IDENTIFICATION OF MARKERS FOR SERODIAGNOSIS OF LEPTOSPIROSIS IN MALAYSIA

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IDENTIFICATION OF MARKERS FOR SERODIAGNOSIS OF LEPTOSPIROSIS IN MALAYSIA

by

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LIST OF ABBREVIATIONS & ACRONYMS

mA	milliampere
µA	micro-ampere
C3	complement
CD14	cluster of differentiation antigen 14
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (a zwitterionic detergent)
DNA	deoxyribonucleic acid
CSF	cerebrospinal fluid
DTT	1,4 dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EAF	eluted antigen fraction
EMJH	Ellinghausen Mc Cullough Johnson Harris
ESI	electrospray ionization
FT	whole leptospiral freeze thaw antigen
G+C	guanine cytosine content
HRP	horseradish peroxidase
IEF	isoelectric focusing
IPG	immobilized pH gradient
pI	isoelectric point
kDa	kilo dalton
cLigA	C-terminal portion of recombinant immuneglobulin-like antigen A
L44	*Leptospira interrogans* serovar Icterohaemorrhagiae
L55	*Leptospira interrogans* serovar Javanica
LPS	lipopolysaccharide
MAT	microscopic agglutination test
MS	mass spectrometry
MW	molecular weight
NCP	nitrocellulose paper
OE	outer envelop
OMP	outer membrane proteins
PBS	phosphate buffer saline
rOmpL1	recombinant OmpL1
rRNA	ribosomal ribonucleic acid
RF	rheumatoid factor
SEB	sequential extraction buffer
SEQ	urea-thiourea sequential extraction
SAP	surface associated protein
SDS	sodium dodecyl sulfate
TBS	tris buffer saline
TEMED	*N,N,N',N'*- tetramethyethylenediamine
TOF	time of flight
TX-114	Triton X-114 extraction

UCDE	ultracentrifugation and detergent extraction
UPM.	Universiti Putra Malaysia
ddH_2O	double distilled water
mW	milliwatt

PENGENALAN PENANDA UNTUK PENDIAGNOSAN SEROLOGI LEPTOSPIROSIS DI MALAYSIA

ABSTRAK

Leptospirosis adalah salah satu penyakit yang semakin menular di kalangan populasi manusia dan haiwan seluruh dunia terutama di kawasan beriklim tropika. Penyakit ini merupakan masalah kesihatan awam yang penting disebabkan ketiadaan ujian yang baik bagi mengesan penyakit ini di peringkat awal. Pengesanan awal adalah penting untuk mengelakkan komplikasi serius pada satu atau lebih organ di dalam tubuh. Walaupun kaedah pengkulturan dan *microscopic agglutination test (MAT)* diiktiraf sebagai *gold standard* bagi diagnosis leptospirosis, namun ia memerlukan masa dan kepakaran dalam pengendaliannya dan tidak membawa sebarang kelebihan dalam diagnosis awal penyakit ini. Oleh yang demikian, kajian ini telah dijalankan bagi mengesan komponen antigenik bagi bakteria *Leptospira* untuk menemukan kaedah pengesanan yang lebih sensitif dan spesifik.

Pendekatan proteomik telah digunakan bagi memperoleh, memisah dan mengenalpasti komponen antigenik bakteria *Leptospira* yang bertindak balas secara spesifik dan sensitif terhadap sampel serum pesakit yang telah disahkan menghidapi leptospirosis akut. Dua jenis *Leptospira* pembawa penyakit di Malaysia iaitu Icterohaemorrhagiae (L44) dan Javanica (L55) telah digunakan bagi penyediaan antigen. Sejumlah 93 sampel serum telah dibahagikan kepada empat kategori berdasarkan keputusan tiga jenis ujian. Beberapa bahan pelarut seperti

acidic glycine, TritonX-114, urea, thiourea, Tris dan *CHAPS* telah digunakan dalam penyediaan lima jenis antigen bagi memaksimumkan jumlah epitop antigenik; dan kaedah SDS-PAGE, elekroforesis dua dimensi (2-DE) dan blot Western telah digunakan bagi mengkaji profil antigen yang reaktif.

Sejumlah enam lajur imunoreaktif yang terdiri daripada dua lajur kembang (*diffuse*) iaitu 10-15, 15-20 kDa serta empat lajur terasing iaitu 15, 44, 63 dan 72 kDa telah dikenalpasti. Pencirian kimia bagi lajur reaktif telah dijalankan menggunakan *periodate* dan *proteinase K*, dan keputusannya menunjukkan lajur kembang tersebut merupakan *lipopolysaccaride* (LPS) dan lajur terasing mempamerkan sifat mirip kepada protin.

