REVIEW OF LITERATURE
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History

Adolf Weil, a German physician, first described the clinical entity of leptospirosis in 1886. He reported the clinical condition of fever presenting with jaundice and renal failure in Heidelberg. In 1887, it was Goldsmidt who gave the name Weil’s disease to a serious form of leptospirosis transmitted by rats and caused by the serovars *icterohaemorrhagiae* and *copenhagenii*. Later in 1907 Stimpson observed Leptospires in silver stained sections in the renal tubules of the kidney of a patient who had died of febrile illness and jaundice. He named it as *Spirochaeta interrogans*, due to its resemblance to a question mark. The spirochetes were also demonstrated in silver stained preparations from the liver of a patient with Weil’s disease. In 1916, Inada and Ido from Japan were able to isolate the spirochetes by injecting blood and urine samples from patients with Weil’s disease into guinea pigs and observed the spirochetes in their blood. These animals also developed the clinical and pathological signs of the disease, with jaundice and hemorrhages. Hence the organism was named as *Spirochaeta icterohaemorrhagiae*. At almost the same time as Inada and Ido, two teams of German physicians comprising of Uhlenhuth and Fromme, Hubener and Reiter demonstrated these spirochetes in the blood of guinea pigs which had been inoculated with the blood of infected soldiers.

In 1918, Noguchi created a separate genus *Leptospira* in the order *Spirochaetales*. Subsequently numerous serovars were isolated from patients and animals suffering from this disease and were classified under the genus *Leptospira*. In the next 15 years until the 1930s many important serovars were discovered. Between 1920 and 1950 many *Leptospira* causing milder forms of the disease were discovered. Several saprophytic leptospires had also been cultured from the environmental sources such as water, sewage etc. Their detailed structure was studied using electron microscopy between 1960 and 1970. Faine and Yanagawa showed that *Leptospira* were similar to other bacteria and that the structural elements have various antigens associated with them.
Later on studies on the antigenicity of the serovars were carried out and many newer diagnostic tests based on different principles were introduced. Galton et al developed the Microscopic Agglutination Test (MAT) in 1958. Subsequently other tests like Complement fixation test, Immunofluorescence Test, ELISA, Immunoperoxidase staining were developed for the diagnosis of Leptospirosis.\textsuperscript{18}

**NOMENCLATURE**

The name *Leptospira* is derived from Lepto meaning thin and speira meaning coil.\textsuperscript{24} Hence they are named as *Leptospira* as they are very thin delicate spirochaetes that are tightly coiled about their long axis.

**Classification**

Taxonomically, *Leptospira* are belonging to the Division of Gracillicutes, the Class of Scotobacteria, Order Spirochaetales, Family Leptospiraceae and Genus *Leptospira*.\textsuperscript{23} The genus *Leptospira* belongs to the family Leptospiraceae which also includes *Leptonema* and *Turneria*. Another family under the order Spirochaetales is Spirochaetaceae which comprises of two genera namely: Borrelia and Treponema.\textsuperscript{24}

As per the earlier classification based on pathogenicity, the genus *Leptospira* was classified into 2 groups, the saprophytic *Leptospira biflexa* and the pathogenic *Leptospira interrogans*.\textsuperscript{25} *L biflexa* is found in different types of surface water and is abundant in soil with a high content of organic matter. These free living saprophytes are morphologically identical to the pathogens. However the saprophytes are non pathogenic to animals, can grow at 13\textdegree C and also in the presence of 8 azaguanine. These two species of *Leptospira* are further classified into serovars by using agglutination techniques and approximately 60 serovars have been identified for *L. biflexa* and more than 225 for *L. interrogans*.\textsuperscript{2, 26}

A new system of classification of the *Leptospira* was proposed by Yasuda and it was based on DNA homology. This classification was proposed in the Subcommittee on the Taxonomy of Leptospiraceae conducted at Quito, in Ecuador in 2007.\textsuperscript{23} Based on 16srRNA homology, DNA-DNA hybridization, studies on pathogenicity, the genus *Leptospira* includes 21 species which are classified
into 3 subgroups.\textsuperscript{27} The Group I and II which included the pathogens and intermediate pathogens and the noninfectious group comprising the saprophytes. As per this system, \textit{Leptospira} are at present grouped into 9 pathogenic species and 6 saprophytic species. In addition, 5 intermediate species have also been identified.\textsuperscript{28}

The pathogens have been classified into more than 225 serotypes. They cause disease ranging from mild to severe illness even leading to death. The pathogenic species included \textit{Leptospira interrogans}, \textit{Leptospira kirschneri}, \textit{Leptospira borgpetersenii}, \textit{Leptospira santarosai}, \textit{Leptospira noguchii}, \textit{Leptospira weilii}, \textit{Leptospira alexanderi} and \textit{Leptospira alstoni}\textsuperscript{23}. \textit{L. interrogans}, \textit{L. borgpetersenii} and \textit{L. kirschneri} are the main pathogenic species of leptospirosis in humans and animals worldwide.\textsuperscript{28} The saprophytic \textit{Leptospira} include the following species: \textit{Leptospira biflexa}, \textit{Leptospira wolbachii}, \textit{Leptospira kmetyi}, \textit{Leptospira meyeri}, \textit{Leptospira vanthelii}, \textit{Leptospira terpstrea} and \textit{Leptospira yanagawae}. The classification based on DNA homology was found to be useful in clearer identification of the serotypes.\textsuperscript{23}

**Morphology**

They are thin, long spirochetes, measuring between 6 \( \mu \text{m} \) and 20 \( \mu \text{m} \) in length and 0.1 \( \mu \text{m} \) in diameter, tightly coiled, and actively motile. The organism consists of a tightly and regularly wound filament tapering at both ends.\textsuperscript{29} One or both ends are hooked like umbrella handles or like a question mark.\textsuperscript{25} The hooks at both ends may be on the same or opposite side. In some \textit{Leptospira} it may be only on one side or they may appear straight.\textsuperscript{29} Pathogens are shorter than the non pathogens.\textsuperscript{23} The \textit{Leptospira} exhibit 2 types of motility, the flexion extension type and rotation about its long axis.\textsuperscript{24} The rotation can occur in both directions alternately. The cytoplasmic cylinder rolls round the endoflagella in a direction opposite to the flagella. This causes the characteristic corkscrew like movement.\textsuperscript{30} Their unique motility, narrow diameter, flexible nature and spiral shape allows them to pass through membrane filters having 0.1-0.45 \( \mu \text{m} \) pore size and they can also migrate inside media which has been solidified using 1\% agar.\textsuperscript{24}
The ultrastructure of the *Leptospira* reveals 3 morphologically distinct structures: a spiral protoplasmic cylinder, a homogeneous axial filament (axistyle) lying external to the cylinder and terminating near the ends of the organism with a knob-like process, and a membranous sheath enveloping the organism. The sheath is 3-5 layered and is called as outer membrane or outer envelope and this encloses the protoplasmic cylinder. This is made up of a peptidoglycan layer and a cytoplasmic membrane. 2 flagella are located, one at either end and are attached to the protoplasmic cylinder in a sub terminal position and the free ends extend to the middle of the cell, without overlapping. The helical cylindrical structure and the endoflagella help the organism to burrow into the tissues. \(^{31}\) The cork screw movement of the spirochaetes is due to the endoflagella present in them. \(^{23, 31}\) The flagellar basal bodies and the surface architecture of the *Leptospira* resemble those of Gram negative bacilli. \(^{24}\) They have a higher content of long chain fatty acids, some of which are
present as lipopolysaccharide (LPS). Though similar to the LPS of Gram negative bacteria, Leptospiral LPS is not toxic or pyrogenic, but they are highly antigenic. The LPS layer is thicker in pathogenic than in saprophytic Leptospira. Variations in the LPS is the cause for the large number of different serovars and serotypes. The Leptospiral outer membrane proteins are classified into transmembrane, lipoprotein and peripheral membrane. The porin protein OmpL1 is a transmembrane protein. The Leptospiral lipoproteins (LipL) include LipL32, 36, 41, 45 and 48 are present in the outer membrane. LipL32 and LipL41 are expressed during infections. The peripheral membrane protein is the protein P31LipL45. They also differ from other spirochaetes in that they lack glycolipids and contain diaminopimelic acid instead of ornithine in its peptidoglycan.

The nuclear body is present in the cytoplasm which also contains the ribosomes, mesosomes and inclusion bodies. The genome is 4500 kb long. A circular plasmid of 350 kb is also present. The DNA base ratio of the Leptospira which are pathogenic ranges from 34.9% to 40.7% (G+C mol %).

**Staining**

As Leptospira are very thin, they cannot be visualised under the light microscope. They can be visualized only under the dark field microscope or a phase contrast microscope.

Even though they are Gram negative, they cannot take up stains by aniline dyes and can be only faintly stained by using Giemsa stain. However, Leptospires are stained by silver impregnation methods like Fontana’s stain for blood films and Levaditi’s staining for tissue sections and the Warthin Finkeldey silver impregnation methods. They stain dark brown against a yellowish brown background.

