Chapter 1

1. INTRODUCTION

Leptospirosis is a zoonotic disease caused by a gram negative spirochete *Leptospira interrogans* [1]. It is categorised under zoonoses, since transmission of the infection causing organism occurs from animals to humans. It is also found to infect a broad range of mammalian species from rodents to humans. Leptospirosis is one of the top ten zoonotic disease of the world [2, 3]. It must be noted that the Leptospiral infection is usually asymptomatic in animals, whereas its infection and pathogenesis is remarkably distinct in humans causing serve and adverse signs [4]. A wide range of symptoms are observable from common cold and myalgia to severe conditions such as jaundice, meningitis, kidney failure, liver damage, conjunctival suffusion, pulmonary haemorrhage, gastrointestinal bleeding, myocarditis and uveitis, to name a few. The symptoms and effects of Leptospiral infection rapidly intensify during the prognosis, which are ultimately lethal. The symptoms of Leptospiral infections overlap with the symptoms of other common infections and diseases such as influenza, jaundice, Weil’s disease, dengue, and thus, it is often misdiagnosed due to this reason [5, 6].

*Leptospira* is found to take a toll on 3,50,000 – 5,00,000 lives worldwide annually, especially in countries with large range of costal coverage, rainfall and countries which are more prone to natural calamities such as floods, earthquake, typhoon, etc., [7].
Figure 1.1: Epidemiology of *Leptospira* outbreaks between 2009 and 2016 throughout the world.

The actual *Leptospira* infection incidents are found to be still under rated due to lack of awareness, diagnostic facility, common and overlapping symptoms with other diseases and also due to lack of notification system in many countries around the globe [8].

*Leptospira* is found to be the leading zoonoses cause of morbidity worldwide with highest number of deaths when compared to other causes of haemorrhagic fever [9]. Even though incidences of Leptospiral outbreaks are high in developing nations, it is also a major concern in developed nations as well [10].

The symptoms usually disappear after a brief period of illness is followed by an overwhelming relapse of the infection. The relapse of infection causes the patient’s conditions to alter drastically and become a critical stage within an incubation period of seven to fourteen days. The diagnosis and treatment of Leptospirosis must be done within this incubation period.

### 1.1. *Leptospira* Virulence

The heightened risk and severity of Leptospiral infection can be attributed to the virulent factors of *Leptospira* which are essential for invasion of the host cells and also
in spread of infection in the host. The virulent factors are distributed on the cell wall as outer membrane proteins and also as secretory proteins which are produced in response to stress conditions during host immune response, during entry into the host cell at the site of exposure such as cuts, wounds or other abrasions which allow easy access for bacteria to the host cells.

The virulent outer membrane proteins of *Leptospira* include *LipL21, LipL32, LipL46*, etc., which elicit a heightened immune response in the host constitutently and are found to be less susceptible to degradation upon host immune response.

The secretory proteins which contribute to virulence of *Leptospira* include; haemolysins, heme binding proteins, heme oxygenase, adhesin, integrins etc., which not only help the organism to adhere to the host cells at the site of infection, but also produce lytic enzymes, which are responsible for host cell lysis, thereby creating a free passage for the entry of the organism into the host body, replicating exponentially during the course of infection and spread through blood. The bacteraemia resulting due to entry of the organism into the blood stream sends the host into a condition of shock due to sepsis caused during chronic infections leading to life threatening conditions with least chance of revivability. The detailed mechanism of action of these virulent factors with respect to *Leptospiral* pathogenesis is explained here under.

**1.2. Haemolysin**

Haemolysin is a well-known pore forming toxin produced by prokaryotes. Haemolysins are not just blood lysing toxins but also pose severe cytotoxicity against other cell types as well. Haemolysins create water filled channels into the adhered host cells leading to hypotonic cell lysis. One of the unique properties of haemolysins include the ability of it to act on the host cells independent of the receptors. Leptospiral haemolysins particularly sphingomyelin dehydrogenase (sph) like haemolysins target the erythrocytes due to high sphingomyelin which constitutes around 50% of the lipids content in the cell membrane.

The fact that *Leptospira* utilize fatty acids as the sole sourced of carbon and energy makes attachment and adherence to erythrocytes, an essential phenomenon for the survival of the organism.
Hence, *Leptospira* adhere to erythrocytes and form pores in them and upon lysis utilize the fatty acid of the cell wall sphingomyelin as their source of nutrition.

Leptospiral haemolysins not only act on erythrocytes but also on other mammalian cells which possess sphingomyelin in their cell wall lipoproteins. This is the reason for high cytotoxicity and multiple organ damage caused by Leptospira, which explains the reason why *Leptospira* harbour liver and kidneys as their safe abodes, likely due to high fatty acid content in the liver due to biosynthesis of fatty acids, and easy entry into the host through intestine wall due to absence of functional immune response in the urethras.

**1.3. Heme Binding Protein**

Heme forms a major source of iron for *Leptospira interrogans*. Hence to acquire good amount of iron for its growth and survival, Leptospiral heme binding proteins which are secreted into the extracellular environment bind to the heme b, followed by ligation of heme iron at histidine/tyrosine pair, followed by heme recognition at the outer membrane protein, and is imported into the bacterial cytoplasm with the help of group of proteins such as TonB, TonB dependent hemin binding protein, TonB dependent outer membrane hemin receptor.

