CHAPTER II

LITERATURE REVIEW

2.1 History

In 1886, Weil described a severe icteric disease which appeared to be a unique clinical illness (30). The etiology of Weil's disease, as it was later designated, however, remained obscure until Inada *et al.* successfully cultivated the causal agent, a spirochete, in 1915 (31). He called this organism *"Spirochaeta icterohaemorrhagiae"*, in deference to the icterus and haemorrhagic phenomena which were prominent in infected patients. Actually, Stimson (32) had identified the same organism unknowingly two years earlier within sections of kidney taken from a patient who had been diagnosed incorrectly as a case of yellow fever.

Shortly after the identification of *Leptospira icterohaemorrhagiae* in man, the significance of the rodent both as a vector and reservoir of icterohaemorrhagiae became apparent. Within a few years, the prevalence of icterohaemorrhagiae in rat populations was recognized in many parts of the world. In 1917 (33), Ido and coworkers published that the rat *(Rattus norvegicus)* was the carrier of *Spirochaeta icterohaemorrhagiae* and the causative agent of Weil's disease. In the United States, Noguchi (34) isolated this organism from a Norwegion rat in 1917 and Wadsworth *et al.* (35) reported the first case of human

Weil's disease associated with rat exposure in 1922. In Thailand, the first case of human leptospirosis was reported by Yunibandha in 1943 (3). For many years, the rat was considered the sole animal host of *icterohaemorrhagiae*, even though Randall and Cooper (36) isolated this agent from a naturally infected dog. Subsequently, *icterohaemorrhagiae* has been associated with many animal hosts, including cattle, swine, goats, hamsters and dogs; however the rat remains the primary reservior in most parts of the world (37). Numerous other serovars, involving human and animals, were discovered throughout the world (33). They are summarized in Table 1.

2.2 Characteristic of *Leptospira*. (4, 39, 67)

Leptospira differ from *Treponema* and *Borrelia* in that their spirals are very fine and close. The genus *Leptospira* comprises thin, 0.1μm in breadth and 6-20μm in length, tightly coiled spirochaetes which are characterized by very active motility, both rotating (Spinning) and bending. Usually one or both ends of the cell are bent or hooked, but straight form also occur which rotate and travel more slowly than the hooked forms. Because of narrow diameter, the leptospires are best visualized by dark-ground illumination or phase-contrast microscopy. Leptospira as short as 4μm and as long as 40μm are occasionally seen (40).

The genus *Leptospira* shares the following basic morphological features with other spirochaetes. Surrounding the leptospiral cell is a 3-5 layered membrane, refered to as the outer membrane or outer envelope (OE). The membrane is thick, almost transparent and appears in the darkground as a narrow clear zone or halo. The term protoplasmic cylinder (PC) describes the cellular components enclosed by the OE,and consists of a peptidoglycan layer and a cytoplasmic membrane. There are generally two axial filaments (flagella) per cell located between the OE and PC, one at each end of the cell (41). They are attached to the PC in a subterminal position and the free ends extend toward the middle of the cell but do not overlap. The flagellar basal bodies resemble those of Gram-negative bacteria. During cellular reproduction, septal wall formation occurs at the middle region of the organism, which leads to transverse division (42). The free-living *(L. biflexa)* and parasitic leptospires *(L. interrogans)* are morphologically indistinguishable.

The unique motility of *Leptospira*, combined with their narrow diameter, flexibility and shape, allows them to pass through 0.1-0.45 μm pore-sized membrane filters and to migrate within media solidified with up to 1% agar.

Table 1 : List of various serovars in *Leptospira* discovered on the world (33).

