CHAPTER II

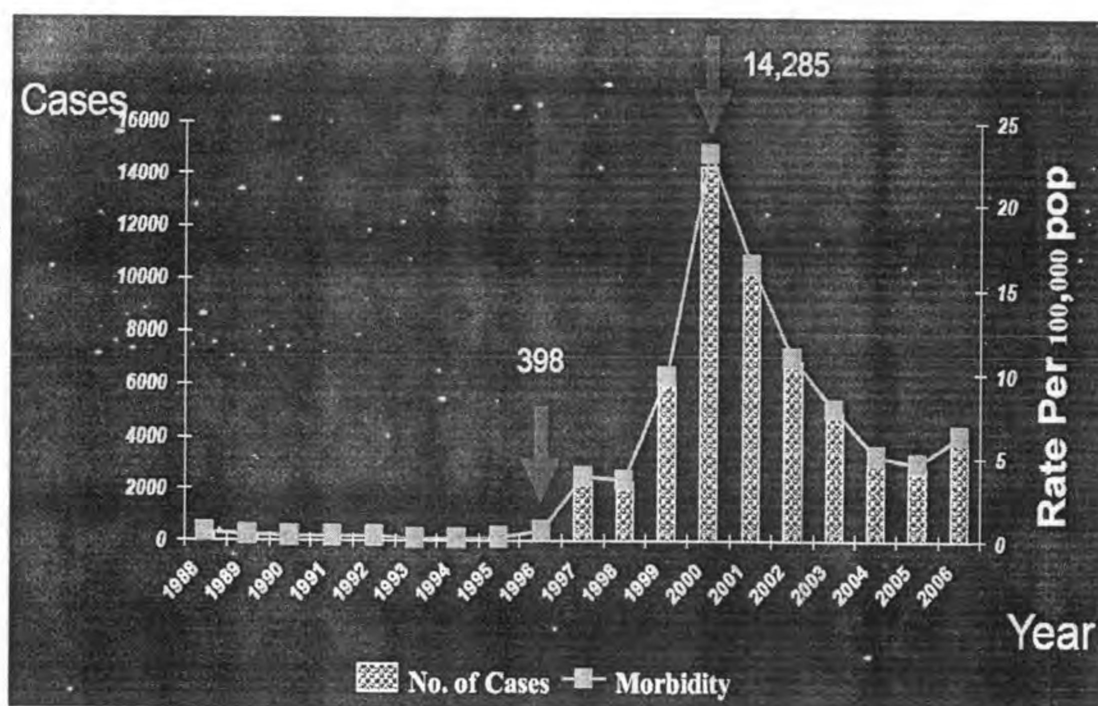
LITERATURE REVIEWS

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human disease caused by leptospires is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality. This syndrome, icteric leptospirosis with renal failure, was first reported over 100 years ago (38). Adolf Weil published in 1886 (39) the first detailed account of the leptospirosis infection and the icteric form of illness he described has been given his name. However the infection was known and reported long before that, with accounts associating fever and jaundice with farming, flooding and livestock all over the world, from ancient China to India and Europe. Hippocrates postulated about it, as did Galen. Weil's disease was noted in medical reports from the Napoleonic campaigns and travelers to the Americas.

The illness Weil described was the severe icteric form with jaundice, and it is not as simple to find descriptions of the milder forms, simply because they are so easily misdiagnosed and weren't accepted as a disease until the advent of bacteriology allowed the causes of illness to be determined. The association of risk was known for decades, even centuries, before the *Leptospira* bacterium was found and associated as a cause. Spirochetes were found to cause a wide range of illness in the latter half of the 19th Century, but the first credited account of a leptospire isolated from a patient was by Stimpson in 1907 (40). Stimpson called the bacteria "Spirocheta interrogans" due to their stained shapes looking like question marks, and the name has remained.

Rats as a carrier (reservoir host) were identified in Japan in the years between discovery of the bacteria and the War (20). Most of the basic pathology and epidemiology we use today was defined before 1940, and in recent years the research has concentrated on the bacterial DNA sequence and internal cellular processes that confer virulence, immunity and may lead to development of better

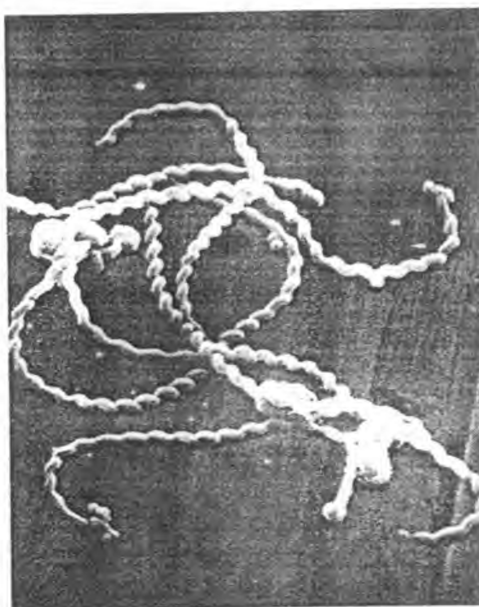
vaccines. In Thailand, the first case of human leptospirosis was reported in 1943 by Yunibandha (41). Subsequently Icterohaemorrhagiae has been isolated with many animal hosts, including cattles, swines, goats, hamsters and dogs: however the rat remains the primary reservoir in most parts of the world. Before 1996, the number of cases reported to the Ministry of publish health was approximately 400 per year. Leptospirosis was sporadic and reported mainly from central and southern regions. A marked change occurred in the subsequent decade, with a year-on-year rise from 398 cases in 1996 to a peak of 14,285 cases in 2000 (Figure 1). This was followed by a continuous decline with 5,000 cases reported in 2006.