**Culture Requirements**

Leptospires are aerobic and microaerophilic. They require an optimum temperature of 28°C to 30°C and a pH of 7.2 to 7.4 for their growth. They can be grown on artificial culture media in contrast to Treponema which cannot be cultured on artificial culture media. They have unique growth requirements, the essential ones are a source of carbon, nitrogen, minerals and vitamins. Long chain fatty acids act as an important major source of carbon and also provide energy. Fatty acids are also
required by the *Leptospira* to synthesise the cellular lipids for which they form an important source. This is because the *Leptospira* cannot synthesise fatty acids. As the fatty acids are toxic, they must be made available to the leptospires, bound to albumen or in a nontoxic esterified form. Most common sources of albumen are 10% rabbit serum and 5% bovine serum. Carbohydrates do not act as a suitable carbon supply. Ammonium salts act as an important nitrogen source. Amino acids to some extent can fulfill the nitrogen requirements. They also require vitamin B1, B12 for their growth. Pyruvate enhances the growth of the parasitic *Leptospira*.¹⁸

*Leptospira* take up purine bases, but do not incorporate pyrimidine bases, into their nucleic acids. Due to this, they are resistant to the antibacterial activity of the pyrimidine analogue, 5-fluorouracil (5FU). 5 FU is an anti metabolite which is added to media, to make it selective for Leptospiral isolation from contaminated sources.²² The generation time at 30°C is 7 to 12 hours and yields 1-2 x 10⁸ cells/ml in 7 to 10 days.

**Culture Media**

Leptospires can be cultured in liquid, semisolid (0.1-0.2% agar) and in solid (0.8 -1% agar) media. The earliest medium used to culture them was the Noguchi medium consisting of ascitic fluid with rabbit kidney. Later on Vervoot developed a liquid medium buffered with phosphates and Fletcher in 1928 used a nutrient broth base with 0.2% agar. Stuart described a simple medium without peptone using phenol red as indicator, glycerol as stabilizer and L asparagine and thiamine hydrochloride as growth promoters.¹⁶ Ellinghausen and Mc Cullough discovered that *Leptospira* required a source of carbon, nitrogen, Vitamin B₂, B₁₂, inorganic salts like iron, calcium, magnesium and zinc for early growth and that these factors were present in rabbit serum, which was also a good source of bound Vitamin B₁₂. Later, Johnson and Harris discovered that bovine serum could be used instead of rabbit serum. Therefore culture media used for the isolation of *Leptospira* can be enriched by adding either 1% rabbit serum or bovine serum albumen or 1% fetal calf serum.

The commonly used culture media used are EMJH (Ellinghausen, McCullough, Johnson & Harris medium), Stuart’s medium, Korthof’s medium and
Fletcher’s medium. Chemically defined media used for culture are Shenberg’s medium & Vegel and Hunter’s medium.  

Liquid media are used for the purpose of isolation of the organism or for preparing antigens for serological tests. The appearance of generalized turbidity is indicative of positive growth in the liquid medium. Liquid media include the Korthof’s, Stuart’s, Vervoot’s media which are enriched with rabbit serum and the liquid EMJH medium enriched by bovine serum albumen.

Semisolid media contain 0.2% agar and evaporate less rapidly than liquid media. They help to maintain the virulence of fresh isolates as lesser subcultures are required. Semisolid media are also used to maintain stock cultures. They include Fletcher’s media and the EMJH media. In these media, leptospiral growth can be visualized just a few millimeters below the surface of the culture medium as as a ring of growth. This ring is called as Dinger’s ring.

Solid media contain 1% agar and these media are used for cloning purposes and for purifying contaminated cultures. In solid media, growth occurs as hemispherical rings just below the surface of the medium, in Petri dishes. They include Cox, Korthof’s and EMJH media.

Leptospiral growth occurs in 7-14 days, but can be delayed up to 4-6 weeks. So no plate or tube should be discarded as negative for Leptospiral culture before 6 weeks.

Actively growing cultures can be preserved in capillary tubes held in liquid nitrogen at -148°C with glycerol (5%v/v) or Dimethyl Sulphoxide (DMSO) as protective agents.

Bacteria can easily contaminate the Leptospiral cultures. Therefore selective media containing 50 to 100 µg/ml 5 fluorouracil or combination of 50 µg/ml of nalidixic acid, 10 µg/ml of vancomycin and 5 Units/ml of polymyxin B sulphate. Alternatively a combination of 100 µg/ml actidione, 40 µg/ml bacitracin, 250 µg/ml 5FU, 2 µg/ml neomycin sulphate or 0.2 µg/ml of polymyxin B sulphate and 10 µg/ml rifampicin have been added to the culture media in order to prevent contamination.
Addition of 5-fluorouracil inhibits other bacteria without inhibiting the Leptospiral growth. Another method of obtaining pure cultures without contaminants is by inoculating the suspected patient’s blood intraperitoneally into guinea pigs and to collect the heart blood 10 minutes later. This is because the *Leptospira* invade the blood stream more rapidly than other bacteria. 24

*Leptospira* can also be grown on the chorioallantoic membrane of chick embryos. They can be demonstrated in the blood of the allantoic vessels, 4-5 days after inoculation.

**Resistance of Leptospira**

*Leptospira* are heat susceptible. They are killed at 50°C in 10 minutes and at 60°C in 10 seconds. They are sensitive to acid and are killed in gastric juice in 30 minutes. They are not usually viable in water or soil if its pH is less than 6.8. But can survive for days in moist conditions at pH 6.8 to 8.0. Their survival in water depends on the temperature, acidity, salinity and the amount of pollution. Pathogenic *Leptospira* are shown to survive for a long period of time when the soil is wet and in fresh water with neutral or slightly alkaline pH. But in salt water, they can survive for only a few hours and die rapidly. They are rapidly killed by bile, trypsin, hypochlorite detergents, antiseptics, disinfectants, desiccation, exposure to pH values outside the range of 6.2 to 8.0, acidic urine and non aerated sewage. 24

**Antigens**

The antigenic structure of the *Leptospira* is complex. The polysaccharide surface antigen is serovar specific. The somatic antigen is genus specific and is present in all the members of the genus. The outer membrane is made up of lipopolysaccharide and is immunogenic. Outer membrane antigens are important and they are the target of antibody and complement activity. Antibodies which develop against the outer membrane antigen are protective in nature.
Natural history and pathogenesis of leptospirosis

Leptospirosis is a zoonotic disease, which occurs in a wide range of animals. Human infection is incidental and is not necessary for maintaining the Leptospires. Humans acquire the infections through direct or by indirect means.

MODE OF TRANSMISSION

Indirect Contact

The most important method of transmission is by contact of an individual with the surrounding environment such as soil, mud, water and foodstuff that has been contaminated with virulent leptospires which have been excreted in the urine of a shedder animal host. These shedder hosts can either be a convalescent (temporary) or a chronic (reservoir) host of Leptospires. Leptospirosis is transmitted to humans when they come in contact with water contaminated with the urine of animal reservoir hosts. Entry of these leptospires into another new host may occur through the skin which has been abraded, macerated, diseased and through intact mucosa of the nose, vagina etc. Leptospira are able to penetrate abraded skin or intact mucosa and from the site of entry, they can disseminate into the bloodstream. No local inflammation is caused by the Leptospira at the site of entry. They can also enter by inhalation into the lungs as droplets or aerosols of liquid containing Leptospires.

Direct Contact

Bites by animals such as rats and mice also pose a risk of acquiring Leptospirosis. Though there is no proof that Leptospira are secreted in the saliva, they may be present in the oral cavity and there is a risk of transmission if the animal had licked its fur or genitals. Another mode of transmission among animals is by venereal contact.

Hosts

The chronic persistent carriers are the reservoir hosts of the Leptospires. In these animals they establish some type of biological equilibrium, so that the
*Leptospira* are present in the lumen of the renal convoluted tubules of the animal reservoir host, but do not cause any damage to the tubular epithelium.

An animal host may become a reservoir for one or more types of *Leptospira*. The same species of host may become infected and shed many serotypes, thereby acting as an incidental host for these serotypes. Many natural animal reservoirs of *Leptospira* are known. They are rodents such as rats and mice, hedgehogs, dogs, foxes, jackals, mongoose, skunks, bandicoots, bats, deer, rabbits and hares. Domesticated animals (dogs, cattle) can also maintain enzootic leptospirosis in their flocks, herds, kennels, and thus infection can get transmitted to other animals and human beings. 

**Spread and Growth**

*Leptospires* spread immediately from their site of entry, via the bloodstream. Non pathogenic organisms do not multiply in the body and are removed from the bloodstream, within 1-2 days of infection. Virulent organisms multiply until they are opsonised and phagocytosed. They are cleared rapidly by the reticuloendothelial system. The growth rate in vivo is equal to the generation time of 6 to 8 hours. In susceptible laboratory animals, growth occurs until a high concentration of the *Leptospira* occurs in the tissues such as blood, liver, kidney, lungs, brain and adrenal gland. In the human host, *Leptospira* have evolved ways of escaping the immune defense system. The pathogenic *Leptospira* can translocate through the cell monolayers at a faster rate than the non pathogens. This rapid rate of translocation between the cells of the mammalian hosts enables them to reach the blood stream quickly and also to spread to the other organs. The primary lesion produced in Leptospirosis is damage to the capillary endothelium leading to capillary leakage and hemorrhages. This is due to the action of a glycoprotein toxin of the organism (GLP). This leads to the extravasation of blood and the leptospires migrate into the tissues. Due to the relative local anoxia, there is ischemic damage to the kidney, liver and adrenals. The virulent *Leptospira* also enter the fibroblasts of the kidney and induce a programmed cell death. Liver cell necrosis also occurs due to ischemia and this leads to jaundice. *Leptospira* also invade the cerebrospinal fluid and the anterior
chamber of the eye. Hemorrhages can occur in any organ or tissue, especially in the muscles and subcutaneous tissue, where there is movement.