Year	Serovar of *Leptospira*	Origin
1923	pyrogenes	Man; Indonesia
1926	bataviae	Man; Indonesia
1925	autumnalis	Man; Japan
1928	grippotyphosa	Man and field mice; Russia
1931	andaman A	Man; Andaman islands
1933	canicola	Dogs; Netherlands
1937	australis A, B	Man; Australia
	pomona	Man; Australia
1939	sejroe	Man; Denmark
1944	saxkoebing	Man; Denmark
	ballum	Man; Denmark
	pomona	Swine; Switzerland
1948	hyos	Man and swine; Switzerland, Australia and Argentina

2.3 Cultivation

Leptospires are obligate aerobes. When cultivated in a suitable aerated medium at 30°c, their generation time varies between 7-12 hours and populations of 6-8 x 10^9 cells per ml may be obtained. The nutritional requirements of *Leptospira* are unique. Vitamins B_1, B_{12} and long-chain fatty acids are the only organic compounds that are mandatory. Fatty acids provide their major source of carbon and energy, and are also required as a source of cellular lipids since *Leptospira* cannot synthesize fatty acids de novo. Owing to the inherent toxicity of free fatty acids, these must be supplied to the leptospires either bound to albumin or in a nontoxic esterified form. Carbohydrates are not a suitable source of energy or of carbon. Amino acids are utilized to a limited extent, they cannot satisfy the nitrogen requirements of these organisms and ammonium salts are effective source of cellular nitrogen. The nonessential nutrient, pyruvate, enhances the initiation of growth of the parasitic leptospires, particularly in serovars such as hardjo and ballum. Leptospires incorporate purine but not pyrimidine bases into their nucleic acids (15). Consequently, they are resistant to the antibacterial activity of the pyrimidine analogue 5-fluorouracil leading to the use of this compound in the selective media for the isolation of leptospires from contaminated sources (43).

The earliest report of the cultivation in vitro of pathogenic leptospires is that of Inada *et al.* (31). Since then, various kinds of media including liquid, semisolid and solid media have been developed and used for diagnostic or research purposes, for instance, special media such as those enriched with rabbit serum (44, 45, 46), bovine serum albumin (BSA) (47, 48) as EMJH medium, and protein-free medium (49). The optimal pH for the growth and survival of the leptospires is around pH 7.4 (50). Liquid media are necessary for growing the cultures (for serological diagnosis of the infection) and for typing the isolates. Semisolid media are generally used for isolating strains and for the maintenance of stock cultures whereas solid media are useful for cloning the strains and for isolating leptospires from contaminated sources (51). The colonies in 1% agar are subsurface and become visible within 7-14 days in most serovars. Media containing pyruvate are required to obtain colonial growth of some of the more fastidious serovars.

2.4 Taxonomy

Since the first isolation of the etiological agent of Weil's disease by Inada and Ido (31) in 1915, many different serovars of leptospires have been isolated all over the world. *Leptospira* is classified in the Order of Spirochaetales, Family Leptospiraceae (42). This family has only one genus, *Leptospira*. In order to make the classification of leptospires conform to the International Code of Nomenclature of Bacteria and Viruses, the WHO Scientific Group on Leptospirosis and the Taxonomic Subcommittee on *Leptospira* have recommended the recognition of two species: *L. biflexa* representing saprophytic strains and *L. interrogans* representing parasitic strains (6, 54). The third species, originally classified as *Leptospira illini* (4),has now been reclassified as *Leptonema illini* on the basis of morphological and DNA differences (28). The best criteria available for the separation of the two species are the ability of *L. interrogans* to infect mammals, their serological differences, and the relative resistance of the saprophytic group to the bacteriostatic action of divalent copper ions (54). In addition, saprophytic leptospires grow readily in simple media without serum, whereas parasitic leptospires usually require serum or other growth factors. Strains of the species *L. biflexa* show a stronger oxidase reaction than do strains of the species *L. interrogans* and *L. biflexa* can grow at 13°c and in media containing 225 μg/ml of 8-azaquanine (4, 55). Serological methods have been used to identify and classify leptospires as serovars. Those serovars that have a close serological relationship and yet show individual antigenic

differences have been grouped together in serogroups although this is not a taxonomic subdivision. The concept of leptospiral serogroups has practical value for the selection of antigens and antisera in the examination of sera and identification of isolates for diagnostic and epidemiological investigations. About 180 serovars in 19 serogroups have been described to date (56).

