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LEPTOSPIROSIS: A SYSTEMATIC REVIEW

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Review

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ABSTRACT

Leptospirosis caused by *Leptospira interrogans* is considered as one of the most important zoonotic infections globally. It affects humans and wide range of animals. It damages various vital organs of the body and can often lead to fatal complications. The various symptoms of leptospirosis are fever, headache, nausea, vomiting and abdominal pain. It may lead to complications like jaundice, hemorrhage, myocarditis, meningitis and in some cases renal failure. Leptospirosis is mainly caused by rodents which are considered as reservoir hosts for leptospires. The other animals such as mammals, birds, amphibians, reptiles and fish are carriers of leptospirosis. Humans acquire leptospirosis accidently by contact with carrier animals or environment contaminated by leptospires. Leptospirosis has a wide geographical distribution including tropical, subtropical and temperate climatic regions. But it is more prevalent in the tropical areas where warm humid conditions and alkaline or neutral soil helps leptospires survive better. This review is an attempt to cover every aspect of leptospirosis in detail. The biology and culture characteristics of leptospires, classification, epidemiology, pathogenesis, host immune response, transmission cycle and different diagnostic methods have been explained in detail.

Keywords: Leptospirosis, *Leptospira interrogans*, Epidemiology, Transmission, Diagnosis

INTRODUCTION

Leptospirosis is the most important zoonosis worldwide **(Sehgal, 2000)**. Due to its infectious nature it has emerged as a matter of major concern in India and many other countries. It has become endemic in some of the South East Asian countries. It is caused by the bacteria (spirochetes) belonging to the genus *Leptospira* **(Waitkins, 1987)**. It occurs in varying environmental regions but is more prevalent in tropics where wet and humid conditions are highly favorable for its transmission. Leptospirosis affects humans and many other species of vertebrates. Human infection is caused by recreational exposure to water contaminated with *Leptospira* and therefore humans are considered to be accidental hosts whereas animals are considered reservoir hosts of *Leptospira*. A large number of clinical manifestations are associated with leptospirosis. It includes respiratory distress, pulmonary hemorrhage, meningitis and renal failure **(Bharti *et al.*, 2003)**.

MORPHOLOGY AND CHARACTERISTIC FEATURES

Leptospires are spirochetes which are highly motile and aerobic (obligate) in nature. They exhibit features common to both Gram-positive and Gram-negative bacteria **(Haake, 2000)**. The size of the cell ranges from 6-20 µm in length and about 0.1 µm in thickness. Leptospires are grown at 28ºC to 30ºC with pH in the range of 7-8 **(Bharti *et al.*, 2003)**. They can survive for many days in wet neutral or slightly alkaline soil and fresh water. Leptospires are poorly stained and therefore cannot be visualized by light microscopy. They can be visualized by dark field or phase-contrast microscopy. They can be stained best by silver impregnation techniques. Under the electron microscope leptospires appear cylindrical, wound helically around an axistyle (0·01– 0·02 µm in diameter), which is comprised of two axial filaments (a spirochetal form of a modified flagellum) inserted sub-terminally at the extremities of the cell body, with their free ends directed towards the middle of the cell **(Hovind-Hougen, 1976)**. A scanning electron microscopic (SEM) image of *Leptospira* is shown in Figure 1.

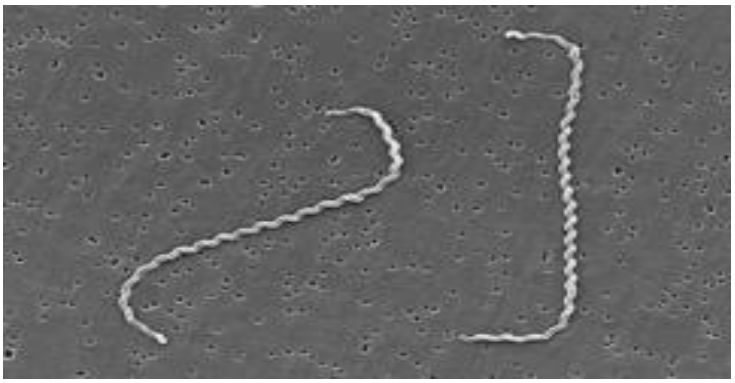

Figure 1 Scanning Electron Microscopy (SEM) images of *L. interrogans* serovar Icterohaemorrhagiae strain RGA **(Weyant *et al.*, 1999)**

CLASSIFICATION

The taxonomical classification of leptospires is given here under:

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Spirochaetes
Division	Gracillicutes
Class	Scotobacteria
Order	Spirochaetales
Family	Leptospiraceae
Genus	*Leptospira*

The family Leptospiraceae consists of thee genera *viz*., *Leptospira*, *Leptonema* and *Turneria*. *Leptospira* consists of many pathogenic and non-pathogenic serovars characterized under it. Inada and Ido in 1915 isolated the first strain of *Leptospira* serovar Icterohaemorrhagiae from the patient suffering from Weil's syndrome. Initially *Leptospira* were classified into two main types namely, namely, the *L. interrogans* (pathogenic) and *the L. biflexa* (non-pathogenic)

(Faine and Stallman 1982). The pathogenic leptospires fail to grow at 13ºC and in the presence of 8-azaguanine whereas non-pathogenic ones grow at 13ºC and are resistant to 8-azaguanine. The species *L. biflexa* fail to exhibit spherical cellular nature in 1M NaCl. There are more than 250 serovars of *L. interrogans* arranged into 25 different serogroups. *L. biflexa* has 65 serovars arranged into 38 serogroups. There are 20 *Leptospira* spp. based on DNA relatedness. This classification system does not correspond with the initial classification of leptospires based on serology because it was observed that both pathogenic and non-pathogenic serovars existed within the same species.

Current phylogenetic classification system of leptospires is based on DNA-DNA hybridization and 16S rRNA sequence analysis **(Picardeau, 2017)**. This system divides the genus *Leptospira* into twenty-two species comprising three different groups. The first group consists of ten pathogenic types that cause disease in humans and animals. The second group is composed of five intermediate types which can cause mild clinical manifestations and the third group which is composed of seven types is saprophytic in nature. A classification of *Leptospira* based on serology, DNA-DNA hybridization and 16SRNA is shown in Figure 2.