Bacterial virulence factors which aid in the pathogenesis are hyaluronidase, hemolysins and cytotoxic factors. Other important factors include attachment to the host cells and extracellular matrix components which enable the *Leptospira* to penetrate, spread and persist in mammalian host tissues.\(^{37}\)

Renal tubular necrosis leads to uremia. Liver cell necrosis leads to disordered liver function and jaundice in severe cases. In severe cases, hemoglobinuria and hemoglobin casts can be found. Hemolytic anemia occurs due to damage to the cell membrane. The membrane of the erythrocytes shows a decrease in sphingomyelin and phosphatidyl ethanolamine, these cells appear thorny and speculated.\(^{16}\) A hemolysin is produced by the serovars *pomona*, but not by others which are capable of producing severe illness. In very severe or terminal hemorrhagic illness, the pathological changes resemble those of Disseminated Intravascular coagulation. Animals which survive become renal carriers, but prolonged renal excretion after convalescence is rare in man. No toxin has been documented to be produced by *Leptospira*.

Carrier state develops 7-28 days after initial infection. Leptospires survive in the proximal renal tubules, adherent to and closely associated with the luminal border of these cells.

**Clinical Features**

The disease may present as Icteric or Anicteric forms.\(^{2}\)

**Anicteric Leptospirosis (or) Febrile Illness**\(^{2,18}\)

Leptospirosis may occur in sub-clinical form, mainly in people who are exposed to a smaller dose of infection over a period of time especially in agricultural workers, sewage workers; animal handlers etc.\(^{35}\) The anicteric type of Leptospirosis can be mild or severe. The mild anicteric Leptospirosis is associated with fever, headache and body pain. Following a 7 to 14 day incubation period, the patient develops a sudden onset of low grade fever, and headache. Anorexia and nausea may
occur. This lasts for 1 day to several days. The features may be nonspecific and so the diagnosis of Leptospirosis may not be suspected and the condition may be labeled as a viral illness.

**Icteric Leptospirosis**

The more severe illness is a biphasic febrile illness (Saddle Type), composed of 2 phases: the First or Septicemic Phase and the Second or Immune Phase.\(^ {2,16,18} \)

The septicemic phase is the period of an acute systemic infection, with large numbers of leptospires in the blood and CSF. In this phase, after a 7 to 14 day incubation period, there is an abrupt onset of rapidly increasing fever with chills and rigors, severe headache, body pain and increasing prostration. Fever, myalgia and conjunctival suffusion are the characteristic clinical features of leptospirosis.

The fever is persistent and high grade, and ranges between 100 and 105 F. Headache is severe and persistent and resistant to common analgesics. Prostration is marked and the body pain is also severe and mostly seen in the lower limbs (calves and thigh). Pain and weakness make walking difficult. The patient also has severe body pain. The pain is more in the neck, back, abdomen and upper limbs. Other common clinical features are anorexia, nausea and vomiting. Constipation may be present. When abdominal symptoms are severe, it mimics an acute abdomen, like acute appendicitis, pancreatitis or cholecystitis. During the early stage, some patients may have epistaxis. Symptoms of pulmonary involvement include chest pain, dry cough, blood stained sputum. Frank hemoptysis is rare but may be massive. Generalized lymphadenopathy, hepatomegaly and splenomegaly occur in 10 % of the patients.

CNS symptoms include restlessness, confusion, delirium, hallucinations and sometimes psychotic behavior occurs. Signs of meningeal irritation- headache, photophobia and irritability can also be seen. A transient rash may appear which can be macular, maculopapular or urticarial rash. This usually occurs on the trunk or may be localized to involve only the upper limb or shin.
The septicemic phase can last from 4 days upto 7 days, following which an afebrile period of 1 to 3 days occurs, when the patient is also asymptomatic. This is followed by a second or immune phase, in which there is a recurrence of fever, meningitis and leptospiuriuria. The immune phase is characterized by the reappearance of the earlier symptoms, as worsening after improvement or the first phase may merge and evolve into the second phase without the biphasic nature. The pyrexia is usually lower or it may be absent. Aseptic meningitis is a characteristic feature of the immune phase. Leptospirosis can also present as fever with symptoms of meningitis such as photophobia, vomiting & with signs of meningeal irritation (neck stiffness, Kernigs, & Brudzinski signs). CSF shows lymphocytic pleocytosis (0.01-1.0 x 10⁹ cells / L), raised protein (1.0 - 2.0 g/L) & normal sugar. Convulsions, focal neurological signs, myelitis, encephalitis & polyneuropathy, peripheral neuritis can also occur.¹⁶

A skin rash may occur which may be macular, maculopapular, urticarial, petechial or purpuric. Hemorrhages into the skin, mucosa, subarachnoid space can occur. The patient may develop pneumonia, pulmonary hemorrhage and jaundice at this stage.

**Severe illness with Hepatorenal Failure**

The septicemic phase worsens to a serious illness with jaundice and renal failure, instead of subsiding, in some patients. The fever may fall slightly, but the biphasic course is rare. The clinical outcome is worse if the period of anemia is longer and the jaundice is deeper. Anorexia, vomiting & hiccups may occur due to uremia. By the end of the 2nd week, the patient develops severe jaundice, anemia and has hemorrhagic tendencies and becomes comatose. This syndrome is also called Weil’s disease. Renal failure is the cause of death, which occurs in the 3rd week, other causes being arrhythmias, cardiac failure, adrenal hemorrhage or a massive bleed from the gastrointestinal or respiratory tract. Weil’s disease with pulmonary hemorrhage has a fatality rate of more than 10%.³⁵

 Patients, in whom the illness is not very severe start recovering by the 2nd or 3rd week. The patient begins to diurese & the blood urea drops gradually. Fever subsides, general condition improves.
The strain of *Leptospira*, the serotype, the host species and the host’s general condition all determine the disease severity. Some Leptospiral serotypes cause severe and fatal illness. Weil’s disease is mainly caused by the serovar *Leptospira icterohaemorrhagiae*. Other species such as *Leptospira australis*, *L.pyrogenes*, *L bataviae* cause a moderately severe illness. Others like *L.canicola*, *L.ballum*, *L.grippotyphosa*, *L.hebdomadis*, *L.pomona* cause a mild and anicteric illness in man. Aseptic meningitis is frequently caused by *L.canicola*.²

Toxins produced by *Leptospira* include Leptospiral lipopolysaccharide (LPS) which exhibits endotoxic activity.¹⁸ Even though Leptospiral LPS is similar to Gram negative bacterial Lipoplysacharide, its toxicity is 10 times less. But it can still activate macrophages and behaves as a B cell mitogen.³⁸ Hemolysin is produced by serovar *pomona*, *ballum*, *hardjo* and *tarassovi*. The nature of this hemolysin is sphingomyelinase. Virulent strains have demonstrated chemotaxis to hemoglobin. Serovar canicola has phospholipidase C activity. Serovars *pomona* and *copenhagenii* produce a cytotoxin.

The general condition of the host may also affect the severity of the illness - immunocompromised patients and those with pre existing liver or kidney dysfunction tend to suffer from a more severe form of the disease.

**Complications of Leptospirosis**

**Renal:** Involvement of the kidneys is a common and grave complication and is also a common cause of death in Leptospirosis patients. The renal complications range from abnormal urine analysis to acute renal failure due to acute tubular necrosis.²

Proteinuria occurs in all patients. Pyuria and hematuria also occur. Decreased urinary output and anuria are usually seen in the 2nd week, but may occur earlier, even on the 4th day of the illness. With prolonged oliguria, uremic symptoms occur and worsen the patient’s condition. Renal failure occurs due to acute tubular necrosis as a result of renal ischemia, due to a direct toxic effect of the *Leptospira*.
Ocular complications include conjunctival injection associated with photophobia and conjunctival hemorrhage. Complications occurring late are iritis, iridocyclitis and rarely chorioretinitis. Unilateral or bilateral uveitis may occur as early as the 2\textsuperscript{nd} week or late up to 6 months to a year.\textsuperscript{16}

Liver: Jaundice is an indicator of the severity of Leptospirosis and it appears between the 4\textsuperscript{th} and the 6\textsuperscript{th} day of the fever, but may appear earlier or later. It deepens rapidly and peaks in 1 week. Hepatocellular damage is the cause of icterus. Mild hepatocellular necrosis is present and in addition to this, the other factors which contribute to the jaundice are increased bilirubin load due the tissue hemorrhage, intravascular hemolysis, intrahepatic cholestasis and decreased bilirubin excretion. The liver enzymes Aspartate transaminases (AST or SGPT), Alanine Transaminase (ALT or SGOT), Gamma Glutamyl Transferase (GGT) are elevated. There is usually complete recovery and death due to hepatic failure is rare.