The imported heme is broken down with the help of heme oxygenase. This gene so obtained is highly essential for normal functioning of the most essential enzymes of *Leptospira*, such as; cytochrome c oxidase, diheme cytochrome c peroxidase, etc., which are not only essential for metabolism in *Leptospira*, but also to evade host immune response.

The synthesis of cytochrome utilizing the heme in *Leptospira* takes place with the help of Herne exporter protein. The iron is further taken up using imelysin. The iron which is unused is stored as bacterioferritin.

Along with a number of virulent factors *Leptospira interrogans* also possess a large number of immune evasion mechanisms which are backed by a large number of proteins, which not only aid the organism in evasion from host immune response but also in evasion from antibiotic therapy. Out of a large number of proteins that
*Leptospira* possess some of the proteins are found to be dedicated to Leptospiral survival and spread in the host niche.

Some of the well-known proteins of *Leptospira* that are involved in immune evasion include: multidrug efflux pumps, heavy metal efflux pump, outer membrane efflux protein Tolc, cation efflux system pumps, Na$^+$ driven multidrug efflux pump, Acriflavine resistance proteins, two component system sensor proteins, antimicrobial peptide ABC transporter ATP-binding protein, catalase, flagellar motor switch, thermolysin, peroxy resoxins and di heme Cytochrome C peroxidase.

These proteins help *Leptospira* to adjust to the host environment and also aid in progression of disease, by preventing the activity of the host immune response on *Leptospira* either by degrading the immune factors or by diluting them. The flagellar motor switch protein present in *Leptospira* aid the organism in binding to the host cells and resulting in apoptosis. *Leptospira interrogans* also possess a wide range of two component system sensor proteins (TCSS). These TCSS proteins sense the host response to Leptospiral infection.

TCSS in *Leptospira* sense the stress response and also is involved in signalling mechanisms in *Leptospira* as a stress response the TCSS histidine kinase phosphorylase signals the molecules downstream through phosphorylation and upregulates the expression of accessory genes based on the conditions observed in the host niche. TCSS play a critical role in adaptability of *Leptospira* in the host during the entry into the host or upon onset of infection and its progression.

The Leptospiral catalase and peroxidase play a vital role in neutralizing the immune assault posed by the host towards the organism by converting the toxic peroxides to water and H$^+$ ions without producing cell damaging oxygen free radicals, this unique ability of *Leptospira* is found to be due to the diheme Cytochrome C peroxidase enzyme, which converts H$_2$O$_2$ to H$_2$O and H$^+$ without forming a O$. The organism further utilizes the H$^+$ ion to pump the proton pump in ATP synthase complex for ATP synthesis. Whereas, catalase converts the H$_2$O$_2$ to H$_2$O and O$_2$.

This synergistic action of catalase and peroxidase makes *Leptospira* resistant to the oxidative burst seen in the host immune cells such as macrophages.
*Leptospira interrogans* possess a plethora of efflux pumps in their cell membrane which are responsible for efflux of toxins from cytosol to cell exterior. They are found to be highly efficient in efflux of antibiotics out of the bacterium, thereby reducing the concentration of residual antibiotic inside the bacterial cell, thereby rendering the antibiotic ineffective in killing the organism. During this process *Leptospira* not only evade the action of antimicrobials but also get acclimatized to antibiotics and develop a mechanism to catabolize the antibiotics, resulting in drug resistance.

Multi drug efflux pumps are found to be capable in exporting varied classes of antibiotics efficiently. This results in development of multi drug resistance in the organism.

Tolc, one of the well-known outer membrane efflux protein involved in efflux of toxins and antibacterial drugs is well characterized in *Leptospira*. The synergistic action of Tolc along with multidrug efflux proteins such as cation efflux pump, heavy metal efflux pump make the organism difficult to kill in the host even in the presence of antibiotics. These efflux pumps also play a critical role in survival of the organism outside the host in diverse environmental/ ecological conditions such as waste disposal sites and various water bodies and still remain viable for prolonged periods.

1.4. Leptospirosis

The current treatment for the infection is possible only with the antibiotics, Benzylpenicillin, Penicillin or doxycycline. Failing to administer treatment in time results in a severely deteriorating condition, with less chances of recuperation and recovery. Hence, there is an urgent and immediate need to find fast and accurate diagnostic and treatment methods which can be relied upon for rapid treatment of Leptospirosis [11].

The commonly used method for diagnosis of Leptospirosis is the microscopic agglutination test (MAT), it is a standard tool used worldwide. The MAT tool requires the presence of IgG antibody in the serum of the infected individual. The IgG antibody usually appears in seven to fourteen days after infection, which coincides with the Leptospirosis incubation period.
During this time *Leptospira* growth occurs at an exponential rate, causing massive damage to the host with irrevocable changes in physiology and attacking various organs in the body. The degree of damage can vary based on an individual’s immune response intensity and the site of infection of *Leptospira interrogans* [10, 12, 13].