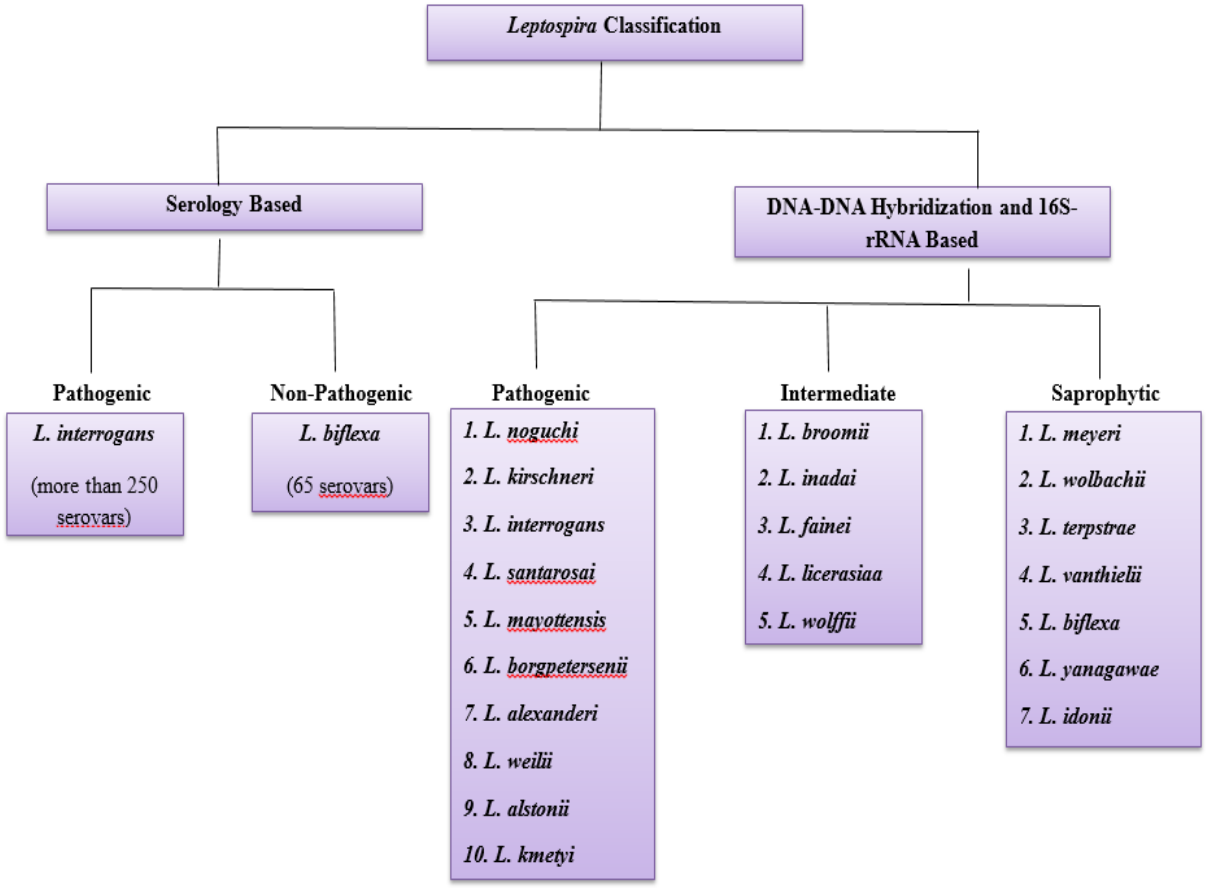

Figure 2 Classification of *Leptospira*

CULTURAL CHARACTERISTICS

Leptospires are aerobic bacteria that require specific and selective media for their culturing. They do not utilize carbohydrates as energy and carbon source. Instead, they depend on long chain fatty acids for their energy and carbon requirements. As free fatty acids have the inherent property of being toxic, they are supplied in esterified form or bound to albumin. Additionally, leptospires require Vitamins B1, B12, ammonium salts and iron for their optimal growth. Pyrimidine analogue 5- flurouracil and neomycin sulphate are used for selective isolation of Leptospires. The other antibiotics that have been used are polymyxin B, vancomycin and rifampicin **(Ellis and Michno, 1976)**. Leptospires are cultured in a wide variety of culture media including liquid, solid and semi-solid media. Liquid media is used for the cultivation of leptospires for various tests. It can be converted into semi-solid media by addition of agar (0.1-0.2%). Semi-solid media is used for isolation of leptospires and their maintenance. Solid media **(Roth *et al.*, 1961)** (0.8-1% agar) is used for research and cloning purposes. The most commonly used commercially available media is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium **(Ellinghausen and McCullough, 1965; Johnson and Harris, 1967)**. In this medium long chain fatty acid is used as a nutritional source and serum albumin as de-toxicant. The other media are liquid or semi-solid in nature and contain rabbit serum. These include Korthoff's (peptone, NaCl, $NaHCO_3$, KCl, $CaCl_2$, KH_2PO_4, Na_2HPO_4), Fletcher's (peptone, beef extract, NaCl, and agar) and Stuart's media ($CaCl_2$, $C_3H_7Na_2O_6P.H_2O$, $C_2H_3NaO_2S$, $C_3H_8ClNO_2S$, methylene blue and agar) **(Faine *et al.*, 1999)**. Rabbit serum contains nutrients including high concentrations of bound vitamin B12 which helps in the growth of leptospires. Several protein free media have been developed which are used for the production of vaccines. The growth rate of leptospires is very slow but once they manage to grow they can be retained in liquid media for several months.

LEPTOSPIRA GENOME

A number of leptospiral genomes have been sequenced so far. In general, the *Leptospira* genome consists of two circular chromosomes which have variable length in different serovars. The variability in the genome helps leptospires to survive and adapt in different conditions **(Picardeau *et al.*, 2008)**. *Leptospira interrogans* serovars Copenhageni and Lai are approximately 4.6 Mb in size whereas *Leptosira borgpetersenii* serovar Hardjo is 3.9 Mb in size. Recently, the complete genome sequence of *L. interrogans* serovar Bratislava having two chromosomes, CI (4.457 Mbp) and CII (358 kbp) has been reported. **Varni *et al.* (2016)** reported that the genome size of *L. interrogans* serovar Pomona is approximately 3.6 Mb with 3763 annotated genes.

EPIDEMIOLOGY

Leptospirosis is widely distributed geographically and occurs mostly in tropical, subtropical and temperate zones **(Ratnam, 1994)**. Though the incidence has decreased in developed countries but in the developing countries there has been considerable increase in leptospirosis cases. In some countries such as Thailand where leptospirosis is under surveillance the occurrence has increased overwhelmingly **(Tangkanakul *et al.*, 1998)**. It is declared endemic in most of the South East Asian countries. A number of leptospirosis outbreaks have occurred in the past in various places across the globe.

Leptospirosis in India

Leptospirosis was reported first in Andaman and Nicobar Islands in the year 1929 **(Kamath and Joshi, 2003)** and has since spread to different parts of India. *Leptospira andamans and Leptospira grippotyphosa* were isolated in the Andamans **(Taylor and Goyle, 1931)**. Several other cases of leptospirosis were reported where leptospires were isolated from humans. Leptospiral serovar