Cardiac: Mild cardiac abnormalities occur in Leptospirosis and return to normal within 2 to 3 weeks. They include ECG abnormalities such as low voltage tracing, ST and T wave changes, conduction defects and arrhythmias. Atrial fibrillation is a common arrhythmia. Cardiac failure is rare.

Pulmonary: Hemorrhagic pneumonia may occur in the 2\textsuperscript{nd} week or even earlier. This is accompanied by chest pain, hemoptysis, respiratory distress and cyanosis. Chest X Ray shows multiple infiltrates or a single patch of consolidation. Lung changes clear in two weeks.\textsuperscript{16}

Vascular: Bleeding is a common feature due to the vascular damage and it is common in severe leptospirosis. Hemorrhages can occur into the respiratory tract, gastrointestinal tract and into the kidneys. Occasionally hemorrhage occurs into the subarachnoid space and adrenals. Damage to the erythrocyte membrane causes hemolytic anemia.
Recovery from infection

Recovery from infection occurs once the lytic and opsonic antibodies appear and the phagocytes clear the *Leptospira* from the blood and tissues. But the *Leptospira* continue to survive in the kidneys, anterior chamber of the eye and brain in some species. The infecting dose of *Leptospira*, multiplication, number of leptospires in the tissues and the extent of vital organ damage determine the rate of recovery. Another factor determining the disease severity includes the host’s immune competence, especially the rate at which the IgM antibody responds.

Immunological Response

Antibodies appear between 2 and 12 days after the onset of symptoms. As in other infections, Leptospiral IgM antibodies are produced first, followed by the appearance of IgG antibodies, which persist for a longer period of time. IgM appears first and is seen early in the disease course. IgM will be detectable within 7 days or may appear as early as the 3rd to 4th day of illness. IgM antibody peak occurs by about 3-4 weeks and start declining slowly by 3-6 months and become undetectable. In an ELISA based study by Silva *et al.*, IgM antibodies could be detected as early as the 2nd day of the illness, in a small percentage of the study population. IgG response is usually higher than the IgM response & lasts for several months. Specific IgG antibodies appear later than IgM. They attain peak levels after few weeks of illness and drop off to low level and become undetectable after months to years. Silva *et al.* demonstrated that IgG were detectable by the 7th day of the illness and persisted even after 12 months. Genus specific antibodies decline more rapidly than serovar specific or homologous antibody. IgA response is irregular & delayed. IgA Anti Leptospiral antibodies could be detected from the 5th day of the onset of symptoms. Though the immune response is effective in clearing the organisms it may also produce inflammatory reactions. Cell mediated immunity also participates in response to Leptospiral invasion. Lymphocytes have an important role to play in eliminating the leptospires from the renal interstitium.
Diagnosis

In view of the heterogeneous clinical presentations of the disease, clinical diagnosis is not easy. However, clinical presentation can be interpreted by taking into account the information about the epidemiological factors and level of endemicity to arrive at a provisional diagnosis, which has to be confirmed by laboratory investigations.

As per the WHO Guidelines on the management of Leptospirosis, a clinically suspected case is defined as suffering from an acute episode of fever accompanied by headache, generalized body ache and associated with any of the following:

- Muscle tenderness
- Cough, haemoptysis and breathlessness
- Conjunctival suffusion
- Meningism
- Jaundice
- Oliguria and/or Proteinuria
- Haemorrhages including sub-conjunctival haemorrhage
- Cardiac arrhythmia

**Confirmed case:** Isolation of leptospires from blood or other clinical specimens or positivity by PCR is a confirmatory evidence of Leptospiral infection. Demonstration of seroconversion or an increase in titre in Microscopic Agglutination Test (MAT) to four times its initial level is also considered as confirmatory evidence. Demonstration of IgM antibodies using ELISA based tests can be considered as an indication of acute infection.

Differential Diagnosis

The differential diagnosis of leptospirosis is as follows:

- Influenza
- Malaria
- Dengue Haemorrhagic Fever
- Viral Hepatitis
- Hanta Virus infection
- Enteric fever
- Brucellosis
- Relapsing fever
- Lyme's disease
- Aseptic meningitis
- Pyelo-nephritis

Conventional diagnostic tests for Leptospirosis are based on the demonstration of *Leptospira* in clinical samples such as blood or urine or isolating the organisms in culture or demonstrating the host’s immune response suggestive of current Leptospiral infection.\(^{40}\)

**Laboratory Diagnosis**

Leptospirosis is overlooked and consequently under diagnosed and under reported in many parts of the world. Its clinical presentation is diverse, nonspecific, misdiagnosis is frequent, and diagnosis is based upon laboratory results.\(^{20}\)

Early diagnosis and initiation of appropriate treatment can help in preventing complications and reducing the mortality.\(^{40}\) For accurate diagnosis, selection of the appropriate sample and timing of sample collection is important. *Leptospira* are present in the patient’s blood during the first week of the disease and is present in the urine and other organs between the second and fourth week. Antibodies appear by the 3\(^{rd}\) or the 4\(^{th}\) day of the illness and are initially predominantly IgM in nature. IgM titre rises by four fold and then starts declining by the third month onwards. IgG antibodies appear later and last longer.\(^{18}\)

**Sample collection:** Blood for culture is collected in the first week of the fever, in a sterile container with either sodium oxalate or liquoid as the anticoagulant. Sodium citrate is toxic for the leptospires and should not be used. Leptospires are excreted in the urine by the beginning of the second week and thus can be
demonstrated at that time. Urine for Leptospiral culture has to be collected in a sterile container and then alkali is added immediately after collection.

Blood for serological tests is collected without anticoagulant towards the end of the first week and a second sample is repeated, seven to ten days later for confirmation in case the serological titres are low.

For molecular diagnostic techniques, serum was reported to be a better sample as when compared to whole blood and plasma was better as when compared to serum. Buffy coat is also reported to be superior to serum and plasma. Heparin was found to be inhibitory to PCR.²⁰

Non specific hematological changes occurring in leptospirosis include leukocytosis, thrombocytopenia and elevated ESR.¹¹,²⁰ The most common hematological change in Leptospirosis is thrombocytopenia, which occurs in 40% to 86.65% of the patients.⁴¹ A study conducted by Turgut et al showed that 87.8% of the patients with Leptospirosis had thrombocytopenia.⁴¹

Liver function tests demonstrate the elevation of enzymes such as aminotransferases and alkaline phosphatase and serum bilirubin. Urine examination shows proteinuria, pyuria and microscopic hematuria. Hyaline casts and granular casts may also be present in the urine. CSF may also show initial increase in polymorphs, but later the lymphocytes may be relatively increased.¹¹

The laboratory tests which are used for the diagnosis of the leptospirosis use either of these methods:

- Direct Microscopy
- Culture
- Antigen Demonstration
- Serology
- Molecular Methods
The tests used in the different phases of the disease are tabulated as follows:

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Demonstration of Leptospires or their products includes the following:

1. **DIRECT MICROSCOPY**

   A. **Dark Field Microscopy (DFM)**

   This is used as a rapid diagnostic tool in early infection and is used to identify *Leptospira* present in the blood or urine of patients.\(^{11}\) DFM is also very useful for checking the Leptospiral culture, where their numbers may be numerous. It is also used in MAT test.\(^2\) However DFM is not 100% reliable.

   This test is technically demanding and if the leptospires are present only in small numbers, they can be missed.\(^2\) Approximately 10\(^7\) Leptospires/L of blood are required for 1 Leptospiral cell to be visible under the DFM per field.\(^{11,\ 20}\) The positivity of dark field microscopy decreases from 100% to 90.9% when the infection is present for more than 1 week.\(^{42}\)
Sometimes, artefacts such as fibrin threads can be mistaken for Leptospires, and can lead to false positives. DFM lacks sensitivity and specificity.\textsuperscript{2} Therefore DFM is a good tool only in the hands of an experienced person.\textsuperscript{2}

Liquoid 1% (sodium polyanethol sulphonate) can be used as an anticoagulant to get plasma from the blood. Double centrifugation helps to concentrate and increase the number of organisms. This involves low speed centrifugation at 1000g per minute for 10 minutes to separate the cellular components and then centrifugation at high speed at 3000g to 4000g per minute to concentrate the Leptospires.