One of the major drawbacks of serological testing using MAT is that the antibodies reach detectable levels in late acute phase of the disease, causing delay in confirmation of the disease, resulting in delay in effective treatment.

Considering the complexity and drawbacks of MAT, rapid screening tests for antibodies against *Leptospira* have been developed. The IgM antibodies are expressed within the first week of infection. Hence Detection of IgM through ELISA has been widely used, with antigens obtained from cultures of non-pathogenic *L. biflexa* but one of the major setbacks with ELISA is false positive results and the IgM antibody cannot be detected in early stages of infection [14]. To diagnose the disease in places with limited laboratory facilities / instrumentation dipstick assay and latex agglutination tests are followed [15]. To overcome the drawbacks of MAT, several molecular detection methods which can be applied in early acute stage of the disease are developed.

Molecular detection and diagnosis methods for detection of *Leptospira* include dot hybridization technique, *in situ* hybridization technique and PCR techniques.

Both dot and *in situ* hybridization techniques use whole genome DNA or segments of DNA as probes and use radioactive isotopes for labelling. The use of radioactive isotopes makes the process unusable in regular pathological laboratories which lack specialized facilities and skills to handle radioactive substances. Hence, enzymatic staining assays using biotin-labelled DNA probes forma more practical tool for normal diagnosis [13].

Due to these limitations, most laboratories worldwide use amplification of specific fragments of *Leptospira* genome using polymerase chain reaction (PCR) as a diagnostic tool. The nucleic acid of *Leptospira* can be isolated at early stage of acute phase infection. PCR is a rapid and efficient technique to diagnose Leptospirosis, where the target for amplification includes pathogenic genes such as 16s rRNA, *LipL32*, *LipL41*, and are specific to pathogenic strains of *L. interrogans*. Previously various types of PCR analysis have been used for molecular detection of *Leptospira* out of which single
strand chain polymorphism (SSCP) PCR, arbitrarily primed (AR) PCR and real time (RT) PCR are the three well known procedures followed in molecular detection. The SSCP PCR can be used to identify and differentiate between the serovars of *Leptospira*, with the ability to detect even a single base substitutions in fragments less than 200 bp length. AR PCR can be used directly without extraction of DNA, using bacterial cells. RT PCR helps in live tracking and visualization of amplification, but is a highly expensive technique.

The drawbacks of conventional PCR based technique is that it is prone to contamination and can result in false positive results. The PCR is incapable of finding the serovar of the infecting pathogen [16].

In the present work four essential genes of *Leptospira* are considered for gene amplification and detection.

The genes selected for PCR in the current study are: *rrs*, Enoyl CoA hydratase, NADH dehydrogenase and Cytochrome C Oxidase subunit II. The 16s rRNA gene, *rrs* of *Leptospira* is an essential and highly conserved gene in the species *Leptospira interrogans*, and hence acts as a molecular marker for detection of *Leptospira*. Enoyl CoA hydratase is a vital protein in fatty acid catabolism in *Leptospira*, without which the organism is incapable of fatty acid uptake and utilization. NADH dehydrogenase is the constituent enzyme of complex I in electron transport chain in *Leptospira*. Cytochrome C Oxidase subunit II which is the part of the holoenzyme Cytochrome C Oxidase, which forms the complex IV in the electron transport chain in *Leptospira*. These four genes are not only essential for *Leptospira* survival and virulence, but are also unique to *Leptospira*, with no similarity between that of other microbial and animal genes, making them ideal choice for molecular diagnosis.

The advanced proteomic strategies in identification of Leptospirosis is employed to fill the lacuna in the above mentioned techniques.

The outer membrane proteins (OMPs) of *Leptospira interrogans* are responsible for eliciting immune response in the host. The outer membrane proteins are therefore used to detect Leptospirosis.
The OMPs are isolated using two dimensional gel electrophoresis (2DGE) and is subject to immunoblotting on a polyvinylidene fluoride (PVDF) membrane, where the patients serum is used as a primary antibody, the secondary antibody conjugated with the enzyme are probed for detection of immunoreactive spots. This advanced technique of two dimensional immune gel electrophoresis (2DIGE) is gaining more weightage with time due to its reliability, accuracy and also the lack of false positive results [16]. The OMP isolation and identification of *Leptospira* have been in rise, with detection of various antigenic OMPs being detected in the past such as *LipL32*, *LipL41*, *LipL32 a, b, c, and d*.

Upon MAT positive test result, Leptospirosis is treated with antibiotics Benzylpenicillin, tetracycline, or doxycycline, the effectiveness of each antibiotic depends upon the patient’s sensitivity towards the antibiotic. Benzylpenicillin is the most effective drug against Leptospirosis, however, it cannot be administered to all Leptospirosis affected individuals due to the high risk of anaphylactic response; the adverse response to the antibiotic is considerably large in the population. Alternatively, tetracycline or doxycycline, are the only treatments available, but an extremely high dosage is required. The added threat of emerging drug resistant strains of *Leptospira interrogans* it has made it a critical challenge to effectively treat Leptospirosis, even after successful diagnosis [17].