Source: Disease notification report, Ministry of publish health, Thailand.

Figure 1 Reported cases and morbidity rate (per100,000 populations) of leptospirosis by year in Thailand, 1988-2006.

2.1 Characteristic of *Leptospira*



(www.dld.go.th/vrd_ne/web/center_lepto.php)

Figure 2 The characteristic of *Leptospira interrogans* visualized by the scanning electron microscope (SEM)

Leptospires are tightly coiled spirochetes. The width sizes of leptospires are usually 0.1 µm and length sizes are 6 to 20 µm (Figure 2). The helical amplitude is about 0.1 to 0.15 µm, and the wavelength is about 0.5 µm. The cells have pointed ends, either or both of which are usually crooked into a hook. The periplasmic space (periplasmic flagella) is the location of two axial filaments (42). The structure of the flagellar proteins is complex (43). Leptospires demonstrate two disparate forms of movement, translational and nontranslational. Morphologically all leptospires are difficult to devide, but the morphology of individual isolates varies with subculture *in vitro* and can be restored by passage in hamsters (44).

Leptospires have a same double membrane structure when compare with other spirochetes. The cytoplasmic membrane and peptidoglycan cell wall are closely related and are overlain by an

outer membrane (45). Leptospiral lipopolysaccharide has lower endotoxic activity (46) but has a composition similar to that of other gram-negative bacteria (47).

Leptospires are aerobic bacterias with an optimum growth temperature of 28 to 30°C. They produce both catalase and oxidase. They grow in simple media abundant vitamins such as vitamin B2 and B12 which are growth factors, long-chain fatty acids, and ammonium salts. Long-chain fatty acids are used as the carbon source and are metabolized by beta-oxidation (48).

2.2 Culture Methods

Growth of leptospires in media containing serum or albumin plus polysorbate and in protein-free synthetic media has been described (49). Fletcher, Korthoff, Noguchi, and Stuart were described several liquid media containing rabbit serum (49). The most widely used medium is based on the oleic acid-albumin medium EMJH (50) which is available commercially from several manufacturers. Some strains are more selective and require the addition of either pyruvate (51) or rabbit serum (52) for initial isolation. the addition of 5-fluorouracil into media can be inhibited the growth of contaminants from clinical specimens (53). Other antibiotics have been added to media for culture of veterinary specimens, in which contamination is more likely to occur (51).

Leptospires is usually grow slowly on primary isolation, and cultures are retained for up to 13 weeks, but the growth of leptospires in pure subcultures in liquid media usually within 10 to 14 days. Leptospiral cultures may be maintained by repeated subculture or storage in semisolid agar containing hemoglobin. In addition, storage with lyophilization or at -70°C is also used for long-term storage of leptospires (54).

Growth of leptospires on solid media with agar has been reported (49, 55). Morphology of colony is dependent on agar concentration and its serovar (56). Media can also be solidified using gellan gum (57). Solid media have been used for isolation of leptospires (58), to segregate mixed

cultures of leptospires and for detection of hemolysin production (59).

2.3 Molecular Biology

Phylogenetic of leptospires are related to other spirochetes (60). The leptospirai genome size is about 5,000 kb (61, 62). The genome is consist of two sections, a 4,400-kb chromosome and a smaller 350-kb chromosome (62). Physical maps have been created from serovars Pomona subtype Kennewicki (62) and Icterohaemorrhagiae (63). Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene (64), and the rRNA genes are widely spaced (65, 66).

A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis (67, 68), rRNA (69), ribosomal proteins (70), RNA polymerase (71), DNA repair (72), heat shock proteins (73), sphingomyelinase (74), hemolysins (4), outer membrane proteins (75, 76), flagellar proteins (77, 78), and lipopolysaccharide (LPS) synthesis (79). The genome of serovar Icterohaemorrhagiae appears to be conserved (63). This conservation allowed the identification of at least one new serovar by recognition of distinct pulsed-field gel electrophoresis (PFGE) profiles (80).

2.4 Taxonomy and Classification

Leptospira is in kingdom Monera, phylum Spirochaetes, class Spirochaetes, order Spirochaetales, family Leptospiraceae and genus *Leptospira*. Prior to 1989, the genus *Leptospira* was divided into two species. *L. interrogans* is all pathogenic strains and *L. biflexa* is the saprophytic strains isolated from the environment (20).

Both *Leptospira* species were divided into numerous serovars, defined by agglutination after cross-absorption with homologous antigen. *L. biflexa* have been recorded over 60 serovars (20) whereas *L. interrogans* over 200 serovars are recognized. Serovars that are antigenically related have

traditionally been grouped into serogroups. Serogroups have no taxonomic standing but useful for epidemiological understanding.

The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of *Leptospira* spp. such as the taxonomic status of the monospecific genus *Leptonema* is confirmed by DNA hybridization (81, 82). Multilocus enzyme electrophoresis data and 16S rRNA gene sequencing supported the genotypic classification of leptospires (20).

The species molecular classification of the genus *Leptospira* is based on DNA relatedness (81-83). The genus is divided into 17 species (84) which shown in the Table 1. It is defined as being at least 70% DNA-related (82). This classification related with the older serological classification in which antisera are used to establish antigenic relatedness between isolates. Leptospiral strains are referred in common to by serovar. Many serovars studied are represented by only one reference strain, and as more strains are studied the number of species is likely to increase (20). Some leptospiral serovars are commonly associated with particular animal reservoirs such as pig as animal reservoirs of *Leptospira* serovar Pomona.