Under the DFM, \textit{Leptospira} can be seen as thin, coiled and rapidly moving organisms in fluids such as blood, urine or culture media.\textsuperscript{2} By observing the typical morphology of the Leptospires (hooked ends, primary and secondary coils) with their characteristic cork screw motility it is possible to differentiate leptospires from the artefacts such as fibrils and extrusions from the cellular elements and fibrin threads. In case of doubts the film should be compared with the film of normal plasma and with one of a Leptospiral culture.\textsuperscript{2} The lower limit of detection (LLOD) is defined as one or two leptospires per counting chamber field (2 × 10\textsuperscript{3} /ml).\textsuperscript{43}

DFM is not recommended as a single diagnostic procedure & should be confirmed by culture or serological methods.\textsuperscript{18}

\textbf{B. Staining Techniques}

\textit{Leptospira} cannot be stained by conventional Gram staining. Staining methods available are: Immunofluorescence, Immuno-peroxidase and silver staining, Warthin-Starry staining, immunohistochemistry and insitu hybridization. But all these techniques have the same disadvantage as DFM: they can produce both false positive and false negative results.\textsuperscript{20} Silver impregnation methods have been used for detecting leptospires in tissues and body fluids. Fontana's method is used to demonstrate Leptospires in blood films. Warthin –Starry technique and Levaditi's method are used for demonstration of \textit{Leptospira} in the tissue sections.\textsuperscript{11} In this stains spirochetes appear brownish black against a yellowish brown background. Staining techniques enable us to directly observe the presence of leptospires in the blood or tissues.\textsuperscript{11}
2. **Immunofluorescence**

Direct Fluorescent Antibody Test: This test is conducted with fluorescein conjugated immunoglobulins in rabbits immunised with specific serovars. In Direct Fluorescent Antibody test, reactions are more pronounced with homologous or serologically closely related serovars and are less intense or negative with heterologous serovars. Many of the technical difficulties of direct fluorescent antibody test can be overcome by using Indirect Immunofluorescence Test.

3. **Culture of Leptospires**

Isolation of the organism in culture, from the patient’s samples is a definitive diagnosis of Leptospirosis. Further the isolate can also be used to identify the prevalent serovars. The blood for culture is ideally collected in the first week of the disease and before the administration of antibiotics. Samples which are collected for Leptospiral culture have to be stored and transported at ambient temperatures. Due to the problem of contamination the samples should be inoculated into the culture media under strict aseptic conditions.

Blood and CSF can be cultured early in the disease (first week) while urine and tissue biopsies are usually cultured later (second to fourth week) in the course of the disease. One or two drops of the patient’s blood is collected and inoculated into 5 ml to 6 ml of liquid or semisolid medium (EMJH) in a screw capped bottle. If blood is added in larger volumes it may be inhibitory to the growth of the leptospires. The culture should be incubated at 28°C to 30°C and examined every week under the dark field microscope, to look for the growth of leptospires. Most Leptospires are detected in culture after 6 to 14 days of incubation. As they are slow growing organisms, cultures should be declared as negative only if no leptospires can be demonstrated after six weeks of incubation. The *Leptospira* grow slowly and the positivity by culture is low. Most of the peripheral hospitals may not have the necessary resources and expertise for the isolation and identification of Leptospires.

Urine can be used for culture from the end of the first week, in suspected patients. As the infected patients shed the organisms intermittently up to 4-6 weeks, repeated samples should be tested. Inoculation should be done immediately after...
collection of the specimen as the leptospires do not survive in acid urine for more than a few hours. Alkalisation of the urine after collection can yield better isolation rates. As undiluted urine may contain growth inhibiting substances, both undiluted urine and a tenfold dilution of urine are recommended for culture. Addition of 5 fluorouracil (5FU) prevents the growth of contaminants.\textsuperscript{18,24} Other samples for culture are CSF, body fluids and tissues.

Although isolation of \textit{Leptospira} by culture confirms the diagnosis of Leptospirosis, it is not commonly used as it is a very strenuous, complicated, expensive and technically demanding technique.\textsuperscript{3} Further sensitivity of culture is low when compared to PCR and serology.\textsuperscript{3} Culture is also time consuming, requiring prolonged incubation (4 to 6 weeks of incubation are required before declaring the sample as negative.\textsuperscript{11} Laboratories with Level 2 Biosafety are required to isolate and to maintain the cultures.

4. \textbf{Animal Inoculation}

This procedure is rarely followed for isolation of leptospires from aseptically obtained specimens like blood or CSF. In specimens like urine which can contain contaminants, animal inoculation is useful. Guinea pigs and hamsters are preferred. They are inoculated by the intraperitoneal route. The patient’s blood sample can also be inoculated intraperitoneally. In the case of \textit{icterohemorrhagiae}, a virulent serotype, the animals develop fever and die in 8 to 12 days, with jaundice and hemorrhages into the lungs and serous cavities.\textsuperscript{24} The organisms can be demonstrated from the heart blood and peritoneal cavity after 3 to 7 days, by microscopy and confirmed by culture.\textsuperscript{24}

5. \textbf{Antigen Detection}

Various antigen detection tests have been developed, but most of them have a low percentage of sensitivity.\textsuperscript{20} Radioimmunoassay, ELISA, Chemiluminescent Immuno Assay (CLIA) and Staphylococcal coagglutination test have been described in other studies. They are more specific than DFM and can detect \(10^4\) to \(10^5\) \textit{Leptospira} / ml of blood.\textsuperscript{11} These immunological methods use fluorescein peroxidase, gold labelled antibodies.
6. Molecular Methods

Alternate methods of early diagnosis rely on DNA based techniques, of which PCR is a popular method.\(^{40}\)

a. Probe hybridization

In clinical samples, Leptospiral DNA can be detected by using the dot blotting and insitu hybridization techniques.\(^{11}\) The earliest hybridization method was developed by Terpstra in 1986 to diagnose of leptospirosis early using DNA containing\(^{32}\)P and biotin labeled probes.\(^{40}\) Here the nucleic acid containing a specific sequence of interest was isolated and then cleaved. This was labeled with a reporter which is either a radioactive molecule (\(^{32}\)P or \(^{35}\)S) or a non radioactive (Biotin or digoxigenin) molecule. This labeled single stranded DNA is then hybridised to the single stranded DNA which is either present in tissues (insitu hybridisation). DNA can also be hybridized to DNA present on paper (southern blot hybridisation) or in a solution (solution hybridisation).

If the sequence of the nucleotide in the probes is complementary to the nucleotide sequence present in the patient’s sample, hybridisation takes place and this leads to the formation of double stranded DNA. This hybridisation can be detected by autoradiography for radio labeled probes or by the colorimetric method for non radio labeled probes. The sensitivity of \(^{32}\)P – labeled probes is around 1000 leptospires/ml.\(^{44}\) This sensitivity is less than that of PCR. The main disadvantage of DNA hybridization is that it uses radio-active isotopes as a label, therefore requiring well equipped Laboratories with special safety facilities. Alternatively, assays which make use of biotin-labeled DNA hybridization probes can be used.\(^{44}\)

b. Nucleic Acid amplification methods: Polymerase Chain Reaction (PCR)

PCR detects DNA sequences specific to the infecting agents and amplifies them exponentially in multiple cycles.\(^{40}\) It can be used to rapidly diagnose many infectious diseases and also for disease surveillance.\(^{40}\) Basically the technique consists of in vitro amplification of target DNA sequences present in the sample, by a chain of polymerization reactions catalysed out by an enzyme DNA polymerase which is
thermostable, and making use of a pair of short DNA fragments (primers) which are designed to bind specifically to the sequence of interest. The amplified DNA produced at the end of this reaction is easily visualised on agarose gel which has been stained with ethidium bromide using a UV transilluminator. PCR and Probe hybridization can be used for the early diagnosis and assays for detecting Leptospiral DNA in blood, serum, CSF and aqueous humour have been developed. Molecular techniques can detect as low as one to ten leptospires per ml, even if only a few Leptospira are present in a clinical sample. In 50 to 90% of the samples (blood, urine and aqueous humour), Leptospiral DNA fragments may be amplified depending on several factors, such as design of the primers, stage of the disease at the time of sample collection and analysis. The advantage of performing PCR on blood samples is that the test rapidly confirms the diagnosis of Leptospirosis during the early phase; even before antibody titers reach detectable levels. PCR can detect Leptospiral DNA in blood during the first 5 to 10 days after the onset of the disease and up to the 15th day. The load of Leptospira in the patient’s blood sample can vary between $10^5$ and $10^9$ leptospires/L. PCR can detect leptospires even if culture of the blood sample is negative and even if the patient has received antibiotics, provided he/she has not cleared the dead Leptospira from the blood.

A disadvantage of PCR is that it cannot detect Leptospira which are present below the detection level. PCR is also expensive and this cost factor may hinder its use in the smaller labs. Another limitation of PCR is that the infecting serovar cannot be identified. This may not affect patient management but may be significant in epidemiological and public health related issues. As the genus diversity of Leptospira has expanded, and new species have been added to this genus, the PCR assays may not pick out the recently added species. Another critical step in PCR is the extraction of DNA from the clinical specimen. The concentration of bacteria is less in serum than fresh blood. Some of the studies comparing PCR and IgM ELISA have reported that PCR, when used alone for the diagnosis of leptospirosis was less sensitive than serological tests, but when used in combination with the MAT & ELISA, it could detect more number of cases. PCR is a supportive tool to serological testing and it complements serodiagnosis, as it is sensitive in the first 5 days after onset of the illness. PCR can be designed and used to amplify target DNA and depending on the
primers used, various levels of specificity can be set up and can therefore be used for the simultaneous detection and characterization of the *Leptospira*.44

Another advantage of PCR is that even those patients for whom antibiotics have been started already can be tested. Blood collection systems using lithium heparin interfered with the sensitivity of PCR.46

Several primer pairs have been described for the diagnosis of Leptospirosis. PCR primers are designed for the detection of universal bacterial genes present in bacteria as gyr B, 32 rrs (16S rRNA gene), 33 secY34; or genes which are present only in pathogenic *Leptospira* spp. such as lipL32, lfb1, 35 ligA, and ligB2.20

Van Eys *et al* detected *Leptospira* in the urine of cattle by PCR which could detect 10 leptospires/ ml of urine.