The identification and classification of newly-isolated strains are performed mostly by agglutination tests and the cross-agglutination-absorption test with immune rabbit sera (57). Two strains are considered to belong to different serovars if, after cross-absorption with adequate amount of heterologous antigen, 10% or more of the homologous titer regularly remains in each of the two antisera in repeated tests (58). This definition is based on the use of antisera prepared in rabbits. A sub-serovar has been defined as "a strain within a serovar where in repeated tests less than 10% of the homologous titre remains in one antiserum but 10% or more in the other antiserum after cross-absorption with adequate amounts of heterologous antigen within the serovar (58).

2.5 Leptospiral Antigens

Many different antigenic preparation have been extracted from leptospires. These antigens have been described and classified according to their anatomical site or serological-reactions. The serologically reactive antigen will be included under "serological tests for leptospirosis". The

present review will concentrated on the study of anatomical antigen of *Leptospira* including axial filament (flagella) and outer envelope antigen.

2.5.1 Axial filament antigen

In 1970, Chang and Faine (41) extracted axial filament antigen using 1% sodium deoxycholate. It was heat labile and appeared to be homogeneous under electron-microscopy (59). Isolated axial filament in purified suspension were 15 nm. wide (ranged 14 nm-16 nm) and varied in length. The filament possessed ultrastructure of both helical and longitudinal types. Immunodiffusion against rabbit anti-axial filament serum or anti-whole-leptospira serum showed one sharp precipitin line which is identifiable with one of the three antigens extracted by trypsin (Graves and Chang, unpublished data), and one of the five antigens from the deoxycholate extract of Yanagawa and Faine (60). Upon immunoelectrophoresis of the axial filament against homologous antiserum, a single antigen line appeared, moving slightly to the cathode at pH 7.2.

In 1969 (61), Nauman *et al.* identified six protein bands when acid-dissociated leptospiral flagella were subjected to polyacrylamide gel electrophoresis (PAGE). This is in contrast to other bacterial flagella which usually possessed a single band. However, the antigenicity of these protein components was not studied, nor was their flagella origin proved. In 1988, Kelson *et al.* (28) studied the composition of leptospiral flagellae by SDS-PAGE and immunoblotting to determine

their immunogenicity and cross-reactivity. Immunoblotting analysis revealed that rabbit antiserum prepared against the doublet band of Mw 33-34 kd of flagellar preparation from serovar pomona reacted strongly with both the Mw of 33-34 and 37 kd bands of flagellar antigen prepared from three *L. interrogans* serovars, as well as with the 35-36 kd and the 33-34 kd doublet of *L. biflexa* serovar patoc and *L. illini* respectively. These results showed that leptospiral flagellae are immunogenic and contain antigens which are conserved among the different genera of the family Leptospiraceae.

2.5.2 Outer envelope antigen

Tightly coiled leptospires have an outer envelope (OE) or enveloping sheath (62, 63, 64, 65). An intact OE is essential for survival of the organism because it encloses the protoplasmic cylinder containing the nuclear and cytoplasmic contents of the leptospire (29). The OE is approximately 11 nm in width and composed of three to five electron-dense layers. Hypertonic conditions cause the leptospire OE to separate from the protoplasmic cylinder and to assume the shape of a sphere (67). According to Palit *et al.* (68), a 90% ethanol precipitate following 50% ethanol extraction of *L. biflexa* strain Patoc 1 contained the erythrocyte-sensitizing substance (E.S.S.). This acted as a genus-specific heamagglutination antigen and was associated with the OE. There has been conflicting results concerning the protective role of OE antigen. According to Auran and Bey (69, 70) OE antigen was both immunogenic and able to confer protection against experimental infection. Furthermore,

anti-OE antibody may be protective since the OE antibody-complement reaction can cause damage in the OE leading to the death of the organism (64).

In contrast, Jost *et al.* in 1988 (29) reported that the OE antigen of leptospire was a protein Mw of 35 kd as revealed in western blotting against monoclonal anti-OE antibodies but this protein antigen was not responsible for protection. These monoclonal antibodies did not agglutinate whole leptospires, could not opsonise homologous leptospires for phagocytosis by mouse macrophages, nor protect new-born guinea-pigs against lethal infection. This is in accordance with Takashima and Yanagawa's finding that the protein fraction of OE was relatively ineffective as a protective antigen (71).