Table 1 Molecular classification of *Leptospira* species

Pathogenic *Leptospira*	**Non-pathogenic *Leptospira***
L. interrogans	Genomospecie 3
L. alexanderi	*L. biflexa*
L. fainei	*L. wolbachii*
L. inadai	*L.parva*
L. kirschneri	
L. borgpetersenii	
L. weillii	
L. noguchii	
L. santarosai	
L. meyeri	
Genomospecies 1	
Genomospecies 4	
Genomospecies 5	

2.5 Epidemiology

Leptospirosis is widespread zoonosis in the world (20). The source of infection in humans is usually either direct by contact with the urine of an infected animal or indirect by contact with the contaminated soil or water. The incidence in tropical countries is higher than in temperate regions (20) because leptospires has longer survival in the warm environment and humid conditions. In addition, most tropical countries are also developing countries, and there are more chance for exposure of the human population to infected animals, whether livestock, domestic pets, or wild animals.

The disease is seasonal. In temperate regions where temperature is the limiting factor in survival of leptospires, the incidence may occur in summer or fall but the peak of incidence occurring

during rainy seasons in tropical regions.

The portal for entry of leptospires is through the skin where is abrasions or cuts or via the conjunctiva. Water-borne transmission has been documented: point contamination of water supplies has resulted in several outbreaks of leptospirosis. Inhalation of contaminated water or aerosols also may result in infection through the mucous membranes of the respiratory tract. Infection from animal bites has been demonstrated rarely (85, 86).

Animals, including humans, can be divided into maintenance hosts and accidental (incidental) hosts. The disease is sustained in nature by chronic infection of the renal tubules of maintenance hosts (87) which usually transferred from animal to animal by direct contact. Humans may become infected with the maintenance host by indirect contact. The most important maintenance hosts are small mammals, which can transfer infection to domestic farm animals and humans. Many factors may affect the transmittion of leptosires in varied extent such as climate, population density, and the degree of contact between maintenance and accidental hosts. The different serovars rodent may have disparate reservoirs species. Icterohaemorrhagiae and Ballum have rats as a reservoir. Ballum has mice as a reservoir. Domestic animals are also reservoirs such as dairy cattle acts as the reservoirs of serovars Hardjo, Pomona, and Grippotyphosa; pigs may act as the reservoirs of serovar Pomona, Tarassovi, or Bratislava; sheeps may act as the reservoirs of serovar Hardjo and Pomona; and dogs may act as the reservoirs of serovar Canicola. Distinct variations in maintenance hosts and the serovars they carry occur throughout the world.

Occupational, recreational, or avocational exposures may relate with human infection. Occupation is an important risk factor for humans (20). Several occupations are risk for leptospiral infections by direct contact such as farmers, veterinarians, abattoir workers (88), meat inspectors, rodent control workers(89), and other occupations which require contact with infected animals. Indirect contact is significant for sewer workers, miners, soldiers, septic tank cleaners, fish farmers, gamekeepers, canal workers, rice field workers, taro farmers, banana farmers, and sugar cane cutters (20). The first occupational risk group to be recognized was miner (90). The occurrence of Weil's

disease in sewer workers was first reported in the 1930s (91). Serovar Icterohaemorrhagiae was isolated by guinea pig inoculation from patients, from rats trapped in sewers (91), and from the slime lining the sewers.

Moreover, fish workers were another occupational group whose risk of leptospiral infection. 86% of all cases were fish workers which occurred in the northeast of Scotland between 1934 and 1948. Recognition of risk factors and adoption of both preventive measures and rodent control have reduced the incidence of these occupational infections greatly. There were 139 cases in coal miners, 79 in sewer workers, and 216 in fish workers in the British Isles from 1933 to 1948. However, in the period from 1978 to 1983, there were nine cases in these three occupations combined (92). More recently, fish farmers have been shown to be at risk (93), almost infected with serovars of serogroup Icterohaemorrhagiae (94), assumed to be infected from rat infestation of property.

Livestock farming is other major occupational risk factor in all directions of the world. The highest risk is related with dairy farming and is related with serovar Hardjo (95) especially with milking of dairy cattle (96). Cattle are maintenance hosts of serovar Hardjo (97) and infection with this serovar occurs throughout the world (98, 99). When leptospires infected into host, leptospires localize in the kidneys (100) and are excreted intermittently in the urine. Serovar Hardjo causes outbreaks of mastitis (101) and abortion (102). Serovar Hardjo is found in aborted fetuses and in premature calves (103). In addition, Hardjo has been isolated from normal fetuses, the genital tracts of pregnant cattle (104), vaginal discharge after calving (105), and the genital tract and urinary tract of 50% of cows (106) and bulls (107). In Australia, both serovars Hardjo and Pomona were demonstrated in bovine abortions, but serological evidence suggested that the incidence of Hardjo infection was much higher (108). In Scotland, 42% of cattle were seropositive for Hardjo, representing 85% of all seropositive animals (109). In the United States, serovar Hardjo is the most commonly isolated serovar in cattle (110) but Pomona also occurs.

Water sports may occur leptospiral infection (111) for example, swimming, canoeing (112, 113), white water rafting (114), fresh water fishing, and other sports where exposure is common, such

as potholing and caving. Pathogenic leptospires have multiplied in freshwater ponds or rivers where hot and dry weather is. Cases of leptospirosis also follow extensive flooding (115, 116).