Icterohaemorrhagiae and Canicola antigens were found in five cases of jaundice in the year 1960 **(Dalal, 1960)**. In 1966, two serovars of leptospirosis viz. Icterohaemorrhagiae and Canicola were confirmed by agglutination lysis test in PUO (Pyrexia of Unknown Origin) cases out of 93 sera samples. **Joseph and Kalra (1966)** reported that out of 43 cases of jaundice in Delhi, two samples tested positive for serovar Icterohaemorrhagiae and one was found positive for both serovars Icterohaemorrhagiae and Pomona. One year later in Bombay in 1966, leptospiral infection due to *Leptospira pyrogenes* was evident in one sample out of 93 sera samples for infective hepatitis **(Bhatnagar *et al.*, 1967)**. In 1983, there was prevalence of leptospiral serovars in 18% and 24% of jaundice and PUO cases respectively **(Ratnam *et al.*, 1983)**. Consequent to bovine leptospirosis outbreak in Chennai, several children in Tamil Nadu tested positive for leptospirosis giving good antibody titer. Leptospirosis was the main cause of acute renal failure in 19 human patients in Madras **(Muthusethupathi and Shivakumar, 1987)**. **Venkataraman *et al.* (1991)** confirmed that 33 (82.5%) patient samples out of 40 had specific leptospiral antibodies as confirmed by MAT. During November and December 1990 to 19991 as many as 54 patients with clinical features such as fever, jaundice, myalgia, conjunctival suffusion and acute renal failure were admitted to Government General Hospital, Madras. Leptospiral antibodies were found in two cases and one patient sample confirmed the presence of *Leptospira* serogroup Autumnalis **(Muthusethupathi *et al.*, 1995)**. **Ratnam *et al.* (1993)** reported 32.9% prevalence rate of leptospirosis during a surosurvey conducted among conservancy workers of Madras as confirmed by MAT. Diglipur, in North Andamans witnessed an outbreak of acute febrile illness involving hemorrhagic and pulmonary associated symptoms during October to November of the year 1993. Leptospiral antibodies were recorded in 66.7% of the patient population **(Sehgal *et al.*, 1995)**. In the year 1994, following severe flooding of Tamil Nadu in the autumn of 1993, a sudden increase in uveitis cases due to epidemic leptospirosis was observed at Aravind Eye hospital, Madurai. Out of 46 patients, 80% of them tested positive for leptospiral DNA and 72% tested serologically positive **(Kathryn *et al.*, 1998)**. In 1995, leptospirosis was reported serologically in 12% patients of jaundice and febrile illness in Pondicherry **(Prabhakar *et al.*, 1995)**. From the month of July to November 1996, thirty-eight acute renal failure cases were tested for clinically suspected leptospirosis in Chennai. Twenty-seven patients (71%) were reported to be seropositive as confirmed by MAT **(Saravanan *et al.*, 1998)**. In 1999, several people suffered from febrile illness with hemorrhagic manifestations after a cyclone hit Orissa followed by floods. Out of 142 patients tested, 28 samples confirmed the serological evidence of leptospiral infection **(Sehgal *et al.*, 2001). Krande *et al.* (2002)** reported the occurrence of an outbreak of leptospirosis in children living in flood areas of Mumbai. The children presented symptoms viz. fever, bodyache, chills, abdominal pain, headache, vomiting, cough, hepatosplenomegaly, edema and crepitations. Out of 93 children samples tested, 30 (32%) were confirmed with acute leptospiral infection, 22 with anicteric leptospirosis and 8 were confirmed with Weil's disease. In 2005, Mumbai witnessed an outbreak of leptospirosis as a consequence of severe flooding. The incidence and fatality rates increased to 7.85 per 0.1 million and 8.7% respectively in contrast to 2.1 per 0.1 million and 7.3% when there were no floods **(Kshirsagar *et al.*, 2006)**. **Sharma *et al.* (2006)** reported the prevalence of *L. interrogans* serovar Hardjo in suspected dairy farm cases in Tirupati region of Andhra Pradesh. **Velineni *et al.* (2007)** reported the occurrence of leptospirosis in humans based on data collected from different hospitals of Hyderabad. *L. interrogans* serovars Icterohaemorrhagiae 68%, Australis 22%, Autumnalis 8% and Javanica 2% were found in 55 patient samples tested by MAT, IgM ELISA and LeptoTek Dri-dot. **Masali *et al.* (2007)** reported the prevalence of leptospirosis in six villages of Karnataka. In 2011, Karnataka and southern part of Gujarat witnessed 130 deaths due to leptospirosis. Leptospirosis struck Surat and Valsad districts of Gujarat in the year 2012. Brihanmumbai Municipal Corporation (BMC) in Mumbai reported 15 leptospirosis deaths within a short span of ten days **(Chaurasia, 2016)**. Before the onset of monsoon in 2016, four persons died due to leptospirosis in Udipi area of Karnataka. Recently in 2018, after the Kerala floods as many as 77 cases of leptospirosis have been reported.

Leptospirosis around the world

Leptospirosis has a global impact on human health and is considered to be burdening the world economy. More than 60,000 people die due to leptospirosis annually and nearly one million are reported to be affected **(Mwachui *et al.*, 2015)**. During the past several years a large number of leptospirosis outbreaks have shook many parts of the world. In addition to Indian sub-continent Oceania, Caribbean and Latin America are considered to be endemic to leptospirosis **(Pappas *et al.*, 2008)**. Leptospirosis has been reported to affect all five continents and many countries because of the favorable climatic conditions **(Sehgal, 1998)**.

An outbreak of leptospirosis struck US army in the year 1961 after exercising in the jungle a few days earlier **(Gale *et al.*, 1966)**. During 1975-1977, leptospiral seroprevalence was reported in different work groups in the Barbados. It was found highest in sanitation workers (42.7%) followed by sugarcane workers (39.4%). In a serosurvey conducted in northern Trinidad between mid-1977 and mid-1978, leptospirosis infection was reported widely in people with a highest prevalence rate of 45% in sugarcane workers. 9% new and 23% re-emergent leptospiral cases were reported during 1977-1982 once again in Trinidad **(Everard *et al.*, 1987)**. A high seroprevelence of leptospirosis was reported in Somalia in the year 1982 **(Cacciapuoti *et al.*, 1982)**. In a serosurveillance study conducted in Italy in the year 1987, leptospirosis was confirmed in 11.34% rural and 3.08% urban area cases of central Italy **(Cacciapuoti *et al.*, 1987)**. In 1987, leptospirosis was confirmed in 14 patients out of 56 patients being hospitalized in Karachi province of Pakistan **(Ahmed, 1987)**. In Barbados and Trinidad region of Caribbean basin leptospirosis was confirmed in 12.5% and 9.5% school children (aged between 9-14 yrs) respectively in the year 1988 **(Everard *et al.*, 1989)**. **Park *et al.* (1989)** reported the occurrence of several outbreaks of leptospirosis in Korea due to flooding of fields before the harvesting season. Within the US the most number of leptospirosis cases have been reported in Hawaii **(CDC report, 1998)**. In 1992 it was reported by Hawaii State Health Department that the incidence rate of leptospirosis in Hawaii was 2.97% per 10000 people as compared to nationwide rate of 0.2% per 100000 people. In Hawaii though the leptospirosis incidence associated with occupational exposure decreased from 56% during 1971-1975 to 29% during 1986-1990 but the incidence associated with recreational, habitational and vocational exposure increased from 43% during 1971-1975 to 71% during 1986-1990 **(Katz *et al.*, 1977)**. Many cases of Acute Renal Failure (ARF) due to leptospirosis have been reported in 1977 and 1993 in Uruguay **(Raul, 1977)**. **Faine (1994)** reported that the incidence of leptospirosis increased from 0.3/100,000 (between1982-1987) individuals to 3/100,000 individuals (between1997-1998) in Thailand. A large number of symptomatic leptospirosis cases viz. 80 cases during 1989 to 1990, 65 cases during 1993 to 1994, and 75 cases during 1995 to 1996 were observed in Seychelles **(Yersin *et al.*, 1998)**. Nicaragua witnessed an outbreak of leptospirosis in October, 1995 following heavy water flooding. As many as 2259 patient samples were analyzed for non-malarial febrile illness with cumulative incidence of 6.1% and 0.7% pulmonary hemorrhagic fatality rate **(Zaki and Sheih, 1996)**.