2.6 Infection and Epidemiology

Leptospirosis is one of the most widespread zoonoses in the world. Pathogenic leptospires are parasites of both wild and domestic animals, the former including rats, dogs, pigs, cattle, deers and foxes (5,72, 73, 74). It has been reported that parasitic leptospires were isolated from reptiles and birds (6). Infection in human is an incidental occurence in which the leptospires are transmitted either by direct contact with blood, tissues, organs or urine of infected animals, or indirect exposure to an environment contaminated with leptospires. The infectious organism can survive in such environment as wet ground and fresh water of low bacterial contamination, neutral or alkali urine or environmental water (5,

73, 75, 76, 77). The site of entry of the leptospire may be skin, mucous membrane such as conjunctivae, nasal cavity and vagina, including unbroken skin if it is softened by long exposure of water (4). Veneral transmission is important in rodents and can occur in livestock. Transplacental infection of the foetus in utero is well-known in livestock and experimental animals.

Leptospirosis has been recognised as occupational health hazard associated with farmers, fish handlers, abattoir workers, garbage collectors, and public-health labourers (79) and also associated with people who walk in contaminated stagnant water. Clinical manifestations may vary from very mild to severe depending upon infected serovar and patients' condition (77, 80). General symptoms consists of fever, myalgia, headache, injected conjunctiva, muscle tenderness, hepatomegaly, renal failure and jaundice etc.(2). In 1984, Faine *et al.* reported a fatal intrauterine human infection by *L. interrogans* serovar hardjo (81). Persons of all ages and sexes are susceptible to infection. Adult men are more frequently infected because they tend to work in high-risk jobs (82).

Leptospiral infection may occur in any month of the year, but the incidence of the disease usually fluctuates with season. In temperate climates, the infections are more common in the warm months. In subtropical and tropical climates more cases may occur in association with period of heavy rainfall and crop-raising cycles. The number of cases in a region often

varied from year to year. This fluctuation may be due to variations in rainfall, flooding, density of rodent population and incidence of leptospiral infection in animals.

The situation of Leptospirosis in Thailand has also been studied. In 1966, Sundharagiati *et al.* (82) reported an incidence of 23-35% leptospiral seropositive agglutination reactions in Thailand with the highest incidence occurring in the south. The prevalence of *Leptospira* among rats and dogs in the provinces of Thailand including Bangkok was also fairly high (83, 84) and 12 serovars of Leptospira have been isolated from man and animals. In 1983, a seroepidemiological study of leptospirosis in man and rodents in the north and northeast of Thailand revealed 0.27% serological positive cases of human population tested (79).

Leptospirosis occurs less frequently as large outbreaks or epidemics over a limited period of time. The large outbreaks typically involve a group of people who have been immersed in flood or who have gathered together to engage in a common activity such as harvesting crops or swimming. For example, in 1988, an outbreak of leptospira was reported in the southern part of Thailand. The incidence of leptospirosis increased between November and December concomitant with seasonal heavy rainfalls and floods (85).

2.7 Immunological Response

The agglutinating antibody response in human leptospirosis has been extensively studied and used for diagnosis. In general, leptospiral agglutinating antibodies appear 7-8 days after the onset of the disease (5, 86); reach their peak after 15-20 days and remain stable for a most variable period, ranging from a few months to as much as 20 years (82, 87); these are followed by a gradual decline with low levels persisting for an indefinite period.

Studies to evaluate the increase of the agglutinating antibodies and their relationships with several immunoglobulins have been undertaken since circulating antibodies seem to play an important role in the defense against leptospirosis (88). In 1965, Pike *et al.* (89) found that the predominant homologous agglutinins in four patients for whom serological test had indicated infection with serovar canicola, grippotyphosa or pomona, were IgM. IgM antibodies appear first, and remain for several weeks before IgG antibodies can be demonstrated (90). Antibodies of both Ig classes, derived from either human (90) and cattle (91) sera, have been shown to be protective in passive protection tests.