In aquatic environment, Pathogenic serovars have been isolated (117). In the United States, serovars Icterohaemorrhagiae, Dakota, Ballum, Pomona, and Grippotyphosa have been recovered (118, 119). Pathogenic leptospires in the environment are survived depend on several factors, including pH, temperature, and the presence of inhibitory compounds. Most studies have used single serovars and quite different methodologies, but some broad conclusions may be drawn. Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8.0 but in river water survival is shorter and is prolonged at lower temperatures (120). The presence of domestic sewage decreases the survival time to a matter of hours (121), but in an oxidation ditch filled with cattle slurry, viable leptospires were detected for several weeks (122).

Avocational exposures that occur during the activities of daily life (123) may cause leptospirosis such as barefooted walking in damp conditions or gardening with bare hands (124). The significant reservoir for human infection in many tropical countries is dog (125) and may be an important source of outbreaks. The contamination of drinking water and handling rodents associated a number of outbreaks of leptospirosis (126).

Faine defined the epidemiological patterns of leptospirosis into three patterns in 1994 (20). The first occurs in temperate climates where few serovars are involved and human infection almost invariably occurs by direct contact with infected animals though farming of cattle and pigs. Control by immunization of animals and/or humans are potentially possible. The second occurs in tropical wet areas, within which there are many, more serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. Human exposure is not limited by occupation but results more often from the widespread environmental contamination, particularly during the rainy season. Control of rodent populations, drainage of wet areas, and occupational hygiene are all necessary for prevention of human leptospirosis. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods, hurricanes, or other disasters

(127). The third pattern comprises rodent-borne infection in the urban environment.

2.6 Clinical Features of Leptospirosis

Leptospirosis has been described as a zoonosis of varied manifestations. The spectrum of symptoms is extremely broad. The severe leptospirosis is called Weil's disease. An explanation for many of the observed associations may be found in the ecology of the maintenance animal hosts in a geographic region. The variety of serogroups in varied animal regions is higher than in a region with few animal hosts. The specific serovars involved depend on the geography of area and the ecology of local reservoirs. Serovars Copenhageni and Icterohaemorrhagiae in common in Europe and carried by rats, while, serovar Lai is common in Southeast Asia.

The clinical presentation of leptospirosis is biphasic. The first is acute or septicemic phase lasting about a week and the last is the immune phase, characterized by antibody production and excretion of leptospires in the urine (128). Most of the leptospirosis complications are associated with where leptospires local within the tissues during the immune phase and thus occur during the second week of the illness.

2.6.1 Anicteric leptospirosis

The majority of infections caused by leptospires are either subclinical or of very mild severity, and patients will probably not see the physician. Other symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often an inflammation of skin. If present, the rash is often temporary, less than 24 h. This anicteric syndrome usually lasts for about a week, and its resolution coincides with the appearance of antibodies. The fever may be biphasic and may return after a remission of 3 to 4 days.

2.6.2 Icteric leptospirosis

Icteric leptospirosis is a much more severe disease and the clinical course is often very rapidly progressive. In the course of the disease, the severe cases present late and have high mortality rate. The icteric form of leptospirosis have between 5 and 10% of all patients (129). The jaundice occurring in leptospirosis is not associated with hepatocellular necrosis, and liver function returns to normal after recovery (130). Serum bilirubin levels may be high, and many weeks may be required for normalization (131). Leptospirosis is a common cause of acute renal failure (ARF), which occurs about 16 to 40% of cases (131-133).

The occurrence of pulmonary symptoms in cases of leptospirosis was first noted by Silverstein (134) and later reports have shown that pulmonary involvement may be the major pathology of leptospirosis in some cases (135-138). The severity of respiratory disease is not related to the presence of jaundice (135). Patients may present with a spectrum of symptoms, ranging from cough, dyspnea, and hemoptysis (which may be mild or severe) to severe respiratory syndrome (20). In the majority of patients, intra-alveolar hemorrhage was detected (139). Pulmonary hemorrhage may be severe enough to cause death (135, 140).

Cardiac involvement in leptospirosis is common and can occur in both icteric and nonicteric cases. Clinical evidence of myocardial involvement, including abnormal T waves, was detected in 10% of 80 severe icteric cases in Louisiana (141), while similar electrocardiographic (ECG) abnormalities were detected in over 40% of patients in China, India, Sri Lanka, and the Philippines (142, 143).

2.7 Diagnosis

2.7.1 Microscopic Demonstration

Visualization of leptospires by dark-field microscopic examination of body fluids such as blood, urine, CSF or peritoneal dialysate fluid is both insensitive and lacks specificity (144). Amount of leptospires which necessary for visible by dark-field microscopy is approximately 10^4 leptospires/ml (49). Leptospires may be detected by histopathological examination in tissues, using either silver staining or immunohistochemical stains.

2.7.2 Molecular diagnosis

Various primer pairs for PCR detection of leptospires have been described. Merien *et al.* (145) described the primers which amplify a 331 bp fragment of the *rrs* (16S RNA) gene of both pathogenic and non-pathogenic leptospires, whereas the G1/G2 primers described by Gravekamp *et al.* (146) do not amplify *L. kirschneri* serovars. Leptospiral DNA has been amplified from serum (147, 148), urine (146-149), aqueous humor (150), CSF (151, 152) and tissues obtained at autopsy (153). In recently, a real-time TaqMan PCR assay was developed, using an 87 bp section of the 16S rRNA gene as its target (154). A real-time SYBR green PCR assay using the LipL32 virulence factor gene as the primers is currently to be described (155). A limitation of PCR-based diagnosis of leptospirosis is not identifying the infecting serovar. Serovar identification requires isolation of the infecting strain from patients or carrier animals.