Ceceroni *et al.* (1995) reported the prevalence of leptospirosis in the Cordillera province of Bolivia. 90 out of 295 samples tested, showed the presence of *Leptospira* antibodies as confirmed by MAT. An outbreak of leptospirosis was reported in the urban areas of El Salvador, Brazil **(Koi *et al.*, 1999)**. An active surveillance system was set up in a hospital in Brazil between March 10 and November 2, 1996. Surveillance identified 326 suspected cases and *Leptospira interrogans* serovar copenhageni was isolated from 87% of the cases with positive blood cultures. The case fatality rate was 15% despite aggressive supportive care. The diagnosis was confirmed using MAT and identification of leptospires in blood or urine samples. Initially 42% of the cases were misdiagnosed as having dengue in the outpatient clinic due to simultaneous dengue outbreak in the region. Leptospirosis was also reported in Turkey where leptospiral antibodies were detected by MAT in 5.48% of patient samples out of 1440 total samples tested **(Leblebicioglu *et al.*, 1996)**. In 1996, leptospirosis outbreak was reported in Rio de Janeiro after heavy rainfall resulting in persistent flooding of the western region **(Barcellos and Sabroza, 2001)**. In the same year an outbreak of leptospirosis was reported in Costa Rica among white water rafters who exhibited unknown febrile illness as reported by a physician to Illinois Department of Public Health **(MMWR June 1997, CDC)**. A random survey was conducted in Seychelles in which 1067 patient samples were analyzed out of which 37% showed prevalence of leptospiral antibodies **(Bovet *et al.*, 1999)**. A survey was conducted in North-eastern Alpine regions of Italy to study the epidemiology of leptospirosis. A seroprevelence rate of 10%-12% was detected in farmers and forestry workers of the region **(Nuti *et al.*, 1993)**. In July, 1998 leptospirosis was reported to cause acute febrile illness in athletes in Wisconsin and Illinois **(MMWR July 1998, CDC)**. During a sporting event (Sabah) in Malaysia in 2000, 80 cases of leptospirosis were confirmed in athletes participating in different water related activities **(Anderson *et al.*, 1978)**. In Dhaka, Bangladesh leptospirosis was confirmed by PCR in 63 patients out of 359 dengue negative patient samples **(LaRocque *et al.*, 2005)**. In Indonesia *Leptospira* serovars Bataviae and Hardjo were reported as a causative agent of leptospirosis infection between 2001 and 2002. A total of 557 (139 in 2001 and 418 in 2002) serum samples were tested during this period and *Leptospira* serovar Bataviae and Hardjo was reported in 18.7% and 12% cases respectively **(Hartskeel, 2002)**.

In China, 1500 confirmed cases of leptospirosis were reported by health department between January 2002 and October 2007 (A report by Medical News Today 2007). In 2007, a total number of 208 cases of leptospirosis were reported in rural areas of Korea in which mostly rice field farmers were affected. According to National Leptospirosis Surveillance report 2008, 112 cases of leptospirosis were reported in Australia. In 2009, leptospirosis was reported in 471 patients in the Philippines following heavy flooding caused by a typhoon **(Shere *et al.*, 2009)**. In 2014, 90 cases of leptospirosis in United States were reported in soldiers when they were attending jungle warfare exercises **(Burns *et al.*, 2015)**. In 2016 in the Dominican Republic, 74 death cases of leptospirosis were reported by Outbreak News today. The same agency recently reported 68% cases of leptospirosis in the Philippines in the year 2017. In the year 2018, a total of 368 leptospirosis cases were reported by The Philippines Department of Health between January 1 and July 3 in Manila Metro.

TRANSMISSION CYCLE AND MODES OF TRANSMISSION

Humans are considered to be the accidental hosts as a result of direct or indirect contact with leptospirosis infected animals. Animal hosts of leptospirosis may be either carrier or reservoir hosts, the latter being the primary source of infection. Though the presence of animal carrier is considered important in leptospirosis transmission but it can occur through various environmental sources. Leptospires being ubiquitous in nature are found everywhere but their primary habitat is renal tubules of carrier animals. Large species of rodent, mammal and marsupial can be carrier or reservoir of leptospirosis. *Leptospira* serovar Icterohaemorrhagiae has been associated with rodents **(Matthias and Levett, 2002)**. Other serovars have been associated with different mammalian hosts **(Farr, 1995)**. Cattle for example, can be infected with *Leptospira* serovars Hardjobovis, Pomona, Bratislava and Grippotyphosa though other serovars have also been reported **(Vijayachari *et al.*, 2008)**. Pigs are commonly infected with serovars Pomona, Tarassovi, Grippotyphosa, Bratislava, Sejroe and Icterohaemorrhagiae whereas serovars Canicola and Icterohaemorrhagiae have been associated with infection in dogs **(Vijayachari *et al.*, 2008)**. Leptospirosis has also been reported in various wild animals such as bats, possums, deer, mongoose and small insectivores **(Ellis, 2015)**.

Leptospirosis has direct and indirect mode of transmission. Direct transmission occurs through leptospires from tissues, body fluids or urine of acutely infected or asymptomatic carrier animals. The most common method of entry is through the skin. They can directly travel in to the blood or lymphatic system through conjunctiva or into the lungs through inhalation as aerosols **(Faine, 1994)**. In animals it can be transplacental, haematogenous, by sexual contact or by suckling milk from infected mother. The direct infection from mother to child via placenta and the occurrence of leptospires in genital tracts have been reported in animals **(Ellis *et al.*, 1986)**. Transmission cycle of *Leptospira* is shown in Figure 3. On the other hand, indirect transmission occurs from the environmental leptospires.

