S1050-4648(02)00178-X
2. Sun Y, Liu CS, Sun L. Comparative Study of the Immune Effect of an Edwardsiella Tarda Antigen in Two Forms: Subunit Vaccine vs DNA Vaccine. *Vaccine* (2011) 29:2051–7. doi: 10.1016/j.vaccine.2011.01.013
3. Mohanty BR, Sahoo PK. Immune Responses and Expression Profiles of Some Immune-Related Genes in Indian Major Carp, Labeo Rohita to Edwardsiella Tarda Infection. *Fish Shellfish Immunol* (2010) 28:613–21. doi: 10.1016/j.fsi.2009.12.025
4. Mohanty BR, Sahoo PK. Edwardsiellosis in Fish: A Brief Review. *J Biosci* (2007) 32:1331–44. doi: 10.1007/s12038-007-0143-8
5. Leung KY, Siame BA, Tenkink BJ, Noort RJ, Mok YK. Edwardsiella Tarda - Virulence Mechanisms of an Emerging Gastroenteritis Pathogen. *Microbes Infect* (2012) 14:26–34. doi: 10.1016/j.micinf.2011.08.005
6. Ling SHM, Wang XH, Xie L, Lim TM, Leung KY. Use of Green Fluorescent Protein (GFP) to Study the Invasion Pathways of Edwardsiella Tarda in *In Vivo* and *In Vitro* Fish Models. *Microbiology* (2000) 146:7–19. doi: 10.1099/00221287-146-1-7
7. Rao PSS, Lim TM, Leung KY. Opsonized Virulent Edwardsiella Tarda Strains Are Able to Adhere to and Survive and Replicate Within Fish Phagocytes But Fail to Stimulate Reactive Oxygen Intermediates. *Infect Immun* (2001) 69:5689–97. doi: 10.1128/IAI.69.9.5689-5697.2001
8. Zheng J, Leung KY. Dissection of a Type VI Secretion System in Edwardsiella Tarda. *Mol Microbiol* (2007) 66:1192–206. doi: 10.1111/j.1365-2958.2007.05993.x