Gravekamp in 1993 derived and designed 2 primer sets G1&G2 and B64 I& II from the genomic DNA library of the *Leptospira*. G1 and G2 amplify a 285 base pair sequence which is present in all the *Leptospira* except *L kirschneri*. B64 I& II amplify a 563 bp sequence present only in *L.kirschneri*.40, 47, 48

Madhurima Roy *et al* in their study have shown that ribosomal gene based (16S and 23S rDNA) PCRs could detect 10 leptospires per ml and that the results were reproducible.49 *omp* gene based (*lipl32, lipl21 and ompl1*) PCRs could detect $10^3$ leptospires/ml of blood sample.

c. **Real time PCR**

Conventional PCR has now being replaced by Real - time quantitative PCR (qPCR). This is a PCR based DNA amplification technique that is monitored during the amplification process.44 In real time PCR, the amplification and the detection of the amplified product occurs in the same reaction vessel. This type of PCR has excellent sensitivity and specificity. The risk of contamination is also low. Detection of PCR products is done by the use of SYBR green, which is sensitive but less specific than the Taqman probes which are based on fluorescent technology. The qPCRs which have been introduced are the SYBR Green qPCR which targets secY or
lipL32; TaqMan qPCR uses lipL32; rss (16S) as the target. A multiplex PCR assay which can simultaneously detect and differentiate pathogenic and nonpathogenic leptospires has also been developed.20

d. Arbitrarily primed PCR

This method uses a single arbitrary primer in a PCR to amplify segments of the genomic DNA. This can produce a highly diverse banding pattern among the different species and in the same species.

e. Characterisation of the Leptospira

Several DNA based techniques help us to study the genetic makeup of the *Leptospira*. They have also helped us to classify the *Leptospira* based on the DNA similarities.40

*Leptospira* spp. is classified into DNA homology categories based on DNA-DNA hybridization technique as the gold standard.44 But DNA hybridization technique is complex and requires the use of high quality isotope labeled DNA. So it is used only in laboratories having the facility to handle radio isotopes. Subsequently, other molecular techniques such as single or multilocus sequence analysis are becoming important molecular tools used to speciate the *Leptospira*.44

The techniques such as restriction endonuclease analysis (REA), ribotyping, random amplified polymorphic DNA (RAPD) fingerprinting, arbitrarily primed PCR (AP-PCR), pulsed field gel electrophoresis (PFGE), ribotyping and DNA sequencing are useful for characterization.40 These methods are also useful in typing the *Leptospira* into different Serovars.2, 44

f. DNA sequencing

Analysis of the nucleic acid strand arrangement can be very useful to identify similarities and differences between bacteria.
7. **Serological Methods**

Antibody detection is the mainstay in the diagnosis of leptospirosis. The various serological methods available for use are:

I) **Genus specific tests** (2)

a) Macroscopic slide agglutination test (MSAT)

b) IgM ELISA

c) Complement fixation test (CFT)

d) Latex Agglutination Test (LAT)

e) Indirect haemagglutination test (IHA)

f) Indirect fluorescent antibody test (IFAT)

g) Lepto-Dipstick

h) Counter immuno electrophoresis (CIE)

i) Micro Capsule Agglutination test (MCAT)

A. **Macroscopic Slide Agglutination Test (MSAT)**

Many rapid slide agglutination tests have been used satisfactorily. The Patoc Slide Agglutination test was the initial test which was developed by Mazzonelli and Maillloux was later modified by Coghlan. This test used *Patoc* (non pathogenic strain) strain as a single antigen. This test was also reported as being sensitive.\(^{16, 50}\) Galton *et al* performed the slide agglutination test using nine Leptospiral cultures which he further divided into 3 groups. It is a simple, quick and reliable test to diagnose leptospirosis at the genus level. It is a valuable and useful test. It is an ideal screening test in determining whether a patient is likely to be suffering from acute Leptospirosis, irrespective of the infecting strains. It becomes negative or weak on recovery from infection. Further, to perform this test, no sophisticated equipments, conjugates and substrates are required.\(^{51}\)

This test methodology is similar to that of other slide tests such as those used for diagnosis of Brucellosis, Salmonellosis or VDRL cardiolipin test for syphilis. A specified quantity of concentrated killed antigen is mixed with the patient’s serum on a slide or a card and this mixture is allowed to react for a specified period, following
which the presence of agglutination is looked for. The antigen can be prepared in the
laboratory or commercially available.\textsuperscript{16}

Antigens used for Slide agglutination tests are usually formalin killed suspensions of \textit{Leptospira}. Antigens killed by thiomersal or by heating in a boiling water bath have also been used. The antigen may be stained for better contrast.

The antigens used in macroscopic slide agglutination test appear to react with a wider range of different antisera. Those antigens prepared from strains of \textit{Leptospira biflexa} species such as \textit{Patoc I}, show a broad genus specific coverage with human sera.

The test may be negative in the first 2 or 3 days of infection. Similarly at the height of infection, diluted serum samples should also be tested as this may give a false positive due to prozone phenomenon. The slide test reveals antibodies in the acute phase up to 1-2 years after infection, where as the MAT test reveals any residual antibodies for many months and longer (up to 20 years) after contracting the disease.

\section*{B. Enzyme Linked Immunosorbent Assay (ELISA)}

This widely used test can detect Leptospiral antibodies of both IgM and IgG classes separately. ELISA kits are commercially available. Here, the pooled Leptospiral antigens are coated in the microtitrewells. To this test serum samples are added after the prescribed dilution. This plate is then incubated for half an hour at 37$^\circ$C. The microplate wells are then washed with wash buffer for a specified number of times. Later anti-human IgG or IgM which has been conjugated to an enzyme is added and then incubated. This is followed by another washing procedure. Then the substrate is added and again incubated. A color reaction occurs during the incubation period, when the substrate is hydrolysed. Then the stop solution is added, owing to which a color change occurs. The intensity of the color is read using a spectrophotometer. The results are read as either positive or negative. The test is always performed using positive and negative controls. These tests have largely replaced the \textit{Patoc} antigen tests as the genus specific tests.
The ELISA test detects IgM antibodies which appear in the blood about 1 day earlier than MAT antibodies. Usually IgM ELISA and MAT do not show a good correlation. The greatest limitation of IgM ELISA is that the IgM antibodies can persist for many months after an infection. Therefore a query is raised as to the interpretation of a positive IgM ELISA result. Another disadvantage is that the IgM antibodies may be negative very early in the disease and may give false negative results.

The advantages of IgM ELISA are that it can detect IgM antibodies earlier than the detection of antibodies by Microscopic Agglutination Test. Further the antigen preparation is single and uniform. Further, the ELISA kit incorporates heat stable antigens thereby making the antigen stable at room temperature for long periods. Many samples can be processed rapidly using this. The disadvantage of ELISA is that the test cannot identify the infecting serovar. Also the calibration of cut off value and knowledge of the significant titre are required. This technique is also comparatively less specific.

Other Diagnostic tests which are available for the serological diagnosis of Leptospirosis are Complement Fixation Test, Latex Agglutination Test, Hemagglutination test, Immunofluorescence Test and Dipstick Immunoassay.

II) Serogroup/Serovar Specific Tests

A. Microscopic Agglutination Test (MAT)

MAT is considered as the gold standard test in diagnosing leptospirosis. This serological reference test was first described in 1918 by Martin and Pettit. The Microscopic Agglutination Test (MAT), which uses a panel of live Leptospiral antigens, has been the cornerstone of the serological diagnosis of leptospirosis. In this test a given volume of live antigen from Leptospiral culture is mixed with an equal volume of serial dilutions of the patient's serum in the wells of a plastic hemagglutination plate or microtitre plate. The serum-antigen mixtures are allowed to react during a period of 1 ½ to 2 hours at room temperature (20° to 30°C). After this incubation time the percentage of agglutination and the end point titre can be determined by examining a sample of the serum-antigen mixture by dark field.
microscopy. The amount of agglutination may be hard to evaluate. The highest
dilution of the serum where there is a 50% agglutination of the Leptospires is taken as
the endpoint titre.\(^2\)

For performing the test, 4-14 day old cultures which have been incubated at
30\(^{0}\)C are used. The density of the culture used should be 1-2 x 10^8 leptospires per ml.
The antigens should not contain “breeding nests” which are clumps or conglomerates
of Leptospires. Live cultures or cultures killed with formalin can be used, but live
antigens are more sensitive than formalin killed antigens.\(^2\)

**Formalinized antigens**

The live Leptospiral cultures which were killed by adding 0.5% formaldehyde
were also used as antigens. These antigens were found to be stable for at least two
months. But the disadvantage of using this killed antigen is that the leptospires
adhere loosely to one another and this can interfere with the interpretation of the
agglutination reaction. Cultures which had grown well and having a minimum density
of 2-3 x 10^8 leptospires per ml were suitable for use as an antigen. The density
can be measured by direct counting, spectrophotometry or by McFarland's
turbidity scale.