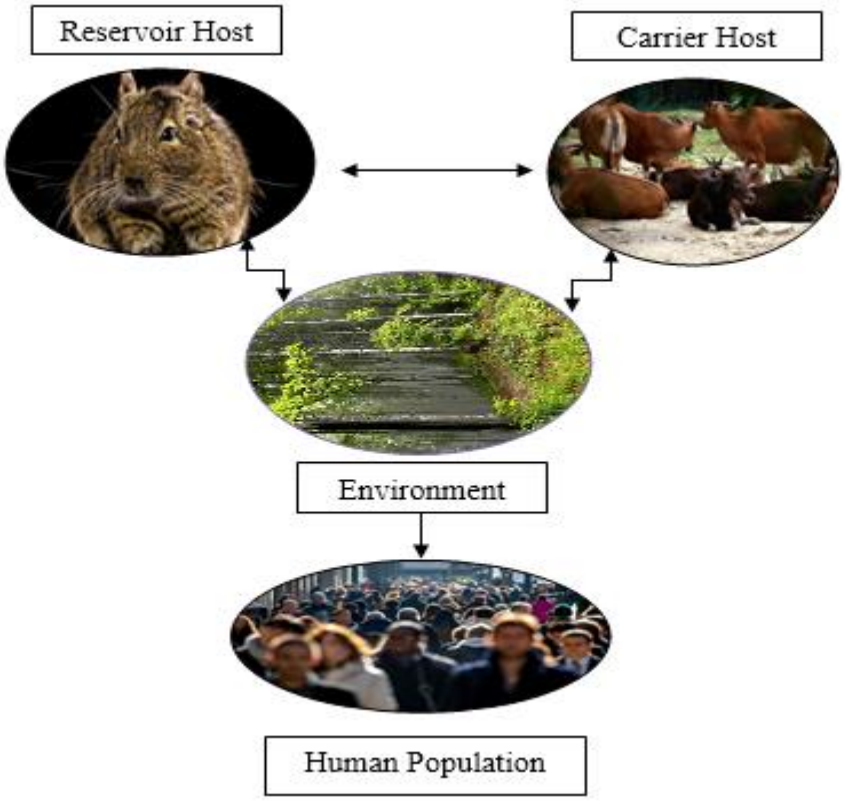

Figure 3 Transmission cycle of *Leptospira*

POPULATION AT RISK AND RISK FACTORS

Leptospirosis in humans is mainly caused due to various occupational and recreational activities. People belonging to different workgroups such as butchers, farmers, veterinarians, rodent control workers and others who are directly involved in any work associated with animals are at higher risk **(Demers *et al.*, 1985; Terry *et al.*, 2000; Thornley *et al.*, 2002)**. Leptospirosis has been considered as an important health hazard of rice farmers in Indonesia and Thailand **(Tangkanakul, 1998)**. Leptospirosis outbreak has occurred in Chonbuk province of Korea due to heavy flooding before harvest **(Park *et al.*, 1989)**. An outbreak of leptospirosis occurred in a dairy farm in east Otago in New Zealand **(Thomas *et al.*, 1994)**. Leptospirosis has also been associated with various water related recreational activities and water sports **(Levett, 2001)**. Leptospirosis infection was reported in participants of Eco-Challenge multi-sport endurance event organized in Borneo, Malaysia in September 2000. Recently in July 2011, leptospirosis has been reported in France. Various other serogroups have been reported to cause infection in humans engaged in different water related recreational activities **(Sejvar *et al.*, 2003)**. A number of cases of leptospirosis have been reported associated with swimming in contaminated water and human to human infection through breast feeding **(Corwin *et al.*, 1990)**. During a scout camp in Belgium in 2012, leptospirosis was reported in boys attending the camp and the source of infection was attributed to be forest rats. Depending on the social behavior, occupation and environmental factors there is a modulated epidemiological risk associated with leptospiral infection.

PATHOGENESIS AND VIRULENCE FACTORS

The mechanisms by which leptospires causes pathogenesis are not clearly understood. Attachment to the host cell surface and production of toxins are the two important characteristics of leptospires. leptospires enter the host by small cuts or openings in the skin, eye conjunctiva, mucous membrane, genital tract and spread quickly through blood and lymphatic system to different parts of the body. They can circulate in the blood stream lasting up to seven days **(Adler *et al.*, 2010)**. Chemotaxis and motility of leptospires play an important role in invasion and spreading of infection **(Charan and Goldstein, 2002)**. The ability to penetrate and move rapidly has been demonstrated on various cell lines **(Liu *et al.*, 2007)**. Outer membrane of leptospires possesses antiphagocytic activity **(Levett, 2001)**. A large number of virulence factors have been associated with the occurrence of leptospirosis. Particularly, outer membrane proteins (OMPs) play a key role in pathogenesis of leptospirosis. OMP LipL32 is a highly conserved virulence factor present in pathogenic leptospires **(Haake *et al.*, 2000)**. Other proteins such as integrin alpha like protein, cell surface polysaccharides and exopolysaccharides are also considered potential virulence factors for leptospirosis **(Isogai *et al.*, 1997)**. Leptospires are believed to interact with extra-cellular matrix (ECM) proteins of the host organism. The proteins such as *LigA, LigB, Lsa21, Lsa24* and *Loa22* are the potential virulence factors that interact with ECM of the host **(Choi *et al.*, 2007; Atzingen *et al.*, 2008; Barosa *et al.*, 2006; Ristow *et al.*, 2007)**.

PRODUCTION OF TOXINS

One characteristic that determines the pathogenicity of leptospires is the production of endotoxins. Several serovars of *Leptospira* are being reported to exhibit endotoxin production. It has been reported that leptospiral lipopolysaccharides (LPS) are believed to show evidence of endotoxin production like other Gram negative bacteria. **Shimiju *et al.* (1987)** reported that biological activity of LPS from *L. interrogans* serovar canicola was lesser as compared to the other Gram negative bacteria. This may be due to differential

immunity activation mechanism in leptospires and other Gram negative bacteria. Glycolipoprotein (GLP) fractions in leptospires are also believed to exhibit cytotoxic effects (Vinh *et al.*, 1986). Such type of activity was first observed in *Leptospira* serovars Copenhaegi and Pomona **(Cinco *et al.*, 1980)**. In serovar Canicola GLP fractions of similar kind were reported to inhibit the activity of enzyme Na^+/K^+ ATPase due to the adsorption of unsaturated fatty acids to GLP fraction **(Burth *et al.*, 1997)**. Leptospires also produce haemolysins that act on red blood cells and other cells having phospholipids in their membranes **(Lee *et al.*, 2002)**. These are believed to be phospholipases that act on substrates having phosphplipids. Some serovars of leptospirosis viz. Pomona and Hardjo also exhibited sphingomyleinase C activity ***(Bernheimer and Bey, 1986; del Real et al., 1989)***. Based on the ability of hemolysins to hydrolyze sphingomylein they are categorized as sphingomyelinase and non-sphingomyelinase hemolysins **(Zhang *et al.*, 2005)**.

HOST IMMUNE RESPONSE

The first line of host defense that helps in the recognition and elimination of leptospires is the innate immune system. The activation of complement system kills the non-pathogenic *Leptospira biflexa* within a few minutes *in vitro* but pathogenic virulent *Leptospira* species are more resistant to the compliment system **(Barbosa *et al.*, 2009)**. The innate response in case of leptospirosis is dependent on the recognition of certain patterns known as pathogen associated molecular patterns (PAMPs) **(Beutler, 2004)**. These PAMPs are recognized by proteins called pathogen recognition particles (PRRs) present on the host immune cells. These PRRs helps the cells to recognize conserved motifs in microbial cells such as lipopolysaccharides and peptidoglycans **(Kawai and Akira, 2007)**. These signaling PRRs can be either Toll-like receptors (TLRs) or Nod-like receptors (NLRs) **(Mogensen, 2009)**. So far in leptospirosis, the role of TLR2 and TLR4 is being studied extensively. LPS in leptospires is reported to activate TLR2 dependent signaling cascade whereas all other Gram negative bacteria activate TLR4 dependent signaling cascade **(Wert *et al.*, 2001)**. This difference in mode of activation is due to the unusual composition of Lipid A in leptospires **(Que *et al.*, 2004)**.