Ujian imunoblot telah mengesan antibodi IgM terhadap antigen LPS 10-15 kDa di dalam 80.4% sampel serum pesakit leptospirosis. Ini merupakan penemuan yang signifikan memandangkan sampel serum pesakit yang diuji mengandungi antibodi *leptospiral heterologous* kerana ianya diambil daripada pesakit di hospital di pelbagai lokasi di Malaysia. Spesifisiti antigen ini melebihi 95% yang menunjukkan tindak balas silang yang sangat rendah dengan serum bagi manusia normal dan pesakit *febrile* lain dari kawasan endemik yang tidak menghidapi leptospirosis. Keputusan yang hampir sama juga dapat diperhatikan dalam *dot enzyme immunoassay (dot EIA)* yang dihasilkan dengan antigen LPS 10-15 kDa yang terelusi. Di antara antigen protin, lajur antigenik 72 kDa daripada sediaan *sequential extraction* (SEQ) dan lajur 15 kDa daripada sediaan *freeze-thaw* (FT) menunjukkan tahap sensitiviti (masing-masing 83.3% and 85.7%) dan spesifisiti (masing-masing 95.2% and 93.3%) yang sama tinggi. Pencirian lanjutan dengan *mass-spectrometry* telah mengenapasti calon teratas adalah seperti berikut: 72 kDa sebagai protin *Leptospira interrogans heat-shock DnaK* dan 15 kDa sebagai protin leptospira *putative* yang belum

dicirikan. Blot Western menggunakan protin rekombinan 72 kDa (r72SEQ) dan panel serum daripada pesakit leptospirosis serta serum kawalan, menujukkan bahawa protin rekombinan tersebut adalah 85% sensitif and 80% spesifik.

Sebagai rumusan, kajian ini telah mengenalpasti satu antigen LPS dan dua protin antigen sebagai penanda jangkitan yang berpotensi bagi pengesanan antibodi IgM *anti-leptospira* secara sensitif dan spesifik di dalam serum pesakit leptospirosis akut terutama di kawasan endemik di Malaysia.

IDENTIFICATION OF MARKERS FOR SERODIAGNOSIS OF LEPTOSPIROSIS IN MALAYSIA

ABSTRACT

Leptospirosis is one of the emerging diseases of both humans and animals worldwide, particularly in the tropics. It is a major public health problem due to the lack of diagnostic facilities for early detection which is key in preventing serious multi-organ complications. Although culture and microscopic agglutination test (MAT) are gold standard, they require time and expertise and are not useful for early diagnosis. Thus this study was performed to identify antigenic components of *Leptospira* for sensitive and specific serodiagnosis of the disease.

Proteomic approach was used for extraction, separation and identification of leptospiral antigenic components that reacted specifically with serum samples of patients with confirmed acute leptospirosis. Two pathogenic *leptospira* serogroups prevalent in Malaysia namely Icterohaemorrhagiae (L44) and Javanica (L55) were used for antigen preparations. A total of 93 serum samples were divided into three categories namely leptospirosis, non-leptospirosis and healthy control sera. Several solubilizing agents such as acidic glycine, TritonX-114, urea, thiourea, Tris and CHAPS were used in five antigen preparations to maximize the number of antigenic epitopes; while SDS-PAGE, two-dimensional electrophoresis (2-DE), and Western blot were performed to analyse the immunoreactivity profiles.

Six immunoreactive bands including two diffuse bands of 10-15, 15-20 kDa and four single bands of 15, 44, 63 and 72 kDa were identified. Chemical characterization of the reactive bands using periodate and proteinase K indicated that the diffuse bands were lipopolysaccharide (LPS) and the single bands were protein in nature. The immunoblot assay detected IgM antibodies against the 10-15 kDa LPS antigen in sera of 80.4% acute leptospirosis patients. This is significant since the patients' samples, from hospitals in various locations in Malaysia, contained leptospiral heterologous antibodies. The specificity of the antigen was above 95% indicating very low cross-reactivity with the serum of healthy people as well as with non-leptospiral febrile patients from endemic regions. Similar results were observed on dot enzyme immunoassay (dot EIA) developed using optimized concentration of eluted 10-15 kDa LPS marker. Among the protein antigens, 72 kDa antigenic band from sequential extraction (SEQ) method and 15kDa band from freeze thaw (FT) method showed similar high sensitivities (83.3% and 85.7% respectively) and specificities (95.2% and 93.3% respectively). Further characterizations by mass-spectrometry identified the top identities of the proteins were as follows: 72 kDa as heat-shock DnaK protein and 15 kDa as a putative uncharacterized protein of *Leptospira interrogans*. Western blot using recombinant protein of the 72kDa antigen (r72SEQ) and a serum panel from patients and controls showed that the recombinant protein was 85% sensitive and 80% specific.

In summary, this study has identified one LPS and two protein antigens as potential infection markers for detection of specific anti-leptospiral IgM antibodies in the

serum of acute leptospirosis patients particularly in the endemic environment of Malaysia.