9. Tan YP, Zheng J, Tung SL, Rosenshine I, Leung KY. Role of Type III Secretion in Edwardsiella Tarda Virulence. *Microbiology* (2005) 151:2301–13. doi: 10.1099/mic.0.28005-0
10. Chen JD, Lai SY, Huang SL. Molecular Cloning, Characterization, and Sequencing of the Hemolysin Gene From Edwardsiella Tarda. *Arch Microbiol* (1996) 165:9–17. doi: 10.1007/s002030050290
11. Janda JM, Abbott SL, Oshiro LS. Penetration and Replication of Edwardsiella Spp. In HEp-2 Cells. *Infect Immun* (1991) 59:154–61. doi: 10.1128/iai.59.1.154-161.1991
12. Sun Y, Zheng WJ, Hu YH, Sun BG, Sun L. Edwardsiella Tarda Eta1, an *In Vivo*-Induced Antigen That Is Involved in Host Infection. *Infect Immun* (2012) 80:2948–55. doi: 10.1128/IAI.00063-12
13. Maiti B, Dubey S, Munang'andu HM, Karunasagar I, Karunasagar I, Evensen Ø. Application of Outer Membrane Protein-Based Vaccines Against Major Bacterial Fish Pathogens in India. *Front Immunol* (2020) 11:1362. doi: 10.3389/fimmu.2020.01362
14. Stock I, Wiedemann B. Natural Antibiotic Susceptibilities Ofedwardsiella Tarda, E. Ictaluri, andE. Hoshinae. *Antimicrob Agents Chemother* (2001) 45:2245–55. doi: 10.1128/AAC.45.8.2245-2255.2001
15. Castro N, Toranzo AE, Núñez S, Magariños B. Development of an Effective Edwardsiella Tarda Vaccine for Cultured Turbot (Scophthalmus Maximus). *Fish Shellfish Immunol* (2008) 25:208–12. doi: 10.1016/j.fsi.2008.05.008
16. Robinson A, Farrar GH, Wiblin CN. *Vaccine Protocols*. Totowa, New Jersey: Springer (2003). doi: 10.1385/0896033341
17. Kawai K, Liu Y, Ohnishi K, Oshima SI. A Conserved 37 kDa Outer Membrane Protein of Edwardsiella Tarda Is an Effective Vaccine Candidate. *Vaccine* (2004) 22:3411–8. doi: 10.1016/j.vaccine.2004.02.026
18. Verjan N, Hirono I, Aoki T. Genetic Loci of Major Antigenic Protein Genes of Edwardsiella Tarda. *Appl Environ Microbiol* (2005) 71:5654–8. doi: 10.1128/AEM.71.9.5654-5658.2005
19. Raja V, Shanmughapriya S, Kanagavel M, Artiushin SC, Velineni S, Timoney JF, et al. *In Vivo*-Expressed Proteins of Virulent Leptospira Interrogans Serovar Autumnalis N2 Elicit Strong IgM Responses of Value in Conclusive Diagnosis. *Clin Vaccine Immunol* (2016) 23:65–72. doi: 10.1128/CVI.00509-15
20. Miller L, Richter M, Hapke C, Stern D, Nitsche A. Genomic Expression Libraries for the Identification of Cross-Reactive Orthopoxvirus Antigens. *PloS One* (2011) 6:e21950. doi: 10.1371/journal.pone.0021950
21. Kihara A, Akiyama Y, Ito K. A Protease Complex in the Escherichia Coli Plasma Membrane: HflKC (HflA) Forms a Complex With FtsH (HflB), Regulating Its Proteolytic Activity Against SecY. *EMBO J* (1996) 15:6122–31. doi: 10.1002/j.1460-2075.1996.tb01000.x
22. Kihara A, Ito K. Translocation, Folding, and Stability of the HflKC Complex With Signal Anchor Topogenic Sequences. *J Biol Chem* (1998) 273:29770–5. doi: 10.1074/jbc.273.45.29770
23. Kihara A, Akiyama Y, Ito K. Host Regulation of Lysogenic Decision in Bacteriophage λ: Transmembrane Modulation of FtsH (HflB), the cII Degrading Protease, by HflKC (HflA). *Proc Natl Acad Sci* (1997) 94:5544–9. doi: 10.1073/pnas.94.11.5544
24. Chiba S, Ito K, Akiyama Y. The Escherichia Coli Plasma Membrane Contains Two PHB (Prohibitin Homology) Domain Protein Complexes of Opposite Orientations. *Mol Microbiol* (2006) 60:448–57. doi: 10.1111/j.1365-2958.2006.05104.x
25. van Bloois E, Dekker HL, Fröderberg L, Houben ENG, Urbanus ML, de Koster CG, et al. Detection of Cross-Links Between FtsH, YidC, HflK/C Suggests a Linked Role for These Proteins in Quality Control Upon Insertion of Bacterial Inner Membrane Proteins. *FEBS Lett* (2008) 582:1419–24. doi: 10.1016/j.febslet.2008.02.082
26. Morrow IC, Parton RG. Flotillins and the PHB Domain Protein Family: Rafts Worms and Anaesthetics. *Traffic* (2005) 6:725–40. doi: 10.1111/j.1600-0854.2005.00318.x
27. Chu CY, Stewart PE, Bestor A, Hansen B, Lin T, Gao L, et al. Function of the Borrelia Burgdorferi Ftsh Homolog Is Essential for Viability Both In Vitro and *In Vivo* and Independent of HflK/C. *MBio* (2016) 7:e00404–16. doi: 10.1128/mBio.00404-16
28. Hinz A, Lee S, Jacoby K, Manoil C. Membrane Proteases and Aminoglycoside Antibiotic Resistance. *J Bacteriol* (2011) 193:4790–97. doi: 10.1128/JB.05133-11
29. Titgemeyer F, Reizer J, Reizer A, Saier MH. Evolutionary Relationships Between Sugar Kinases and Transcriptional Repressors in Bacteria. *Microbiology* (1994) 140:2349–54. doi: 10.1099/13500872-140-9-2349
30. Hinderlich S, Stäsche R, Zeitler R, Reutter W. A Bifunctional Enzyme Catalyzes the First Two Steps in N- Acetylneuraminic Acid Biosynthesis of Rat Liver. Purification and Characterization of UDP-N-Acetylglucosamine 2-Epimerase/N-Acetylmannosamine Kinase. *J Biol Chem* (1997) 272:24313–8. doi: 10.1074/jbc.272.39.24313
31. Plumbridge J, Vimr E. Convergent Pathways for Utilization of the Amino Sugars N- Acetylglucosamine, N-Acetylmannosamine, and N-Acetylneuraminic Acid by Escherichia Coli. *J Bacteriol* (1999) 181:47–54. doi: 10.1128/jb.181.1.47-54.1999
32. Wang C, Hu Yh, Chi H, Sun L. The Major Fimbrial Subunit Protein of Edwardsiella Tarda: Vaccine Potential, Adjuvant Effect, and Involvement in Host Infection. *Fish Shellfish Immunol* (2013) 35:858–65. doi: 10.1016/j.fsi.2013.06.021
33. Sun Y, Liu C, Sun L. Identification of an Edwardsiella Tarda Surface Antigen and Analysis of Its Immunoprotective Potential as a Purified Recombinant Subunit Vaccine and a Surface-Anchored Subunit Vaccine Expressed by a Fish Commensal Strain. *Vaccine* (2010) 28:6603–8. doi: 10.1016/j.vaccine.2010.07.050