The acquired immune response which is based on the production of antibodies requires the activation of classical compliment system. The main mechanism of immunity in leptospires is considered to be humoral which is believed to be serovar specific **(Alder and Faine, 1999)**. The immunization mostly with anti-LPS antibodies confers immunity against leptospirosis **(Jost *et al.*, 1986)**. The outer membrane proteins of leptospires play an important role in determining the host immune response. *LipL32* happens to be the most dominant outer membrane protein that triggers immune response against leptospirosis in humans **(Hauk *et al.*, 2008)**. Another outer membrane protein *LipL21* which belongs to serovar Lai of *Leptospira interrogans* is also reported to be having immunogenic potential **(He *et al.*, 2008)**. Immunoglobulin M (IgM) antibodies are the first to occur followed by immunoglobulin G (IgG). Humoral immunity studies involving LPS and seven other proteins were carried out which showed the IgM antibodies specific to LPS and IgG antibodies to all seven proteins selected as targets **(Guerreiro *et al.*, 2001)**.

Cell mediated immunity (CMI) is also exhibited by the leptospiral host in addition to humoral immunity. It has been reported that cattle immunized with killed *Leptospira borgpetersenii* vaccine developed increased interferon-γ (IF- γ) associated with CD4+ T cells and γδT cells **(Brown *et al.*, 2003)**. *Leptospira interrogans* glycolipoprotein (GLP) activated peripheral blood mononuclear cells (PBMCs) that resulted in high amount of TNF-α and IL-6 **(Dorigatti *et al.*, 2005)**. In another study, GLP extracted from *Leptospira interrogans* serovar Copenhageni activated PMBCs by secreting TNF-α and IL-10 but non-pathogenic *Leptospira biflexa* serovar Patoc failed to activate PMBCs **(Diment *et al.*, 2002)**. Though humoral immunity remains to be the choice of immune response in *Leptospira* host but other mechanisms cannot be ruled out. The efficient vaccination can only be developed after understanding the varied host immune response mechanisms of leptospirosis.

CLINICAL MANIFESTATIONS

The clinical presentation of leptospirosis is highly variable. The incubation period is generally 7-14 days but it may vary ranging from 2 days to one month. Leptospirosis signs and symptoms mimic several other diseases and therefore the diagnosis becomes difficult. The most cases of leptospirosis recover without any complications. But some are misdiagnosed and this makes leptospirosis an under-reported disease **(Heuer *et al.*, 2008)**. Leptospirosis course in humans is considered to be having two phases- septicemic and immune. The acute or septicemic phase lasts up to a week and is characterized by the presence of leptospires in blood, cerebrospinal fluid etc. Various signs such as fever, myalgia, chills, headache etc. occur during this phase. The septicemic phase is followed by the immune or delayed phase characterized by the production of antibodies and occurrence of leptospires in the urine **(Levett, 2001)**. The disease at this stage is transferred from the blood vessels to various vital organs of the body. At this point the disease can be characterized as either anicteric or icteric. The anicteric leptospirosis is the milder form and occurs in 90% of the cases. Icteric form of leptospirosis is known as Weil's disease and is the severe form of disease which occurs in 5-10% patients with 5-40% mortality **(Izureita *et al.*, 2008)**. It is characterized by the involvement of many vital organs of the body **(Vinetz, 2001)**.

The disease onset in leptospirosis is very quick and is characterized by fever ranging from 100-105°F along with consistent headache. The pain is also witnessed in the lower part of the body such as thighs, calves etc. but the involvement of other parts of the body is also prominent **(Kelley, 1998)**. Prostrations, anorexia, nausea, vomiting along with constipation or diarrhea are other markers of anicteric leptospirosis. A person may experience hallucinations and stays in confusing state of mind. The signs of aseptic meningitis are also observed in younger patients). Some patients may suffer from gastroenteritis discomfort and pain in the abdominal region. Encephalitis and uveitis has also been reported in some cases (Guerra 2009). Conjunctival suffusion is also observed during initial phase in some cases with no inflammatory discharge ruling out the chances of true conjunctivitis. Hemorrhagic pneumonia with acute respiratory syndrome has also been reported recently in anicteric form of leptospirosis **(Grzeszczuk, 2007)**. Fatality rate in anicteric leptospirosis is almost zero **(Edwards and Domm, 1960)** but deaths in some patients were observed in China **(Wang *et al.*, 1965)**.

Icteric form of leptospirosis (Weil's disease) occurs in very small proportion of people. *Leptospira* serovar Icterohaemorrhagiae happens to be associated with most cases of icteric leptospirosis **(Katz *et al.*, 2001)**. The most common symptom of this form is jaundice which may occur in the initial days or later during the progressive phase of the disease. Hepato-cellular necrosis occurs and increased amount of bilirubin in the serum is also observed. Mild increase in transaminases and alkaline phosphatases may be observed. Death may also occur but at the latest stage of icteric leptospirosis due to renal failure. Most patients of kidney failure also have hepatic involvement. Cardiac involvement has also been observed in the form of myocarditis and pericarditis **(Chukurkar *et al.*, 2008)**. Pulmonary hemorrhage associated with leptospirosis has also been reported **(Vijayachari *et al.*, 2003)**. In the pregnant women leptospirosis infection can cause miscarriage in most of the cases **(Carles *et al.*, 1995)**.

LEPTOSPIROSIS DIAGNOSIS

Efficient laboratory diagnosis of leptospirosis becomes very essential because its clinical signs and symptoms mimic those characterized in various other diseases and disorders. There are several conditions that present febrile illness as in leptospirosis *viz.* influenza, meningitis, liver infections, virus invasion etc. **(Levett, 2001)**. Leptospirosis cannot be diagnosed alone on the basis of clinical manifestations it exhibits but an array of laboratory diagnostic methods is required for correct diagnosis. Understanding the course of the disease, right specimen selection and the choice of diagnostic test are the essential factors that help in precise leptospirosis diagnosis. Some of the common diagnostic procedures used during different stages for leptospirosis are given in Table 1.