2.7.3 Serologic diagnosis

Serology is the most frequently used diagnostic approach for leptospirosis. The microscopic agglutination test (MAT) is the standard test for serological diagnosis of leptospires. In the microscopic agglutination test (MAT), patients' sera are reacted with live antigen suspensions of

leptospiral serovars. After incubation the mixtures are examined microscopically for agglutination and the titers are determined. The MAT is demonstrated by dark-field microscopy. The end-point is the highest dilution of serum in which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end-point is determined by the presence of approximately 50% free, unagglutinated leptospires, by comparison with the control suspension (156).

2.8 Pathogenesis

The molecular mechanisms by which leptospires cause disease are not well understood. Several virulence factors such as lipopolysaccharide, various secretory proteins and outer membrane proteins may associated in pathogenesis of leptospirosis studied (4-11). Moreover, immune response to leptospires has been increasingly explored since it may be involved in induction of leptospirosis pathogenesis. Several virulence factors of leptospires such as lipopolysaccharide, hemolysin, sphingomyelinase C, Sphingomyelinase H have been studied. Lee et al. found that sphingomyelinase H could induce pores and lysis of sheep erythrocytes (7). In addition, human liver cell lines treated with sphigomyelinase-like hemolysin displayed apoptosis morphological feature (11).

Outer membrane proteins (OMPs) have also been suggested to be virulence factors of leptospires. The scientists studied about OMPs related to leptospirosis pathogenesis by demonstrated the OMPs expression in 30°C and 37°C *in vitro* and *in vivo*. The components that could express in 37°C *in vitro* and *in vivo* may be essential for leptospiral survival in host cell and may involve in leptospirosis pathogenesis. Moreover, the conservation among pathogenic leptospires was studied to confirm the involvement of pathogenesis. In addition, OMPs of pathogenic *Leptospira* were investigatged as antigens for leptospirosis diagnosis and vaccine development.

Development of leptospiral vaccine has been focused on bacteria motility, lipoproteins, outer membrane proteins and potential virulence factors. Inactivated and attenuated *Leptospira* were used (157-159); however, the immunity induced by this type of vaccine does not last very long (160). LPS

vaccine can induce side effect in hosts (161). Another disadvantage of both inactivated/attenuated and LPS vaccine was serovar-dependent (162). They do not induce cross-protection among different serovars. For these reasons, researchers have been searching for components that are conserved among pathogenic serovars that can induce cross-protection (37, 163). OMPs are the most interesting and widely studied for this purpose.

Various components of *Leptospira* have been characterized, identified and suggested to be virulence factors (4-11) however; the most widely studied components are outer membrane proteins (OMPs). OMPs are the components exposed to the environment. It is likely that OMPs can play roles in host invasion and attachment. In addition, their potentially expose to immune system on the leptospiral surface make them good candidates for the development of antigens for antibody detection and vaccine for protection. Various OMPs of *Leptospira* have been identified.

OmpL1

OmpL1 was the first leptospiral OMP to be described. It is a transmembrane OMP which functions as a porin in the leptospiral outer membrane (164). Based on crystallography studies of *E. coli* OMPs and analysis of its amino acid sequence, OmpL1 is predicted to contain transmembrane segments that traverse the outer membrane in beta conformation (165). OmpL1 was highly conserved among various pathogenic *Leptospira* strains, which have been identified by epidemic and molecular analysis (166). The structure of OmpL1 is predicted to contain at least 10 β-sheet transmembrane segments, which probably accounts for its heat-modifiable electrophoretic mobility. Its transmembrane structure is also supported by evidence that purified OmpL1 creates porin channels in the planar lipid bilayer assay (167).

LipL32

The major protein of the leptospiral outer membrane is a 32-kDa lipoprotein designated LipL32. LipL32 is one of the most highly studied leptospiral proteins. Genetic data showed that

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LipL32 gene is highly conserved in pathogenic species and is absent in closely related saprophytic species. LipL32 is expressed not only during cultivation but also during mammalian infection. Proteomics studies by Nally, J. et al. (168) using leptospires isolated from an individual with the severe pulmonary form of leptospirosis showed that LipL32 was expressed during infection. Haake DA et al. (30) demonstrated the intense LipL32 expression in *L. kirschneri* infecting proximal tubules of hamster kidneys. LipL32 is also a prominent immunogen during human leptospirosis.

The involvement of LipL32 in host immune response induction has been reported. Pathogenic leptospiral outer membrane proteins activated nuclear factor-KB (NF-KB) and downstream gene expression in mouse medullary thick ascending limb cells (18). LipL32 induced a robust inflammatory response in cultured renal proximal tubule cells through a nuclear factor-kappaB-related pathway. Addition of the LipL32 preparation from a serovar *shermani* strain to mouse proximal renal tubule cells *in vitro* caused a dose-dependent expression of MCP-1 (monocyte chemoattractant protein-1), RANTES (Regulated on Activation Normal T Cell Expressed and Secreted), nitrite, tumor necrosis factor alpha, inducible nitric oxide synthase, and augmented nuclear binding of nuclear factor kappa B and AP1 transcription factors (33). These studies demonstrated that LipL32 was involved in cytokine and chemokine induction which may be involved in the pathogenesis of tubulointerstitial nephritis.