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Bothammal, Ganesh, Vigneshwaran, Anbarasu, Ponmurugan, Al-Dhabi and Natarajaseenivasan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Comparative Immunology, Microbiology and Infectious Diseases 79 (2021) 101698

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Prevalence of leptospirosis among animal herds of north eastern provinces of India

Perumal Saranya [a], Chandrani Goswami [b], Kalimuthusamy Sumathi [c], Alankar Hitlar Balasundareshwaran [d], Palanisamy Bothammal [a], Lakshya Jyoti Dutta [e], Gangatharan Muralitharan [a], Durlav Prasad Bora [b,**], Kalimuthusamy Natarajaseenivasan [a,*]

[a] Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India
[b] Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati, 781022, Assam, India
[c] Department of Parasitology, Tamilnadu Veterinary and Animal Sciences University, Tirunelveli, 600051, India
[d] Department of Geography, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India
[e] Department of Animal Reproduction Gynaecology& Obstetrics (ARGO), College of Veterinary Science, Assam Agricultural University, Guwahati, 781022, Assam, India

ARTICLE INFO

Keywords:
Leptospirosis
Ballum
Grippotyphosa
Risk factors
Cattle
Pigs
Goats

ABSTRACT

Serum samples from 840 animals were examined for *Leptospira* spp. antibodies by microscopic agglutination test (MAT) to assess the risk factors and the prevalence of leptospirosis among animal herds of Assam, Meghalaya, Mizoram the north eastern (NER) provinces. They were compared with Tamilnadu (TN) the southern province of India for the serovar and risk factor inconsistency. Serovar Ballum was reported to be prevalent (28.1 %) in Assam and Grippotyphosa (16.1 %) in Tamilnadu. The overall seropositivity observed was 36.8 %(206/560) from NER and 30.7 %(86/280) from TN. In this study, the higher seroprevalence was observed in pigs (42.6 %), cattle (39.8 %) and goats (26 %) in NER. Cattle (36.4 %) and goat (17.6 %) showed higher prevalence in TN. The presence of rodents in pig herds was found to be significant (P = 0.0088) in NER and it was for cattle in TN (P = 0.0063). We suggest that a program of rodent control should be included in the flock management practices aiming to reduce transmission of the leptospires.

1. Introduction

Leptospirosis is an infectious zoonotic disease caused by the genus *Leptospira* along with its more than 260 serovars [1], infecting a variety of domestic and wild animals [2]. Leptospirosis is one of the sixth diseases enlisted by World Organization for Animal Health (OIE) with reference to "animal diseases and climate change" [3]. Leptospirosis has long been recognized as one of the causes of acute febrile illness in those parts of the country [4,5]. Though similar conditions exist in several regions of India, but few reports were evidenced, and the probable reason might be due to lack of awareness, clinical suspicion, and active surveillance [6–8].

Leptospirosis also been established as one of the most important infectious diseases and cause economic losses in livestock, particularly in cattle, due to negative impacts on reproduction (abortion, embryonic death, stillbirths, infertility), mastitis, weak pregnancy, decreased milk production and growth rates, as well as indirect costs associated with treatments [9]. The common clinical signs of pyrexia, anorexia, depression, jaundice, agalactiae, conjunctivitis and hemorrhagic syndromes are present in goats and pigs [10], because of ill habits with dirty and muddy environment where leptospires live abundantly in pigs. To understand the epidemiology of the disease in a region where there are no reports so far, it is important to identify that the circulating serovars of *Leptospira* and their animal hosts. Therefore, the purpose of the present study was to investigate the circulating *Leptospira* and its association with potential risk factors in animal herds of Assam, Meghalaya, Mizoram from the northeastern provinces and compared with Tamilnadu the southern province of India.

* Corresponding author at: Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, 620 024, India.
** Corresponding author.
E-mail addresses: drdpbora@gmail.com (D.P. Bora), natarajaseenivasan@gmail.com (K. Natarajaseenivasan).

https://doi.org/10.1016/j.cimid.2021.101698
Received 3 December 2020; Received in revised form 20 August 2021; Accepted 23 August 2021
Available online 25 August 2021
0147-9571/© 2021 Elsevier Ltd. All rights reserved.