Table 1 Various diagnostic methods of leptospirosis

A. Leptospiraemic Phase <7days

Sr. No.	Test	Sample Required	Reference
1	Isolation (Bacterial Culture)	Blood, Urine, CSF	O'Keefe, 2002
2	**Direct Antigen Detection**		
	1. Dark Field Microscopy	Urine	Levett *et al.*, 2006, Turner, 1970 Yener and Keles, 2001
	2. Histological Staining	Kidney lysate	Skilbeck, 1986
	3. Immunological Staining	Kidney lysate	Elliott, 1998
	4. Monoclonal Antibody	Urine	

3	**PCR Based Methods** 1. PCR 2. Real Time PCR 3. REA 4. RFLP 5. PFGE 6. PCR-RFLP 7. RAPD 8. FAFLP 9. MLVA 10. MLST 11. LAMP 12. LSSP-PCR 13. Nucleic Acids probes 14. AP-PCR 15. Ribotyping	DNA from Isolates or Clinical Samples	Terpstra *et al.*, 1986 Ahmed *et al.*, 2009 Marshall *et al.*,1981 Li *et al.*, 2009 Galloway and Levett, 2008 Li *et al.*, 2009 Ramadass *et al.*,1997 Vijyachari *et al.*, 2004 Pavan *et al.*, 2011 Wiess *et al.*, 2016 Notomi *et al.*, 2000 Bomfim and Koury, 1991 Terpstra *et al.*, 1986 Perolate *et al.*, 1994 Perolate *et al.*, 1994

B. Immune Phase >7days

Sr. No.	Test	Sample Required	Reference
1.	**Serological Based Method** 1. CAAT	Viable isolates of *Leptospira*	Dikken and Kmety, 1991
2.	**Antibody Based Methods** 1. MAT 2. MSAT 3.MCAT 4. CF 5. HL 6. IFA 7. IHA 8. Dipstick 9. ELISA 10. Lepto tri-dot 11. Lepto lateral flow 12. LAT 13. Flow Cytometry 14. Lepto dri-dot	Serum,Urine,CSF, Kidney Lysate	Dutta and Christopher, 2005 Sumathi *et al.*, 1997 Arimitsu *et al.*, 1982 Terzin, 1956 Cox, 1957 Appassakij *et al.*, 1995 Sulzer *et al.*, 1975 Vijayachari and Sehgal, 2006 Wasinki and Pajsek, 2010 Smits *et al.*, 1999 Sehgal *et al.*, 2003 Senthilkumar *et al*, 2008 Yitzhaki *et al.*, 2004 Sehgal *et al.*, 1999

Direct examination of body fluids for leptospires using dark field microscopy

Various body fluids such as blood, cerebrospinal fluid (CSF), urine and dialysis solution can be examined directly under the dark field microscope for the rapid detection of leptospires **(O'Keefe, 2002)**. They may appear coiled and thin with active motility when examined in fluids under the dark field microscope **(Levett *et al.*, 2006)**. Apart from body fluids they can be examined from tissue samples extracted from various sources such as necrotic tissues, abortion products, carcasses etc. **(Faine *et al.*, 1999)**. The concentration of leptospires during the initial stage of infection is low for direct microscopic examination. Their concentration in blood or urine can be increased by differential centrifugation. Approximately 10^4 leptospires/ml are required for one cell per field to properly visualize them in urine under dark field microscope **(Langston and Heuter, 2003)**. Leptospires are not stained or poorly stained by Gram staining. Silver impregnation methods work well for staining leptospires. Various other staining methods such as immunohistochemical staining, immunofluorescent staining, Warthin-Starry staining etc. have been used to increase the sensitivity of dark field microscopic examination of leptospires **(Yener and Keles, 2001)**. Though dark field microscopy becomes the only choice for the examination of leptospires due to non-availability of other resources but it is not always considered the best option. Table 3 shows various diagnostics methods used for *Leptospira* detection. Due to its low sensitivity and specificity it cannot be used for routinely diagnosis of leptospirosis **(Levett, 2001)**. In addition, it requires high expertise and very careful examination of the specimen along with cross confirmation with other tests **(Musso and Scola, 2013)**.

Isolation and culture-based methods

Leptospires can be isolated from various sources such as blood, urine, CSF and lysis solution. The isolation source and culturing of leptospires mainly depend on the stage and course of the disease. The most important source for leptospires during the initial phase (leptospiraemic phase) of leptospirosis is considered to be blood but culturing should be done within ten days from the disease onset **(Schreier *et al.*, 2013)**. Blood may contain 10^2-10^6 leptospires per milliliter **(Agampodi *et al.*, 2012)**. Ellinghausen- McCullough Johnson-Harris (EMJH) media and Fletcher's media can be used for culturing leptospires from blood but most common use is of EMJH media **(Levett and Haake, 2010)**. Fastidious growth can be achieved by adding 0.1%-0.15% agarose and 0.4-1% of fetal calf or rabbit serum. Regular examination using dark field microscopy should be done till the last week. The culture media can be made selective to avoid contamination by the use of 5-Flourouracil and a combination of antibiotics such as vancomycin, nalidixic acid, polymyxin B, rifampicin and bacitracin but use of selective media can reduce the chances of isolation of pathogen of interest. The use of blood culture for regular determination of leptospires cannot be done because of disadvantages such as long incubation period required, cumbersome to perform and slow sensitivity. Apart from blood, leptospires can also be isolated during the initial stage of the disease from CSF and dialysate **(Ahmed *et al.*, 2005)**. The cultures should be taken in initial 5-10 days of the infection and the same procedure as used for blood culture should be followed. Culturing from urine can be done during leptospiruria phase of the disease. The ideal time for urine culture is between 10-30 days from the onset of leptospirosis though the duration of excretion in urine may vary **(Bal *et al.*, 1994)**. Urine culture becomes less sensitive due to intermittent leptospire shedding in humans and animals, acidic nature of urine and high chances of contamination **(Fearnley *et al.*, 2008)**. Leptospires can also be cultured from fatal cases in human and animal postmortem tissues such as brain, liver, kidney and aborted animal fetuses **(Faine *et al.*, 1999)**. Leptospires require varied time periods for growth after culturing. Less fastidious serovars such as Grippotyphosa and Pomona may exhibit good culture growth after inoculation with but some serovars such as Hardjo may take even several months to grow. Though culture-based methods are considered to be

the most reliable and definite methods for leptospirosis diagnosis but they cannot be used for quick routinely disease diagnosis because of several shortcomings. Being time consuming, less sensitive and labor intensive makes them unsuitable for laboratory diagnosis of leptospirosis however they are still being used in research.

Serology based methods

Serology based methods for leptospirosis diagnoses are based on the detection of specific antibodies against *Leptospira* in blood sera. The tests may not work during the initial stage of infection because the antibody production against *Leptospira* takes a while. Once produced, they may persist in the body for long period of time. Though the initial antibody titer may be low, re-sampling after 3-4 days is recommended for definitive diagnosis. Serological tests for the diagnosis of leptospirosis are both genus and serogroup specific **(Levett, 2004)**. The current serology-based methods emphasize on the detection of IgM and IgG antibodies. IgM antibodies are the first class of antibodies to appear followed by IgG antibodies **(Silva *et al.*, 1995)**. IgM antibody based diagnostic tests for leptospirosis are used to diagnose leptospirosis at an early stage whereas IgG antibody-based tests are used for later stage leptospirosis diagnosis. This is because of the fact that IgG antibodies are reported to persist for a longer period of time after infection as compared to IgM antibodies.