The MAT test is a serovar specific test. For testing human sera, one or more
strains of saprophytic serovars (Patoc I) should be included. They tend react as genus
specific antigens and agglutinate with human antibodies produced against other
infecting serovars. In addition to the saprophytic strain the patient's serum should also
be tested against a battery of serovars, known to be endemic to that area. Though
MAT is a serovar/serogroup specific test, cross-reactivity with other Serovars can
occur at times. The MAT test is very sensitive and specific. Further an infected
individual will remain MAT positive for years. So this can be made use of
for epidemiological studies.

A titre of >1/100 or more is considered be positive.\(^{16, 20, 50}\) A single high titre
or a fourfold rise in antibody titer demonstrated by testing paired serum samples
collected about 10-15 days apart is considered evidence of current Leptospiral
infection. Rise in MAT titres is considered to be a definitive criterion in the diagnosis
of leptospirosis. Therefore testing a repeat sample is important but it may be difficult to follow up the patient and collect a second sample. When using single serum samples it is necessary to calibrate the significant titre. False negatives can occur during the early phase of the disease.

The test has several drawbacks that prevent it from being used as a routine diagnostic test for leptospirosis. The procedure is complicated and time consuming. Many reference strains of leptospires have to be maintained in culture for use as antigens in the test. This also poses a threat to the laboratory safety, as the technicians handling the culture may get infected during the process of sub culturing the strains. Selection of the panel of antigens is crucial and requires an accurate knowledge about the locally circulating serovars. Moreover in a single MAT test, the significant titer varies from one geographical area to another. There is a high possibility of inter-laboratory variations in readings of the results. Since live antigens are used for this test, MAT cannot be standardized. MAT may be negative when the infecting strain is not represented in the panel. Sometimes the MAT titres may be low, when the infecting serovar resembles the serovar in the panel. The panel may not always be complete and capable of detecting infections by all Serovars, as new or unidentified species of Leptospira can cause the disease.

Another difficulty is in determining the degree of agglutination, which can be assessed by determining the number of free non-agglutinable leptospires. Hence variations can occur between personnel while reading and interpreting the test results. Reading results requires experienced personnel.

MAT detects both IgG and IgM antibodies and cannot differentiate between the present infection and previous infection.

Even if the MAT test is serovar specific, cross reactions between different serovars are observed. These cross reacting antibodies may appear first. This paradoxical reaction is seen when the patient's serum is obtained during the second or third week after the onset of the disease. But these titres drop and usually the homologous titre finally predominates. If strains of two serovars continue to react strongly during the first four weeks, a double infection might be present.
B. Serologic Typing

The basic unit or taxon in Leptospiral taxonomy and classification is the serovar. Therefore it becomes necessary to characterize and identify the unknown strain of *Leptospira* up to the serovar level. The serotyping methods available and which can be utilised are cross agglutination absorption test (CAAT), the factor sera analysis and typing based on Monoclonal antibodies. The molecular methods have already been mentioned earlier.18

Epidemiology and Prevalence of Leptospirosis

Leptospirosis occurs throughout the world but the disease is most prevalent in the tropics and subtropics which are areas where heavy rainfall occurs.2 It is a widespread zoonosis, with more than 500,000 severe human cases and 100,000 deaths per year. It is also a big economic burden on the nation as it affects both farm and pet animals every year. The reason for the higher number of cases in the tropical countries is due to the following: first of all the warm and humid climate favors the survival and multiplication of the organisms; secondly there is a close contact between the animal reservoirs and susceptible human hosts, which favor the direct transmission of *Leptospira* from animals, to man. Further in India, the practice of walking barefoot and working in the fields without protection allows indirect transmission of the *Leptospira* from the contaminated environment. Rodents (rats, mice, voles, gerbils) are very important wild animal source of human disease and a major reservoir of infection.23

Each species of *Leptospira* is usually associated with a reservoir host. *Leptospira* (L) *icterohaemorrhagiae* and *L ballum* are found in rats, *L ballum* is associated with mice, *L grippotyphosa* and *L hardjo* are seen in dairy cattle, *L pomona* and *L tarassovi* in pigs, *L pomona* and *L hardjo* in sheep and *L canicola* is usually associated with dogs.26

A wide variety of animals act as sources of human infection. Most domestic animals like cattle, sheep, goats, water buffalo, pigs, horses, dogs and cats may be infected. Wild animals can also be infected. Rabbits, deer, jackals, skunks, bandicoots, hedgehogs are also reservoirs.24 These infected animals shed large
numbers of Leptospires in their urine. Domestic animals often shed Leptospires for weeks to months following infection and wild animals can shed these organisms for more than a year. As they can remain alive in the soil and water for weeks, the level of infectivity and contamination of the environment will be very high in the areas where the animals urinate frequently.

In the tropics, Leptospirosis is a common cause of fever and numerous non-specific manifestations. The associated factors are poverty, poor hygiene and sanitation and exposure to contaminated environments.5

This disease appeared inconsequential in the past but has now gained significance as an emerging public health problem in our country. Though viewed primarily as a disease of animals, a large number of human cases occur every year throughout the world.

In India the first case of human leptospirosis was reported by Taylor and Goyal in 1929. This outbreak occurred in bund construction workers in Andaman.7 Most outbreaks of leptospirosis in India are reported from the coastal regions of the states of Gujarat, Maharashtra, West Bengal, Orissa, Kerala, Tamil Nadu, Karnataka and the Andaman Islands. Of these states, Kerala, Tamilnadu, Gujarat and Maharashtra report more than 500 cases per year.23,50 Recently West Bengal, Punjab, Haryana and Himachal Pradesh have also reported leptospirosis cases.50 The prevalence rate of Leptospirosis reported is 38.1% from Calicut, 52.7% from Andaman and Nicobar Islands and 32.9% from Chennai.53 More than 100 deaths occur per year due to leptospirosis in the states of Gujarat, Kerala and Maharashtra.

Leptospirosis occurs in any time of the year and the number of cases fluctuates with the season. In temperate climates infection is more common in the warmer months. In tropical and sub tropical regions seasonal changes in the incidence may be associated with other factors like heavy rainfall and crop raising cycles. In India the highest rates occur during October to November which coincides with the monsoon season in these parts.13
The lifestyle and environment which makes one susceptible to this disease is the same as that is seen in the developing countries. However the disease exists in most parts of the world, virtually in every place where it has been looked.\textsuperscript{16}

Human leptospirosis results from direct or indirect contact with the urine of infected animals. Other methods of infection are by handling of infected animal tissues, ingestion of contaminated food and water.\textsuperscript{2} Person to person transmission is rare.

*Leptospira* can enter the human body through breaks in the skin (even small scratches) and through intact mucosal lining of the mouth, nose, conjunctiva, vagina, etc. Persons of all age groups and genders can get the infection. It has been noted that adult men are more commonly infected, as they work in higher risk jobs. The male is to female ratio is 4:1. Peak age incidence in men is between the age group of 30 to 39 years.\textsuperscript{33} Socio-economic factors are also important in the spread of the disease. Limited sanitation facilities, lack of pipe water supply for daily use, lack of proper drainage facilities, poor abattoir and animal handling techniques, inadequate public health supervision also play a role in disease transmission.

The optimal temperature for survival in the environment is 28°C to 32°C. Tropical unpolluted non saline water with a slightly alkaline pH (7.2 to 8) provides an ideal environment for survival of these organisms. In regions of high temperature and heavy rainfall such as seen in the tropics, leptospires survive in fresh water ditches, drains, ponds, soil and mud. Flooding after the heavy rains is favourable to the Leptospires and saturates the water with these organisms, enabling them to infect cattle, rodents, or humans, coming in contact with the contaminated water.

Leptospiral infection tends to occur as single cases or a small group of. Less frequently it occurs as large outbreaks or epidemics over a limited period of time.

**Prevalent Serovars in India and neighbouring countries**

*Icterohaemorrhagiae* is the most predominant serogroup which causes epidemics and is responsible for more than 60% of Leptospirosis cases in China.\textsuperscript{28} Other prevalent strains include *L. borgpetersenii* and *L. kirschneri*. 
Sara Chandy reports that the serovars predominantly causing infections are *Leptospira icterohaemorrhagiae*, *L australis*, *L autumnalis*, and *L javanica* in Andhra Pradesh and *L icterohaemorrhagiae*, *L bataviae*, *L tarassovi*, *L canicola*, *L australis*, and *L pomona* in Maharashtra and the Serovars *L australis*, *L pyrogenes*, *L canicola*, and *L hebdomadis* have been reported from Tamil Nadu. Serovars *L copenhageni*, *L autumnalis*, *L pyrogenes*, *L grippotyphosa*, *L canicola*, *L australis*, *L javanica*, *L sejroe*, *L louisiana*, and *L pomona* have been reported from other states.\(^5\)^

**Leptospirosis as an occupational disease**

Humans can get infected by virtue of their occupation also. Thus it can also be called an "Occupational disease". Prior to the discovery of spirochetal etiology, various names have been given for this illness such as swamp fever, rice field fever, swineherd’s disease, cane cutters disease, seven days fever and autumnal fever.\(^1\)\(^6\) Most of these names are given based on the occupation associated with the disease.