Microbial Pathogenesis 152 (2021) 104738

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Biochemical analysis of leptospiral LPS explained the difference between pathogenic and non-pathogenic serogroups

Shanmugam Vanithamani [a,1], Charles Solomon Akino Mercy [a,1], Murugesan Kanagavel [a,1], Krishnamoorthi Sumaiya [a], Palanisamy Bothammal [a], Perumal Saranya [a], Muthu Prasad [a], Karuppiah Ponmurugan [b], Gangatharan Muralitharan [a], Naif Abdullah Al-Dhabi [b], Ashutosh Verma [c], Paluru Vijayachari [d], Kalimuthusamy Natarajaseenivasan [a,e,*]

[a] Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, 620024, India
[b] Department of Botany & Microbiology, College of Science, King Saud University, P.O.Box 2455, Riyadh, 11451, Saudi Arabia
[c] Lincoln Memorial University, College of Veterinary Medicine, Harrogate, TN, 37752, USA
[d] WHO Collaborating Centre for Diagnosis, Reference, Research and Training in Leptospirosis, Regional Medical Research Centre (ICMR), Port Blair, 744103, India
[e] Department of Neuroscience, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, 19140, USA

ARTICLE INFO

Keywords:
Leptospira
Lipopolysaccharide (LPS)
LAL assay
FTIR
NMR
HOG

ABSTRACT

Lipopolysaccharide (LPS) is the major surface antigen of *Leptospira*. In this study, the genes involved in the LPS biosynthesis were analyzed and compared by bioinformatics tools. Also, the chemical composition analysis of leptospiral lipopolysaccharides (LPS) extracted from 5 pathogenic serovars like Autumnalis, Australis, Ballum, Grippotyphosa, Pomona, and the nonpathogenic serovar Andamana was performed. Methods used were Limulus amebocyte lysate assay (LAL), gas chromatography-mass spectrometry (GC-MS), fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance spectroscopy (NMR). LAL assay showed a significantly higher level of endotoxicity among pathogenic serovars (~0.490 EU/mL) than that of nonpathogenic Andamana (~0.102 EU/mL). FAMES analysis showed the presence of palmitic acid (C16:0), hydroxy lauric acid (3-OH-C12:0), and oleic acid (C18:0). Palmitoleic acid (C16: 1), and 3- hydroxy palmitate (3-OH-C16:0) was detected only in pathogenic serovars. In contrast myristoleic acid (C14:1) and stearic acid (C18:0) were present in Andamana. FTIR analysis revealed C–O–C stretch of esters, 3°ROH functional groups and carbohydrate vibration range were similar among pathogenic serovars. The NMR analysis reveals similarity for 6 deoxy sugars and methyl groups of Autumnalis, Australis, and Ballum. Further, the presence of palmitoleic acid and 3-hydroxy palmitate may be the significant pathogen-associated predisposing factor. This mediates high osmolarity glycerol (HOG) mediated stress response in leptospiral LPS mediated pathogenesis.

1. Introduction

Leptospirosis is a common, globally important neglected zoonotic infectious disease, caused by spirochetes of the genus *Leptospira*. Leptospirosis is a disease of public health importance in tropical and subtropical countries [1]. Conventionally the genus *Leptospira* was classified into 24 serogroups that have been further classified into ≥250 pathogenic serovars based on the immunological characterization of surface lipopolysaccharide (LPS) by microscopic agglutination test. Recently, phylogenetic analysis revealed that *Leptospira* can be divided into three lineages that correlate with the level of pathogenicity of the species: pathogenic (P1), intermediate (P2), and saprophytic (S1 and S2), [2]. The emerging serodiversity among leptospires relies on the changes in the genetic organization of the LPS biosynthesis genes [3]. LPS are complex, amphipathic biomolecules that offer biological protection to the pathogen from lysozymal degradation and anti-microbial effects of many peptide antibiotics. The acyl chains present in the LPS molecules are highly saturated thus serving as tight and an efficient barrier for most of the hydrophobic moieties [4]. LPS increases the negative charge of the cell membrane and maintains membrane

* Corresponding author. Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, 620024, India.
E-mail addresses: natarajaseenivasan@gmail.com, natarajaseenivasan@bdu.ac.in (K. Natarajaseenivasan).
[1] Authors contributed equally.

https://doi.org/10.1016/j.micpath.2021.104738
Received 16 October 2020; Received in revised form 2 January 2021; Accepted 7 January 2021
Available online 30 January 2021
0882-4010/© 2021 Elsevier Ltd. All rights reserved.