In addition, several reports suggested that LipL32 may act as an optimal antigen molecular candidate in the serodiagnosis of leptospirosis since it is the most abundant OMP and it is conserved among pathogenic *Leptospira*. Xiang-Yan Zhang et al. (32) reported that LipL32 coding gene was highly conserved among all the pathogenic leptospires including three epidemic species; *L. interrogans* serovar Lai, *L. interrogans* serovar Copenhageni and *L. kirschneri*. It was demonstrated by immunoblot assay that this gene was expressed conservatively in most cultured epidemic leptospires. Furthermore, mRNA expression of the conserved LipL32 gene was detected in six virulent *Leptospira* strains tested including five *L. interrogans* strains and one *L. borgpetersenii* strain. Boonyod D. et al. (31) demonstrated LipL32 gene expression in pathogenic *Leptospira* serovar Autumnails, Ballum, Bataviae, Bratislava, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa,

Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Panama, Pomona, Pyrogenes, Sejroe but not in non-pathogenic *L. biflexa* serovar Patoc. In addition, they demonstrated that LipL32 protein could be used as an antigen in a dipstick method for detection of *Leptospira* antibody in sera from leptospirosis patients. Sensitivity and specificity of the Lip32 dipstick assay, when compared to those of MAT, were 100% and 98.33%, respectively. Barnett JK et al. (21) found that LipL32 seroreactivity had sensitivity levels of 37 and 84% in detecting leptospiral infection during the acute and convalescent phases of illness, respectively.

Tahiliani P. et al. (35) compared the utility of three antigens; a gel-purified recombinant protein (rLipL32), secreted proteins and whole organism sonicate of *Leptospira spp.* in an ELISA assay. Their data suggested that gel-purified LipL32 is a valuable antigen for early and accurate diagnosis of leptospirosis since it gave the highest sensitivity and specificity of detection compared with other two types of antigens. Fernandes C.P. et al. (169) immunized BALB/c mice with recombinant LipL32 to develop murine monoclonal antibodies (MAbs). Their results suggested that the MAbs produced can be useful for the development of diagnostic tests based on detection of LipL32 leptospiral antigen in biological fluids.

Molecular diagnosis detecting LipL32 gene has been reported. Bomfim M.R. et al. (170) demonstrated the detection of LipL32 gene in urine from naturally infected cattle by using polymerase chain reaction. In addition to diagnostic tool development, the study of LipL32 as a vaccine candidate has been reported. Seixas F.K. et al. (163) demonstrated that a monoclonal antibody specific to LipL32 was inhibited the growth of *Leptospira in vitro*, indicating potential protection induced by the LipL32 antigen. Moreover, DNA vaccine encoding LipL32 induced protection in an animal model challenged with *Leptospira* (171).

LipL36

LipL36, a 36-kDa leptospiral outer membrane lipoprotein is a lipid modified at its amino-terminal cysteine residue (172). Another unusual feature of the LipL36 sequence is the abundance of

alanine residues. Almost 16% (55 of 344) of the residues in the mature LipL36 protein are alanines, and 25 of these alanine residues are arranged in pairs or triplets. Alanine containing polypeptides form unusually stable alpha-helices. Roughly 38% of the LipL36 sequence is predicted to be alpha-helical by Chou-Fasman analysis, and 30% (39 of 131) of the residues in the alpha-helical regions are alanines.

LipL36 was shown to be expressed by leptospires growing *in vitro* but not within the mammalian host. Haake et al. (172) analysed the humoral immune response to LipL36 during *L. kirschneri* infection and suggested that LipL36 expression is downregulated during mammalian infection. Nally et al. (173) observed the decrease of a 36-kDa detergent phase protein when the organisms were shifted from 30 to 37°C. Immunoblotting with LipL36 antiserum was performed to assess whether this thermoregulated protein was LipL36. LipL36 protein was expressed at 30°C but was switched off when cultures were shifted to 37°C. LipL36 remained switched off at 37°C, and shifting the cultures from 37 back to 30°C restored its expression.

Paul A. Cullen (5) reported that LipL36 was expressed at 20°C and significantly downregulated at 30°C but were not apparent at temperatures above 30°C or after iron depletion at 30°C. On the other hand, lipoprotein LipL36 was found not suitable as leptospirosis vaccine. LipL36 is a 36 kDa leptospiral outer membrane lipoprotein, which is synthesized at 30°C, but not at 37°C *in vivo* (173). These reports demonstrated that production of LipL36 was downregulated in host adapted leptospires, suggesting that it may not be involved in pathogenesis after mammalian host entry.

LipL48

LipL48 sequence encodes a membrane lipoprotein with a 25-amino-acid signal peptide, a lipoprotein signal peptidase cleavage site, and an amino-terminal cysteine. The mature protein would be 436-amino acid long, with a predicted molecular mass of 44.1 kDa, similar to the observed molecular mass of 48 kDa. Peal A. Cullen (5) reported that the expression of LipL48 significantly downregulated at 37°C in the presence of fetal calf serum which suggest that the expression of LipL48

was downregulated during mammalian infection. For this reason, similar to LipL36, LipL48 may not be a good candidate for diagnostic tool and vaccine development.

LipL41

Natarajaseenivasan et al. (164) investigated the presence of LipL41 in pathogenic *Leptospira* by using the PCR. It was revealed that LipL41 gene is conserved among the pathogenic leptospires. Ellen S. Shang et al. (174) studied the level and distribution of LipL41 expression using immunoblot analysis performed with a panel of *Leptospira* species and with antiserum from a rabbit immunized with purified His6-LipL41. They found that the LipL41 antiserum is reactive with a single band with a molecular mass of 41 kDa and the molecular weight and amount of LipL41 expressed among pathogenic *Leptospira* species are highly conserved.

LipL41 has also been interested as a candidate protein for serodiagnosis and vaccine development for leptospirosis. Mariya et al. (175) studied the efficiency of the recombinant LipL41 antigen for the detection of bovine anti-leptospires antibodies using ELISA and found that the sensitivity of recombinant LipL41 protein based ELISA was 100%. This suggested that LipL41 ELISA was as effective as MAT in detecting true positive samples. However, the specificity of LipL41 ELISA was only 85.3%.