In 1964, Faine (92) demonstrated the major role of fixed phagocytes of the reticuloendothelial system in clearing pathogenic leptospires from the blood of mice and the opsonizing role of specific antibody. This was also confirmed in the study of macrophages and polymorphonuclear neutrophils by Faine *et al.*

(93) although, no in vitro opsonizing effect of antiserum on the phagocytosis of leptospires by human monocytes was observed by Rose (94). In 1984, Wang *et al.* (95) revealed that human neutrophils were able to ingest and kill nonpathogenic *L. biflexa* but not pathogenic *L. interrogans* even in the presence of normal serum. Only when the organisms have been opsonized by specific antibodies did human monocytes, macrophages and neutrophils possess the capacity to ingest and kill pathogenic *L. interrogans* serovar icterohaemorrhagiae (96). These results indicate that the opsonizing effect of specific antibody may play an important role in the mechanism of host defense against leptospirosis.

Apart from the humoral immune response, the cell mediated immune response (CMIR) to leptospirosis was shown to appear earlier and might be useful in the early detection of the disease (97).

2.8 Laboratory Diagnosis

2.8.1 Isolation of Leptospirae

Leptospires can be isolated from the blood, cerebrospinal fluid, urine or organs of dead or sacrificed animals. Generally the organisms are present in the blood and cerobrospinal fluid only during the first 10 days of clinical illness, although isolation has occasionally been made from samples obtained as late as the 19th day (78, 98). Leptospires usually appear in the urine at the beginning of the second week of the disease and may persist for 30 days or longer.

Many different media have been developed for the cultivation of leptospires (5). For routine use, Fletcher's semisolid medium or EMJH semisolid medium is recommended (100). For blood culture, the specimens should be collected during the leptospiremic stage, prior to treatment with antimicrobial therapy. It is advisable to vary the number of drops of blood inoculated in each tube of cultivation medium since an excessive amount of blood may inhibit growth of leptospires. If the seeding cannot be affected immediately after taking the sample, it is preferable to add the blood to an anticoagulant buffer solution. Cultures are then incubated at 28 to 30°c in the dark for 5 to 6 weeks or longer, and examined by dark-field microscopy. Cerebrospinal fluid culture is carried out in the same manner.

For urine culture, it is essential that urine be taken under sterile conditions by means of a catheter. If the urine is acid, the culture medium must be checked to ensure that it contains sufficient buffer to maintain its neutral or slightly alkaline reaction when the urine is added. Even with care, cultures from urine very often become contaminated. The contaminated specimens or suspension of primary cultures in which contaminants are suspected must be cultured indirectly utilizing a test animal in order to isolate leptospires. Generally, guinea-pigs and hamsters are the animals of choice and are inoculated via the subcutaneous or intraperitoneal routes. The test animals are observed for the development of illness or death. In either event, immediate phlebotomy or necropsy should

be performed and sections of liver, kidney and brain obtained. Suspensions of these tissue in buffered saline and blood samples are then recultured in an appropriate semisolid medium for several weeks in the usual manner.

2.8.2 Serological Tests

Since cultures may be positive only during the first 10 days of illness and may require several weeks to cultivate, laboratory diagnosis often depends on the demonstration of antibodies to leptospires in the sera. Thus, a variety of tests have been developed (Table 2). At first, the agglutination tests were performed. They are classed as microscopic (read by a low-power dark field microscope) or macroscopic (read by naked eyes) agglutination. The microscopic agglutination test (79, 82) requires multiple serovars of live leptospira responsible for the infection as the antigens. The maintenance of a battery of live cultures to provide the antigens are necessary (6). Thus, the risk of infection and time-consumption limit the usefulness of this test for routine application in diagnostic laboratories. However, the microscopic agglutination test is still the standard reference test because of its high specificity and sensitivity (101, 102).