AMERICAN SOCIETY FOR MICROBIOLOGY | mSphere®

RESEARCH ARTICLE
Clinical Science and Epidemiology

MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis

Charles Solomon Akino Mercy,[a] Natarajaseenivasan Suriya Muthukumaran,[a,b] Prema Velusamy,[c] Palanisamy Bothammal,[a] Krishnamoorthi Sumaiya,[a] Perumal Saranya,[a] Dianne Langford,[f] Santhanam Shanmughapriya,[c,d,e] Kalimuthusamy Natarajaseenivasan[a,f]

[a]Medical Microbiology Laboratory, Department of Microbiology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India
[b]Department of Biotechnology, School of Biotechnology and Genetic Engineering, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India
[c]Heart and Vascular Institute, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania, USA
[d]Department of Medicine, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania, USA
[e]Department of Cellular and Molecular Physiology, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania, USA
[f]Department of Neuroscience, Lewis Katz School of Medicine, Temple University, Philadelphia, Pennsylvania, USA

ABSTRACT Leptospirosis remains a significant human health issue due to its systemic complications. Therefore, biomarkers that are more effective are urgently needed for the early diagnosis of leptospirosis. MicroRNAs (miRNAs) are evolutionarily conserved regulatory RNAs that have shown the potential to be used as biomarkers for diagnosis, prognosis, and therapy of infectious diseases. In this study, we performed an unbiased screen using the miRNome miRNA array to identify circulating miRNAs with the potential to serve as authentic biomarkers for early diagnosis of leptospirosis. Because leptospiral lipopolysaccharide (LPS) is the predominant leptospiral antigen and plays a vital role in immunological and biological activities, we used LPS treated and untreated *in vitro* (THP1 cells) and *in vivo* (BALB/c mice) surrogate models to identify the LPS-specific miRNAs. Differential expression analysis revealed 18 miRNAs to be associated strongly with LPS stimulation in THP1 cells. Of these, three (miR-let-7b-5p, miR-144-3p, and miR-21-5p) were observed to be present at increased levels *in vivo*. The identified miRNAs were validated for their biomarker potential using serum samples from leptospirosis-negative patients and patients with confirmed cases of leptospirosis. Identified miRNAs were able to discriminate the acute leptospiral infection from other febrile diseases with a test sensitivity and specificity of 93.2% and 88.19%, respectively. Gene functional enrichment and protein-protein interaction (PPI) network analysis revealed that the identified miRNAs play important roles in disease signal transduction, signaling by interleukins, the stress-activated protein kinase signaling cascade, the mitogen-activated protein kinase (MAPK) signaling pathway, and the cellular response to a transforming growth factor β (TGF-β) stimulus with a notable interconnection between these biological processes.

IMPORTANCE Here, we used miRNAs that are differentially regulated by the LPS/TLR2 immune axis to devise a miRNA-based diagnosis for leptospirosis. The study established the role of the circulating stable miRNAs (miR-21-5p, miR-144-3p, and miR-let-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs can be used to diagnose acute leptospirosis and also to differentiate leptospiral infection from other bacterial and spirochetal infections, as proved by the use of human clinical samples. Thus, our findings indicate that miRNAs can play a crucial role in the diagnosis of infectious diseases, like leptospirosis, that are generally misdiagnosed.

KEYWORDS leptospirosis, LPS, microRNA, TLR2, biomarkers, PPI network

Citation Akino Mercy CS, Suriya Muthukumaran N, Velusamy P, Bothammal P, Sumaiya K, Saranya P, Langford D, Shanmughapriya S, Natarajaseenivasan K. 2020. MicroRNAs regulated by the LPS/TLR2 immune axis as bona fide biomarkers for diagnosis of acute leptospirosis. mSphere 5:e00409-20. https://doi.org/10.1128/mSphere.00409-20.

Editor Sarah E. F. D'Orazio, University of Kentucky

Copyright © 2020 Akino Mercy et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Santhanam Shanmughapriya, ssanthanam@pennstatehealth.psu.edu, or Kalimuthusamy Natarajaseenivasan, tuf29518@temple.edu.

Received 1 May 2020
Accepted 19 June 2020
Published 15 July 2020

Downloaded from https://journals.asm.org/journal/msphere on 28 September 2021 by 14.139.186.110.

Microbial Pathogenesis 149 (2020) 104407

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Leptospiral protein LIC11334 display an immunogenic peptide KNSMP01

Muthu Prasad, Palanisamy Bothammal, Charles Solomon Akino Mercy, Krishnamoorthi Sumaiya, Perumal Saranya, Gangatharan Muralitharan, Kalimuthusamy Natarajaseenivasan *

Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India

ARTICLE INFO

Keywords:
Leptospirosis
Leptospira
Virulent proteins
Epitopes
Peptide
Subunit vaccine