The occupations at risk of contacting leptospirosis include agriculture, dairy and fish farming, forestry, mining, conservancy work, cleaning sewers, laboratory workers, nurses, veterinarians, animal handlers and abattoir workers. Those handling fish offal and the meat inspectors are also at risk of leptospirosis.

Agricultural manual workers are an important risk group. Raising wet land crops like rice is particularly hazardous. These workers work with their bare feet and hands immersed in water for a prolonged period of time. The skin changes resulting from prolonged immersion in water and small cuts and abrasions, provide portals of invasion for leptospires. The relative risk of infection for these field workers varies from area to area, depending on factors such as water pH, soil type and rodent density in the fields.

Persons involved in raising dry land crops such as sugarcane and vegetables, are also affected. With these crops the risk of infection is greatest during harvesting where the workers have considerable hand and barefoot contact with moist soil.

Persons taking care of or raising cattle may be infected by exposure to the animal urine, directly (urine splattering during milking) or indirectly (walking
barefoot in contaminated wet or muddy soil). Infection also results from helping an animal to give birth, by contact with discharges of the reproductive tract of the infected females.

Raising fish and prawns in fresh water ponds has also been associated with human Leptospirosis. Infection is acquired while wading through water during maintenance of the ponds, or while harvesting its contents.

Leptospirosis is also an occupational disease among workers in slaughter houses, poultry, and fish processing plants and meat inspectors. Rat infestation of the processing plants can contaminate the working areas during non working hours. The wet environment of the processing plants allows the Leptospires to survive and infect the workers. In the slaughter houses infection also results from contact with infected animal tissues. Contact with internal organs like liver, kidneys, urine and blood of the animals can also lead to Leptospiral infection.16, 55, 56

The overcrowded cities also provide an environment conducive for disease transmission. Here the sewage canals and drainage outlets of most of the cities are choked with vast amounts of waste generated by their unmanageable population. The underground canals are not covered properly in many places, resulting in overflow of waste water into the roads and house compounds. Also heaps of garbage found in most cities attracts a large number of rodents. In the developing countries, Leptospirosis has become an urban slum health issue.57 The habit of entering sewage canals for maintenance ark without any protective clothing and footwear puts these workers at risk. In Sewer workers in Pune the prevalence rate was 16.6%.58 Similarly the conservancy workers, the sweepers whose work includes street workers are also at risk of developing the infection.59

Veterinarians can also get infected by virtue of their occupation, while handling sick (infected) animals, their discharges etc.60 Laboratory animals may also be infected and transmit the infection to in contact laboratory personnel attending on them.

Military and Forestry personnel 61 are also included under the high risk group, along with miners.61 Miners are at risk due to the constant water seepage and cooling
water leakages in the mines, which are infested with rats. The main infectious source here is rat urine contaminating the ground water.\textsuperscript{62}

Leptospirosis is also associated with recreational activities like hunting and swimming, fishing and kayaking in waters contaminated with Leptospires.

**TREATMENT**

**Chemotherapy**

For the antibiotic treatment to be effective it has to be started within the first 1 week to 10 days of infection. Treatment should commence immediately on diagnosis or suspicion. The aim of chemotherapy is to get rid of leptospiremia and avert complications. *Leptospira* are very sensitive to many antibiotics. Penicillin is the most useful and effective antibiotic if it is started early, during the first 4-5 days of the illness. 6 to 8 million units of benzyl penicillin can be administered in divided doses, by the intravenous route, for 5-7 days.\textsuperscript{2, 16, 17} Fever subsides within 1-2 days of starting treatment. It may cause a temporary reaction, but its use should not be discontinued. Patients with penicillin hypersensitivity can be prescribed erythromycin 250 mg 4 times a day for 5 days. Doxycycline 100 mg twice daily for 10 days is also recommended.\textsuperscript{6,3} Tetracyclines are also effective but contraindicated in patients with renal insufficiency, in children and in pregnant women. Ampicillin, Amoxycillin, Moxalactam and Cefotaxime are all effective in the treatment of leptospirosis.

**Supportive Therapy**

This aims to maintain fluid and electrolyte balance. Hypovolemia and hypotension are to be treated promptly. In patients with oliguria and prerenal azotemia, prompt diuresis is attempted with fluid therapy.\textsuperscript{16, 17} In Leptospiral acute renal failure, peritoneal dialysis is a simple safe and effective procedure. Hemodialysis can also be done. Analgesics can be used to treat headache and myalgia are treated with analgesics, Antipyretics for fever, sedatives for restlessness and anxiety, blood transfusion for anemia.
PROPHYLAXIS

The chemoprophylactic agent of choice is doxycycline for short-duration and should not be used for long-term occupational exposure.\textsuperscript{2}

CONTROL AND PREVENTION

Prevention of Leptospirosis is not an easy task. Occupational hygiene should be followed, such as use of protective clothing and avoiding splash from urine or water. But these steps are hard to execute as they hamper work and are not easily accepted by both workers and employees. It is not feasible to advice dwellers in tropical villages, to avoid contact with animals and walking or working in wet conditions including in soil or water contaminated by the animal's urine. Vaccination of livestock can reduce urinary excretion and thereby reduce the risk to human handlers. Chemotherapy of livestock with didydrostreptomycin has been used but isexpensive and not practical for wide spread control.

Control of sources of infection among Domestic Animals.\textsuperscript{2}

The following measures are important in controlling Leptospirosis among the Domestic animals:

**Isolation:** Domestic animals, once identified as either diseased or subclinically infected, should be separated from uninfected animals. The infected animals are to be treated with chemotherapeutic drugs. Floors, soil, tools, which are contaminated with the urine, fetus, or fetal membranes of the animals, must be washed with disinfectant.

**Slaughter:** Under certain circumstances where the only source of enzootic infection is the continued presence of a carrier, the slaughter of these carrier animals is carried out. Carcasses of animals that have died of acute leptospirosis or have been slaughtered during the acute infection should be incinerated
Control of Source of Infection among free living animals

Environmental control

Agricultural products frequently allow the commensal and endemic rodent population to reach high densities. Such rodent populations act as sources of disease for animals and humans working in those areas.

The most effective way of preventing the rodent population is by denying access to their food and shelter. Farm buildings should be made of concrete or brick with solid foundations, to prevent burrowing. Animal feeds should be kept in rodent free buildings. Stored, harvested cereals should also be kept in rodent proof areas.

In both urban & rural environments, untidy areas aid in rodent settlements, (such as rubbish heaps, buildings, & material debris etc.). Steps should be taken to keep areas clean.

Chemical & Biological control of Rodents

Use of rodenticides is a very efficient means of urban and rural rodent control. Poisons are of two kinds: acute, quick acting & chronic poisons. Quick acting poisons include zinc phosphide & red squill, arsenious oxide, thallium sulfate, alpha naphthylourea (ANTU) and alpha chlorasol.

Chronic poisoning is done by using anticoagulant drugs like warfarins, liphendione, puval. Rats living in burrows away from buildings can be gassed by a powder that produces hydrogen cyanide, or by aluminium pellets which produce phosphine.

Biological control can be done by habitat manipulation and habitat aeration.

Control of Transmission

Disinfection of Drinking Water: Standard hygiene methods for treating of drinking water to render it microbiologically safe will kill leptospires.
Physical Protection

Those working with animals likely to be infected with leptospires, such as veterinarians, dairy farm workers, should avoid contact with infected urine and tissue fluids. All cuts and abrasions are to be kept covered with a waterproof dressing. Urine contamination of skin, particularly of face and eyes, should be immediately washed off with clean water. Wearing protective boots, gloves and irons are necessary for sewer, conservancy, and abattoir workers. Doctors and nurses should wear gloves and aprons while carrying out procedures on infected people. Technicians in the laboratories must also be careful to avoid contact with blood or urine of patients.

Immunisation

Vaccines have been shown to be effective in preventing infection in susceptible animals, provided that they are prepared from serovars which are endemic in the area where they are to be used. Polyvalent vaccines are useful, when more than one serovar is prevalent. It is a challenge for the immunologist to develop a safe and effective leptospirosis vaccine. Human vaccines need further studies on efficacy and standardization. Current vaccines focus on bacterial motility, lipopolysaccharides, lipoproteins and outer membrane proteins. The different classes of vaccines are:

Recombinant virulence factor vaccines – Fla A, Fla B, Hsp58, SphH, ChpK. Fla A and B are the components of the periplasmic flagella (PF). Hsp 58 AND SphH are important virulence factors.

Health Education

The Public must be made aware of the disease, its clinical presentation, mode of transmission and the methods of prevention. The Occupational risk groups must be made aware of the risks involved in their occupation and should be stressed upon the need for wearing protective clothing, gloves, boots, etc. Care should be taken to see that they follow these precautions. The health care personnel must also be educated about the myriad presentations of the disease and its complications, the available diagnostic methods, and of the importance of early commencement of therapy.
Many studies have been conducted to evaluate the various diagnostic tests in the diagnosis of Leptospirosis. But they compare either DFM with serology or any 2 serological techniques or MAT with PCR. This study proposes to comparatively evaluate phenotypic methods such as dark field microscopy, silver staining, serological methods such as MAT, MSAT IgM ELISA along with molecular methods such as Polymerase Chain Reaction in the diagnosis of Leptospirosis.