Ruan P. et al. (176) constructed prokaryotic expression systems of ltB/ctB-lipL41/1 fusion genes. LtB-lipL41/1 and ctB-lipL41/1 fusion genes and their prokaryotic expression systems were successfully constructed and the two expressed fusion proteins showed immunogenic and adjuvant activities. They concluded that rLTB-rLipL41/1 or rCTB-rLipL41/1 seemed to have good potential to serve as an antigen in *L. interrogans* genus-specific vaccine.

LipL21

The pL21 amino acid sequence was found to contain a 17-amino-acid signal peptide and a

putative lipoprotein signal peptidase (LSP) cleavage site. In accordance with standard nomenclature for leptospiral lipoproteins, the protein was renamed LipL21 (6). LipL21 is a surface-exposed, abundant outer membrane lipoprotein that is expressed during infection and conserved among pathogenic *Leptospira* species. It could not be detected in saprophytic strains. Cullen et al. (6) used a 400-bp *lipL21* probe to detect the presence of similar DNA sequences in a panel of pathogenic and environmental leptospires by high stringency Southern Hybridization and found that single copy of the *lipL21* gene was present in all five pathogenic *Leptospira* species analyzed (*L. interrogans* serovar Lai, *L. interrogans* serovar Pomona, *L. kirschneri* serovar Grippotyphosa, *L. kirschneri* serovar Mozdok, *L. borgpetersenii* serovar Hardjo, *L. santarosai* serovar Bakeri, and *L. noguchii* serovar Proechymis). However, no hybridizing bands were detected when genomic DNAs from the environmental leptospires *L. biflexa* serovar Patoc and *L. wolbachii* serovar Biflexa were probed. However, the expression of LipL21 did not appear to vary between the virulent and culture-attenuated strains included in the Western blotting experiments. The uniform expression of LipL21 may indicate that this protein endows the pathogenic members of the genus *Leptospira* with a selective advantage both in the mammalian host and in laboratory cultures. They also determined whether LipL21 is expressed during infection and is immunogenic. Sera were collected from four hamsters inoculated with leptospires from infected hamster liver, three hamsters challenged with virulent *L. kirschneri* RM52, and one uninfected hamster. In Western blotting experiments, all seven infected hamster sera recognized recombinant LipL21, while the uninfected control sera showed no reaction. In addition, MAT positive human sera recognized recombinant LipL21. No reactivity with LipL21 was observed when sera from non-leptospirosis patients. The study suggests that LipL21 is expressed during infections of both hamsters and humans. Development of LipL21 PCR for leptospirosis has been reported (177). According to reports mentioned above, LipL21 is another attractive OMP for further developing a novel universal vaccine as well as detection kit of leptospirosis.

2.9 Immune response in *Leptospira* infection

The second stage of acute leptospirosis is also referred to as the immune phase, in which the disappearance of the organism from the bloodstream coincides with the appearance of antibodies. The clinical severity of the disease often appears to be out of proportion to the histopathological findings. Immunemediated disease has been proposed as one factor influencing the severity of the symptoms. Levels of circulating immune complexes were correlated with severity of symptoms (178), and in patients who survived, circulating immune complex levels fell concurrently with clinical improvement. However, in experimental infections in guinea pigs, leptospiral antigen localized in the kidney interstitium, while immunoglobulin G (IgG) and C3 were deposited in the glomeruli and in the walls of small blood vessels (179). The pathogenesis of equine recurrent uveitis appears to involve the production of antibodies against a leptospiral antigen which cross-react with ocular tissues (180, 181). Retinal damage in horses with uveitis is related to the presence of B lymphocytes in the retina (182). Antiplatelet antibodies have been demonstrated in human leptospirosis (183, 184). In leptospirosis such antibodies are directed against cryptantigens exposed on damaged platelets and do not play a causal role in the development of thrombocytopenia. Other autoantibodies have been detected in acute illness, including IgG anticardiolipin antibodies (185) and antineutrophil cytoplasmic antibodies (186). However, the significance of antineutrophil cytoplasmic antibodies in the pathogenesis of vascular injury in leptospirosis has been questioned (132). Virulent leptospires induce apoptosis *in vivo* and *in vitro*. In mice, apoptosis of lymphocytes is elicited by LPS via induction of tumor necrosis factor alpha (TNF-α) (187).

The several reports suggested that pathogenic *Leptospira* might be associated with the cytokine induction. Elevated levels of inflammatory cytokines such as TNF-α have been reported in patients with leptospirosis (188). Vernel-Pauillac F. found that *Leptospira. interrogans* serovar Icterohaemorrhagiae could be trigger the induction of IL-4, IL-10, IL-12p40, TNF-α, IFN-γ, TGF-β in blood of infected hamsters (15). Dorigatti F. et al. suggested that Glycolipoprotein of *Leptospira interrogans* serovar Copenhageni could induce the production of TNF-α and IL-10 in peripheral blood mononuclear cells (16).and could be induced IL-6 in whole blood (17). De Fost M. et al.

reported that *Leptospira interrogans* serovar rachmati induced the production of gamma interferon (IFN-γ) and the IFN-γ-inducing cytokines interleukin-12p40 (IL-12p40) and tumor necrosis factor alpha (TNF-α) in human whole blood *in vitro* (13). When they studied about the level of IP-10 in the plasma concentrations in leptospirosis patients, they found that the level of IP-10 in leptospirosis patients were higher in leptospirosis patients than in healthy blood donors (29). Tian Y.C. et al. found that *Leptospira santarosai* serovar Shermani OMP could be increase collagen type I and type IV by increase TGF-β1 secretion in human renal proximal tubular cells (19). Yang C.W. suggested that LipL32 which was the outer membrane protein of pathogenic *Leptospira* could be induced the production of chemokine MCP-1, RANTES, iNOS, and TNF-α at both the mRNA and the protein levels in mouse proximal tubule cells (33). Several cytokines are involved in immune response against infection. Four cytokines, TNF-α, TGF-β, IL-10 and IP-10, were chosen to study in this study.