ABSTRACT

Leptospirosis is considered as a neglected tropical disease which is caused by pathogenic *Leptospira* spp. The precise mechanisms of leptospirosis pathogenesis are unclear and hence, the progress in development of treatment modalities has been dismal. The present study aimed to identify novel virulent factors of leptospires to understand the disease pathogenesis and to develop treatment modalities. *Leptospira interrogans* contains two chromosomes and encodes for ~3703 genes, but the functions of several open reading frames have not yet been explored. Among them, novel virulent associated leptospiral proteins (LIC11334, LIC11542, LIC11436, LIC11120 and LIC12539) were identified using VirulentPredict and the antigenicity of these targets was explored by VaxiJen server. Domain architecture of the pathogen specific proteins revealed that LIC11334 had potential to evoke significant immune response against leptospiral infection and LIC11436 contains four folds of immunoglobulin-like domain and plays a vital role in pathogenesis. Therefore, B-cell epitopes were predicted and the epitope of high virulence (and VaxiJen score from LIC11334) was chemically synthesized as peptide (KNSMP01) and labeled with Biotin (Biotin-SGSGEVENPDPKVAQEC). Binding affinity of KNSMP01 with MHC molecules was predicted and the molecule was discovered to have potential to elicit both humoral and cell mediated immune responses and found to interact with host components via hydrophobic interaction, hydrogen bonding and salt bridges. Rabbit antisera was raised against KNSMP01 and found to elicit antigenicity using Western, ELISA and dot blot assays. *In silico* and *in vitro* experiments show KNSMP01 to be a promising immunogen and may be a better vaccine candidate for leptospirosis.

1. Introduction

Leptospirosis is a re-emerging zoonotic infectious disease, which causes major public health problems worldwide. Recent outbreaks and reports have surveyed the global burden of this disease. Around 60,000 deaths and 1.03 million leptospirosis cases are registered annually [1]. Leptospirosis is an environment dependent tropical disease, caused by pathogenic *Leptospira* spp. It infects humans by direct contact with infected animal urine or with contaminated environments and disseminates haematogenously causing diverse clinical symptoms [2]. Leptospirosis is diagnosed by immunological, molecular and immunochromatographic methods. The disease is confirmed by the gold standard microscopic agglutination test (MAT). Due to non-specific early symptoms, early diagnosis and treatment of the disease has not been entirely successful [3]. Hence, elucidation of the molecular mechanisms of infection is crucial for developing methods to successfully diagnose and treat the disease.

The cell adhesion molecules of virulent *Leptospira* interact with host surface-exposed proteins to establish the infection. The surface associated proteins such as outer membrane proteins (OMPs), *Leptospira* OmpA-like lipoprotein (Loa22), LipL32, immunoglobulin-like proteins (LigA and LigB), *Leptospira* endostatin-like proteins (LenA-LenE) and lipopolysaccharides (LPS) are potentially involved in virulence mechanism and studied extensively to find vaccine targets [4]. Especially, the OMPs are being explored deeper insights to understand the pathogenesis of leptospirosis [5] The immune response elicited by OMPs has been evaluated using animal models and aid in the vaccine development. Recombinant proteins of pathogenic *Leptospira* spp. were shown to bind with host extracellular matrix components such as fibronectin, plasminogen, collagens, laminin and elastin [6]. But the molecular

* Corresponding author.
E-mail address: natarajaseenivasan@gmail.com (K. Natarajaseenivasan).

https://doi.org/10.1016/j.micpath.2020.104407
Received 8 May 2020; Received in revised form 8 July 2020; Accepted 17 July 2020
Available online 4 August 2020
0882-4010/© 2020 Elsevier Ltd. All rights reserved.

Journal of Microbiological Methods 156 (2019) 20–22

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Silver enhanced nano-gold dot blot immunoassay for leptospirosis

Veerapandian Raja, Muthu Prasad, Palanisamy Bothammal, Perumal Saranya, Krishnamoorthi Sumaiya, Charles Solomon Akino Mercy, Kalimuthusamy Natarajaseenivasan*

Medical Microbiology Laboratory, Department of Microbiology, Center for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

ARTICLE INFO

Keywords:
Leptospirosis
Diagnosis
Nanogold
Recombinant protein
Dot blot

ABSTRACT

Leptospirosis is a widespread zoonotic disease and lacks in efficient diagnostic tools. In the present study, a nanogold based dot blot immunoassay was developed and evaluated for the detection of leptospirosis in human urine samples. This method was found to be rapid (< 4 h) with higher sensitivity (> 4.2–14.6%) than horse radish peroxidase (HRP) conjugated dot blot assay.