Microscopic agglutination test (MAT)

Several tests based on antigen-antibody agglutination are being used currently for the diagnosis of leptospirosis. The most common method based on serology used for the diagnosis of leptospirosis is MAT. It is considered to be serogroup/serovar specific when carried out on paired sera. MAT involves the treatment of patient serum sample containing leptospiral antibodies with live antigen. The agglutination between antibody and live antigen is observed under dark field microscope **(Mulla *et al.*, 2006)**. When no free leptospires are observed under the dark field microscope maximum agglutination has occurred. The patient serum samples are continuously diluted till maximum agglutination has occurred and no free leptospires are available. The final serum dilution is the one at which 50% or more leptospires have undergone agglutination. A number of studies have been carried out which exhibits the variable sensitivity and specificity of MAT in leptospirosis diagnosis. The sensitivity of MAT in diagnosing leptospirosis in the acute phase is considered very less **(Limmathurotsakul *et al.*, 2012)**.
MAT is considered to be highly sensitive, specific and reliable test for leptospirosis diagnosis. But it requires high expertise to perform and is a cumbersome test for routinely disease diagnosis. Also, for optimal sensitivity a large number of serovar/serogroups residing in a local population has to be included as antigens **(Dassanayake *et al.*, 2009)**. A high degree of cross-reactivity between serovars can also produce misleading MAT results. This limits the usefulness of MAT in determining infecting serovar in highly endemic areas. A number of studies have been carried out which revealed the inability of MAT to predict the infecting serovar, given was the antigen pool of that particular region **(Subharat, 2010)**. Another problem with MAT is that it requires working with live antigens which can be bio-hazardous **(Ahmed *et al.*, 2005)**. The regular sub-culturing is required to keep a check on the authenticity of serovars. The culture maintenance and quality control can be tedious and time consuming.

Enzyme linked immuno sorbent assay (ELISA)

Enzyme linked immuno sorbent assay is considered to be the easy and simplest method used for leptospirosis diagnosis. It is considered to be more sensitive than most of the serology based tests used for conventional diagnosis of leptospirosis **(Wasinki and Pajsek, 2010)**. ELISA requires minimal training to perform and can give result in a short period of time (usually 2-4 hours). ELISA is based on the detection of antibodies against leptospiral lipoproteins and the outer membrane proteins such as *LipL32*, *LipL21*, *LipL41*, *ompL1* and *LigB* which are highly conserved markers in leptospires. A number of antigen specimen can be used in ELISA such as formalin treated leptospires, sonicated whole cells, polystyrene micro-titer plate coated leptospiral cells, purified and recombinant antigens.
IgM ELISA has been found suitable for the human and animal leptospirosis diagnosis during the acute phase of infection. It is considered to be better than MAT as it can detect IgM antibodies in advance before cut-off MAT titers are reached **(Winslow *et al.*, 1997)**. If antibody titer is low or negative another serum sample should be collected for examining sero-conversion **(Vasconcellos *et al.*, 2010)**. The other advantage of ELISA over MAT is the use of killed antigen which poses no bio-hazardous threat to laboratory personnel. Due to low specificity of ELISA as compared to MAT for a single sample, convalescent sample testing with MAT is recommended for confirming the results obtained **(Cumberland *et al.*, 1999)**.

Other serological tests

A number of serological tests have been developed for leptospirosis diagnosis though they are rarely used for routinely diagnosis because of low sensitivity and specificity **(Picardeau, 2013)**. Tests such as hemolysin test (HL), complement fixation (CF) test, indirect haemagglutination assay (IHA), Microcapsule Agglutination test (MCAT), latex agglutination test (LAT), indirect immunofluorescence antibody assay (IFA), lepto dipstick assay, macroscopic slide agglutination test (MSAT), lepto lateral flow lepto dri-dot have been developed **(Picardeau, 2014)**. All these tests are rapid, simple to perform and require no special equipment. A large number of diagnostic kits based on these tests are commercially available in the market.

Molecular methods

Molecular methods based on DNA-DNA hybridization are considered to be highly specific, sensitive and rapid methods for pathogen detection and disease diagnosis. These methods make use of DNA probe that binds to its complementary counter-part found in clinical samples. PCR has been used in the diagnosis of various infectious diseases caused by bacteria (fastidious organisms). PCR has been used for the detection of leptospires since 1990s. PCR assays based on *ompL1*, *flab*, 23S rRNA, *LigA*, *LigB*, *LipL32* etc. genes have been used extensively for leptospirosis diagnosis. Multiplex PCR has also been used for leptospirosis diagnosis **(Kositanont *et al.*, 2007)**. PCR can detect leptospiral DNA even in culture negative serum samples containing un-cleared non-viable leptospires. Real time PCR or quantitative PCR (qPCR) has been recently used in leptospirosis diagnosis. It has been used for the rapid and sensitive diagnosis of leptospirosis along with quantification of leptospiral DNA **(Dezhbord *et al.*, 2014)**.
Though PCR is considered more sensitive and specific than other tests used for leptospirosis diagnosis it still has got some shortcomings. It uses sophisticated instrumentation and expensive reagents that limits its usefulness for routinely disease diagnosis **(Faine *et al.*, 1999)**. Another limitation of PCR is that it cannot identify the infecting serovar. A newer method known as loop mediated isothermal amplification (LAMP) has been reported to show lesser sensitivity to contaminants present in PCR **(Kaneko *et al.*, 2007)**. Molecular typing using restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), fluorescent amplified fragment length polymorphism (FAFLP) and variable number tandem repeat (VNTR) has also been used in the diagnosis of leptospirosis **(Gerritsen *et al.*, 1995; Vijayachari *et al*, 2004; Majed *et al.*, 2005; Slack *et al.*, 2005)**. However, it is not very specific and reproducible.

TREATMENT

The treatment of leptospirosis depends on the severity and stage of the infection. The patients with symptomatic leptospirosis are advised to keep a check on the progression of the infection and are suggested symptomatic treatment options. The severe form of leptospirosis requires immediate intensive care unit treatment accompanied by the administration of antibiotics. The various antibiotics recommended for leptospirosis treatment are penicillin, doxycycline, cefotaxime, ceftriaxone and azithromycin **(Levett, 2001)**. In milder cases of leptospirosis infection, oral treatment with tetracycline, doxycycline, ampicillin, or amoxicillin is recommended and penicillin G and ampicillin are recommended for severe cases. Some cases of leptospirosis require intravenous administration of antibiotics such as penicillin G, erythromycin, amoxicillin and ampicillin. Doxycycline is the best pre-exposure prophylaxis antibiotic but it is not recommended in majority of cases.

CONCLUSION AND FUTURE PROSPECTS

Leptospirosis is one the important zoonotic infections worldwide. It exhibits diverse clinical manifestations and involves various vital organs of the body. The clinical form of leptospirosis is biphasic in nature involving the initial acute phase and late immune phase characterized by the development of antibodies. The non-availability of efficient diagnostic procedures keeps it underdiagnosed. With the advancement of technology new and efficient methods for leptospirosis diagnosis can help in the management of this disease. Recently, biosensors have gained high importance in disease diagnosis and pathogen detection due to their high sensitivity and specificity. Biosensors for leptospirosis diagnosis will surely help in easy and efficient diagnosis of leptospirosis and thus will play a major role in the management of the disease.

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