Table 2 : Serological tests for determination of leptospiral antibodies

Tests	Antigen	Comments
1. Microscopic agglutination (MA) test* Bubudieri(5)	live leptospires	- high specificity and sensitivity of test. - need a battery of live leptospires - risk of infection - time-consuming
2. Macroscopic agglutination test Galton(7) and Stonner(8)	pooled formalinized leptospires	- stability of antigen - simplicity of test to perform - less sensitive and not suitable for epidemiological survey
3. Complement fixation(CF) test Randall(103)	sonicated leptospiral antigen	
Schneider(104)	serologically active "fraction I" (heat stable and non-protein antigen)	- technical complexity of test - instability of reagents
Ezell(105)	ethanol soluble antigen (type-specific, heat stable and non-protein antigen)	
Terzin(9)	acetone-extracted antigen	

Tests	Antigen	Comments
4. Hemolysis(HL) test. Cox(11, 106)	ethanol-extracted antigen (Erythrocyte sensitizing substance, E.S.S. antigen)	- high sensitivity, broad specificity and simplicity of test - useful in epidemiological survey - need complement activity and serum absorption with sheep erythrocytes.
5. Heamagglutination (HA) test* Chang and McComb(107)	ethanol-extracted antigen (E.S.S.Antigen) sensitized on fresh human erythrocytes	- sensitivity and reliability of test - showing genus-specific reaction - stability of sensitized erythrocytes - ease of performance - suggesting that only IgM antibodies took part in the reaction
Baker(108)	ethanol-extracted antigen (E.S.S.Antigen) sensitized on glutaraldehyde-fixed erythrocytes	
Palit(13)	ethanol-extracted antigen (E.S.S.Antigen) sensitized on fresh sheep erythrocytes and formalinized sheep erythrocytes	
6. Latex agglutination (LA) test Murachi(109)	formalinized leptospires	- non-infective antigen - high sensitivity, specificity, and rapidity of test - easily employing - stability of reagents.
Kelen(110)	pyridine-extracted antigen	
7. Microcapsule agglutination test Yoshiko(101)	sonicated leptsopiral antigen	

Tests	Antigen	Comments
8. Indirect immunofluorescent (IF) assay* Torten(12)	fixed-whole cell leptospires	- showing genus-specific reaction - the simplicity and rapidity of assay - suitable for antibody screening - need of fluorescent microscope and specific training
9. Enzyme-linked immunosorbent assay (ELISA)* Adler(111)	sonicated leptospiral antigen	- highly sensitive and specific serological assay - detecting of both IgM and IgG antibodies
Hartman(112)	outer envelop antigen	- showing genus-specific reaction
Terpstra (14, 113)	heat-extracted antigen	- stability of antigen - highly sensitive and specific serological assay - detecting of both IgM and IgG antibodies

*These serological tests are still used for determination of leptospiral antibody at present.

The antibody response is only detectable after the first week of leptospiremia (5, 16) or it may remain negative even in fatal case (17). This poses problems in diagnosis. Consequently, Adler *et al.* (18) in 1982 attempted to develop tests for the detection of leptospiral antigen (*L. interrogans* serovar hardjo) in which the best sensitivity obtained by ELISA was 10^5 leptospires per ml, and 10^4 to 10^5 organisms per ml by radioimmunoassay (RIA). Nonetheless, this has not been performed in the clinical specimen.

In 1987, an immunoperoxidase (IP) staining procedure was applied to detect leptospiral antigen on human livers and kidneys collected from autopsies or biopsies. IP stained leptospiral antigen was demonstrated in portal spaces of the liver, engulfed by cells of the mononuclear phagocyte system, in the interstitium of the kidney, and living vessel walls of both organs (17).

In 1985 and 1988, a newly IgM-specific dot ELISA test using ethanol-extracted leptospiral antigen was shown to be comparable to the classic MA test in its ability to detect antibody against leptospires (15, 114). It appeared to be sensitive and specific for the serodiagnosis of acute leptospirosis. In addition, it is inexpensive, simple to perform, utilizes only minute volumes of killed antigen and is easily adapted to field use.