Leptospirosis is a re-emerging public health problem that is generally under diagnosed. The early and accurate diagnosis is still challenging and often leads to a fatal outcome (Bharti et al., 2003; Pappas et al., 2008). Annually, 500,000 cases of severe leptospirosis are reported, with fatality rates < 5 to 30% (WHO, 2011). The misdiagnosis of the disease can damage multiple organs and hence the development of a sensitive and reliable diagnostics is important. The gold standard diagnosis of leptospirosis is the microscopic agglutination test (MAT) that is often used for detection of leptospires. The other serological techniques including enzyme linked immunosorbent assay (ELISA), lepto dipstick, leptodridot and latex agglutination test are used but their sensitivity is dubitable during the acute illness. Advanced diagnostic formats including quantitative PCR and recombinant protein based ELISA have been developed (Raja and Natarajaseenivasan, 2015) but they have several drawbacks, including restricted availability; requiring skilled technicians and lack of uniformity. Moreover, seroconversion must be demonstrated with paired sera to confirm diagnosis. To overcome these limitations, there is a stringent demand for the development of new diagnostic formats for a simple and rapid method to perform in any diagnostic laboratory. Recently, the nanogold based diagnostic format has been evolving and showed significant outputs to resolve the above routes. Chirathaworn et al., 2011 reported detection of *Leptospira* in urine using anti-*Leptospira*-coated gold nanoparticles with high sensitivity. Furthermore, highly sensitive markers can be identified for rapid and prompt diagnostics and these lacunas will be empowered by nanogold based dot blot assay. The present study demonstrated the detection of human leptospirosis using a nanogold conjugate based dot blot immunoassay.

A total of 112 human urine and blood samples was collected from patients with febrile illness and suspected for leptospirosis at Government Hospital of Tiruchirappalli, Tamil Nadu, India between November 2010 and April 2012. Patients fulfilling any of the following criteria were considered as clinically suspected and laboratory confirmed cases of leptospirosis: (1) positive isolation of leptospires from blood/urine, (2) seroconversion or a 4-fold rise in titre in paired serum samples (collected with a mean interval of 23 days) by MAT; and/or (3) a seropositivity (> 1:400) in a crude leptospiral antigen based IgM ELISA. Diagnosis of leptospirosis was confirmed in 48 samples (group I) and 64 did not meet the diagnostic criteria and hence considered as discarded cases of leptospirosis (group II). Similarly, 45 seronegative healthy individuals matching for age (± 5 years) were recruited from patients attending the same hospital for complaints other than febrile illness (group III). Individual urine samples were centrifuged at 12,000 ×*g* for 10 min at room temperature; the supernatant was discarded leaving an aliquot of 50 µl, boiled for 30 min and centrifuged at 1000 ×*g* for 10 min. The supernatant obtained was subjected for protein estimation by bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO) and desired concentration was dotted individually onto NC strips (Kanagavel et al., 2017). Individual informed written consent was obtained from both cases and controls. This study was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University, India (Reference No.: DM/2010/101/14).

Several leptospiral proteins are reported to be used for diagnosis and LipL32 and LigA are being widely used for diagnostics. But the

* Corresponding author at: Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, India.
E-mail address: natarajaseenivasan@rediffmail.com (K. Natarajaseenivasan).

https://doi.org/10.1016/j.mimet.2018.11.021
Received 26 October 2018; Received in revised form 27 November 2018; Accepted 28 November 2018
Available online 28 November 2018
0167-7012/ © 2018 Elsevier B.V. All rights reserved.

EDWARDSIELLA TARDA BIOFILM FORMATION AND INHIBITION BY SECONDARY METABOLITES OF ACTINOMYCETES

K.Sivasankari[1], M. Ganesh[2], P. Bothammal[3] & Dr. K. Natarajaseenivasan[4]
[1,2,3 & 4]Department of Microbiology, Centre for Excellence in Life Sciences,
Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India.

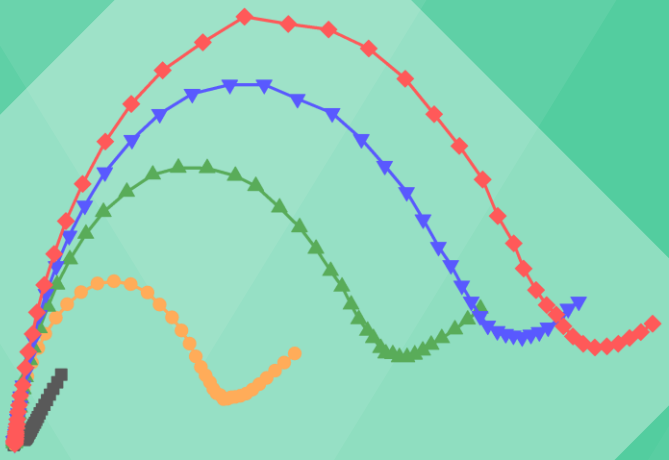
Received: August 25, 2018 Accepted: October 14, 2018