Tumor necrotic factor alpha (TNF-α)

TNF-α is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages. Although macrophages are the major source of TNF-α, activated T cells, NK cells, mast cell, lymphoid cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neuronal tissue also secrete this cytokine. At low concentrations, TNF-α has diverse biologic properties. These include the ability to increase the expression of many cell adhesion molecules, such as ICAM-1, which is important in causing leukocyte extravasation, leading to their migration to sites of tissue damage. TNF-α stimulates endothelial cells and macrophages to secrete cytokines called chemokines that induce leukocyte chemotaxis and recruitment. It also acts on mononuclear phagocytes to stimulate secretion of IL-1 which functions much like TNF itself. A locally increasing concentration of TNF will cause the cardinal signs of Inflammation to occur: Heat, swelling, redness and pain. At higher concentrations, TNF-α enters the bloodstream and becomes a potent mediator of inflammatory responses to bacteria. However, very high concentrations of TNF-α can cause severe pathologies resulting from disseminated intrasascular coagulation, shock, and tissue damage that can lead to death.

Transforming growth factor beta (TGF-β)

TGF-β constitutes a superfamily of multifunctional cytokines with important implications in morphogenesis, cell differentiation, and tissue remodeling (189). It is a homodimeric protein that is synthesized as a precursor and is activated by proteolytic cleavage. TGF-β is secreted by T cells, B cells, monocyte/macropahge, and many other cell types. It has effects on a wide variety of cell types such as macrophages, endothelial cells and T and B lymphocytes. The principle function of TGF-β is to inhibit immune and inflammatory response. It inhibits T cell proliferation and effector function, B cell proliferation and IgA production, and macrophage activation (189). TGF-β has many diverse actions outside the immune system. It may inhibit proliferation of some cell types and stimulate others. TGF-β causes synthesis of extracellular matrix proteins such as collagens. This action may promote tissue repair after local immune and inflammatory reactions have been controlled. Currently, TGF-β has been increasingly studied as a cytokine secreted by regulatory T cells.

Interleukin-10 (IL-10)

IL-10, also known as a human cytokine synthesis inhibitory factor (CSIF), is anti-inflammatory cytokine. It is produced primarily by monocytes and to a lesser extent by lymphocytes (mainly regulatory T cells). IL-10 has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines and MHC class II, and costimulatory molecule expression on macrophages. It also enhances B cell survival, proliferation, and antibody production (190). IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines like IFN-γ, IL-2, IL-3, TNF-α and GM-CSF made by cells such as macrophages and the Type 1 T helper cells (191).

IL-10 also displays potent abilities to suppress the antigen presentation capacity of antigen presenting cells. However, it is also stimulatory towards certain T cells, mast cells and B cells. A study in mice has shown that interleukin-10 is also produced by mast cells, counteracting the inflammatory effect that these cells have at the site of an allergic reaction. IL-10 is released by cytotoxic T-cells to inhibit the actions of NK cells during the immune response to viral infection

(192). IL-10 is usually considered along with TGF-β since both are anti-inflammatory cytokines and are expressed by regulatory T cells.

Interferon-gamma-inducible protein 10 (IP-10)

Migration of immune cells at sites of antigenic challenge or lesions is mainly mediated by chemotactic cytokines called chemokines. Interferon-gamma inducible-protein- 10 (IP-10) is a C-X-C cytokine that belongs to the subfamily of chemokines and is secreted by T cells, monocytes, endothelial cells, fibroblast and keratinocytes. IP-10 exerts a chemotactic effect on activated T lymphocytes and, monocytes and NK cells. It also has antitumor activity mediated by its angiostatic features, inhibiting tumor neovascularization and promoting damage in established tumor vasculature followed by necrosis *in vivo* (193).

Four cytokines are selected for investigation in this study since their involvement in leptospirosis has been reported. The study of cytokine expression following infection can provide information on host immune response to that infection. The understanding of immune response to certain infection can lead to the development of vaccine that induces better immune response. Induction of immune response, especially cytokine production, not only results in eradication of organisms but also, in some diseases, can lead to pathogenesis at the organs where robust immune response occurs. Pro-inflammatory cytokines initiate immune response and chemokines are involved in recruitment of immune cells to the sites of infection. The expressions of these two groups of cytokines at the infected sites indicate immune response. TNF-α and IP-10 were pro-inflammatory cytokine and chemokine, respectively, chosen to investigate in this study. TNF-α expression was induced by *Leptospira* or *Leptospira* components (15-18, 21, 169). Its expression was also detected in blood of *Leptospira*-infected hamsters (15). The level of IP-10 has been shown to increase in leptospirosis patients (29). Anti-inflammatory cytokines control the immune response. Down-regulation or deficiency of this group of cytokines can result in uncontrolled immune response. Both TGF-β and IL-10 are inflammatory cytokines and both are secreted from regulatory T cells. Although they both regulate immune response, as mentioned above, the mechanisms of these two

cytokines in regulation of immune response are different. Their expressions were detected in blood of infected hamsters. However, TGF-β expression was increased since 8 hours post infection whereas IL-10 expression was increased on day 3 post infection (15). This study investigated both IL-10 and TGF-β expression to demonstrate whether there is the difference between these two anti-inflammatory cytokines in kidneys and livers of *Leptospira* infected hamsters.