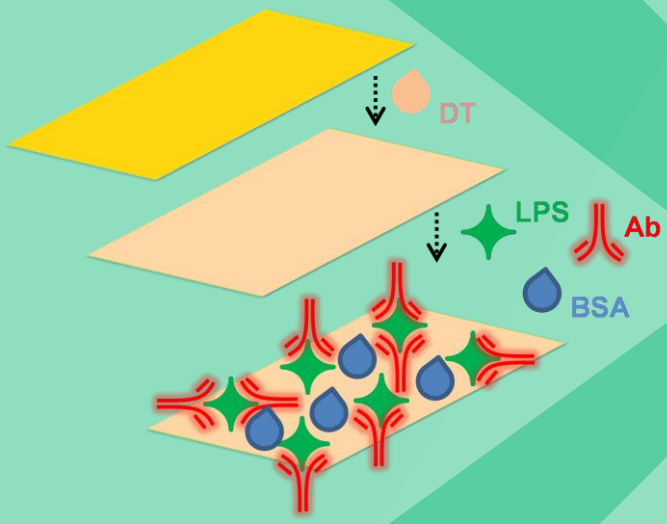
ABSTRACT *Edwardsiellatardais a most virulent fish pathogen that causes extensive economic losses in the aquaculture industry worldwide. The antibiotic resistance status of E.tardais high, especially in the biofilm status; however, the mechanisms underlying its resistance remain largely unknown.New broad spectrum antimicrobial agents are urgently needed to combat frequently emerging multidrug resistant pathogens. In this study, the 40 commensal actinomycetes was isolated and primary screening was done using cross-streak method in which A76 isolate showed a good activity and its secondary metabolites was used against the E.tardabiofilm inhibition. The results of the present study revealed that the commensal Actinomycetes have immense potential activity against the Edwardsiella biofilm at 100µg.*

Keywords: *Edwardsiellatarda, Biofilm, Actinomycetes.*

Introduction

*Edwardsiellatarda*is a Gram-negative bacteria pathogen with a broad host range including fish and humans (Jandaand Abbott 1993a). *E. Tarda* infection is responsible for tremendous economic loss of a variety of cultured fish in Asia, especially Japan and India, and also in the United States (Herman and Bullock 1986; Castro *et al.,*2006; Lima *et al.,* 2008). In India, edwardsiellosis has been found to affect many fish species, such as *Anabas testudineus* (Bloch) (Sahoo*et al.,*2000), *Clariasbatrachus* (L.) (Sahoo, Mukherjee &Sahoo 1998) and *Channapunctata* (Kumar *et al.,* 2007).*Edwardsiellatarda*was also isolated from Indian major carp rohu, *Labeorohita* Hamilton (Acharya et al. 2007), and it causes mass mortality in stocks of Asian catfish, *Clariasbatrachus* (L.) during the observation of hatchery-rearing operations (Sahoo*et al.,* 1998; Mohanty&Sahoo 2007). *Edwardsiellatarda*is widely distributed in nature and has been found to affect reptiles, birds and mammals, including humans (Bullock & Herman 1985).Antibiotic resistance has been referred to as "the silent tsunami facing modern medicine" (Cox, 2015). We now take for granted that any infectious disease is curable by antibiotic therapy. Antibiotics are manufactured at an estimated scale of about 100,000 tons annually worldwide, and their use had a profound impact on the life of bacteria on earth. More strains of pathogens have become antibiotic resistant, and some have become resistant to many antibiotics and chemotherapeutic agents, the phenomenon of multidrug resistance. Many types of microorganisms cause infection in humans and animals, so disease prevention and treatment strategies must be adapted to reflect infection risk factors and available treatment options. Over the past decades, most pathogenic species have developed resistance to one or more antimicrobials.. Some reports have observed that resistance emerge Antibiotics resistance has been reported in shrimps (Tjahjadi*et al.,* 1994), eels (Alcaide*et al.,*2005), and aquaculture environments (Chelossi*et al.,*2006) (Kim*et al.,* 2004) within few years of treating infections with antibacterial drugs (Sorum H 1999) and this is a factor, which limits their value in the control of bacterial fish diseases (Smith*et al.,* 1994). Apart from any public health concerns, recognition of the resistance issue has led to calls for intensified surveillance of antibiotic use and antibiotic resistance (Aoki T 1992) .Actinomycetes from the genera Actinoplane, Streptomyces and Actinopolyspora have been reported to produce a number of broad- spectrum antibiotics. Apart from production of antibiotics, actinomycetes have been looked upon as potential sources of bioactive compounds and they are the richest sources of secondary metabolites. Actinomycetes population has been identified as one of the major group of soil population, which may vary with soil type. Apart from soil, they are found in marine and terrestrial environments and also exist in symbiotic association with plants and other living organisms. The important genera of actinomycetes are Streptomycetes, Nocardia, Micromonospora, Thermomonospora, Actinoplanes, Microbispora, Streptosporangium, Actinomadura, Actinosynnema, Dactylosporangium, Rhodococcus, ActinosynnemaKitasatospora, Gordona, Intrasporangium and Streptoalloteichus. Actinobacteria from terrestrial origin produce hundreds of antibiotics which are widely used at present.




DT
LPS
Ab
BSA