CHAPTER 1

INTRODUCTION

1. 1 Background

Leptospirosis is recognized as the most widespread zoonotic infection in the world, with over 500,000 severe human cases and over 10% death reported annually (WHO, 2003). It is caused by spiral-shaped bacteria (spirochetes) of the genus *Leptospira*, referred to as leptospires, which infect a variety of wild and domestic animals. Infected animals are capable of carrying the bacteria and spreading the disease among others of their own kind, and to other species including man. It is an important cause of abortion, stillbirth, infertility, decreased milk production and death in animals reared for its milk and meat, thus may be responsible for great economic losses in some countries (Haake *et al.*, 1998; Bharti *et al.*, 2003).

The disease is endemic in tropical countries particularly in Southeast Asia including Malaysia. As much as 80% of the population in these areas was seroconverted indicating either past or present infection. Awareness of its public health importance has increased following reports of outbreaks associated with recreational exposures in USA, Costa Rica and recently in several other countries as a result of the Eco-Challenge Sabah 2000 competition in Malaysia (Sejvar *et al.*, 2003). During these outbreaks, confusion between the wide range of clinical

presentations associated with leptospirosis and other hemorrhagic fever (e.g. dengue) caused delay in the early diagnosis required for the timely administration of antibiotic therapy (Sanders, *et al.,* 1999*;* Levett *et al.,* 2000; Ellis *et al.,* 2008). The need for rapid and appropriate diagnostic tests has become ever more urgent to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations.

In Malaysia most cases are acquired by direct or indirect exposure to urine of reservoir animals through contaminated soil, mud and water particularly during flood seasons. A number of outbreaks have occurred during events such as the Eco-Challenge Sabah 2000, and recently among National Service trainees in Melaka, and in Johor following severe rainfall and flooding (Sejvar *et al.,* 2003; Koay *et al.,* 2004). There were also reports of mortalities in those outbreaks causing serious public health concern.

1.2 History of leptospirosis

Leptospirosis was first described by Adolph Weil in 1886 when he reported an "acute infectious disease with enlargement of spleen, jaundice and nephritis". Weil published the first description of leptospirosis in man in 1886 (Weil, 1886). Although the human form of leptospirosis was given the name Weil's disease, it was several years later that the disease was recognized in livestock. In 1907 Stimson demonstrated the spirochete in silver stained renal tissue section for the

first time from a patient who died of a so-called “yellow fever” (Stimson, 1907). He proposed the name *Spirochaete interrogans,* as the organism with its hooked ends resembled a question mark. The spirochete was first isolated in Japan by Inada and co-workers in 1915 (Inada *et al.,* 1916).

The importance of occupation as risk factor was recognized much earlier. In ancient China the disease was known as “wei ni” or rice-harvest jaundice, in Australia it was called “cane-cutter’s disease” and other names such as “swine-herd’s disease” and “mud fever”. The role of the rat as a source of human infection was discovered in 1917 (Ido, 1917).

In Malaysia, Fletcher was the first person who began working on human leptospirosis. He reported the first fatal case of human leptospirosis in Malaysia in 1925 due to *Leptospira interrogans* serovar Icterohemorrhagiae (Fletcher, 1928). During this early period he identified serovar Icterohemorrhagiae, Hebdomadis and Pyrogenes from 21 patient samples (El Jalii and Bahaman, 2002). In 1926, Galloway detected four cases of human leptospirosis in Singapore, which at that time was a state in Malaysia. In 1927, Fletcher and Kanagarayer were able to demonstrate leptospires in four patients from Kuala Lumpur General Hospital. Typical cases with jaundice were well recognized in Malaysia particularly among military personnel and farmers (Danaraj, 1950). These cases had drawn attention to leptospirosis as an emerging disease in Malaysia (Broom, 1953). Robinson and Kennedy (1956)

detected cases of leptospirosis among British army personnel in Malaysia. In 1957, McCrumb *et al*. studied febrile illness in military personnel operating in the jungle during the Malayan Emergency where they indicated leptospirosis was the most common cause of fever in soldiers, with up to 35% of cases admitted to a military hospital.

1.3 Bacteriology

1.3.1 Structure and Morphology

Leptospires are tightly coiled spirochetes, usually 0.1 µm by 6 µm to 0.1 µm by 20 µm, but cultures may occasionally contain much longer cells (Faine *et al*., 1999). The cells have pointed ends which are usually bent into a distinctive hook shape (Figure 1.1). A pair of axial filaments lying in the periplasmic space and around which the bacterium is 'coiled' acts as endoflagella enabling it to move by turning and wriggling. Leptospires are gram negative but too thin to be visible under the ordinary microscope. Dark-field microscopy is most often used for leptospires observation. All leptospires look alike with only minor differences; therefore, morphology does not help to differentiate between pathogenic and saprophytic leptospires or between the various pathogenic leptospires. Leptospires have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane (Haake 2000). Leptospiral lipopolysaccharide has a composition similar to that of other gram-negative bacteria but has lower

endotoxin activity (Priya *et al.*, 2008). The most outer layer of leptospires consist of outer envelope (OE) which is composed of protein, lipid and lipopolysaccharide (LPS) moieties.Various antigens,notably those associated with the LPS are located in the outer envelopOE region (Haake, 1991).

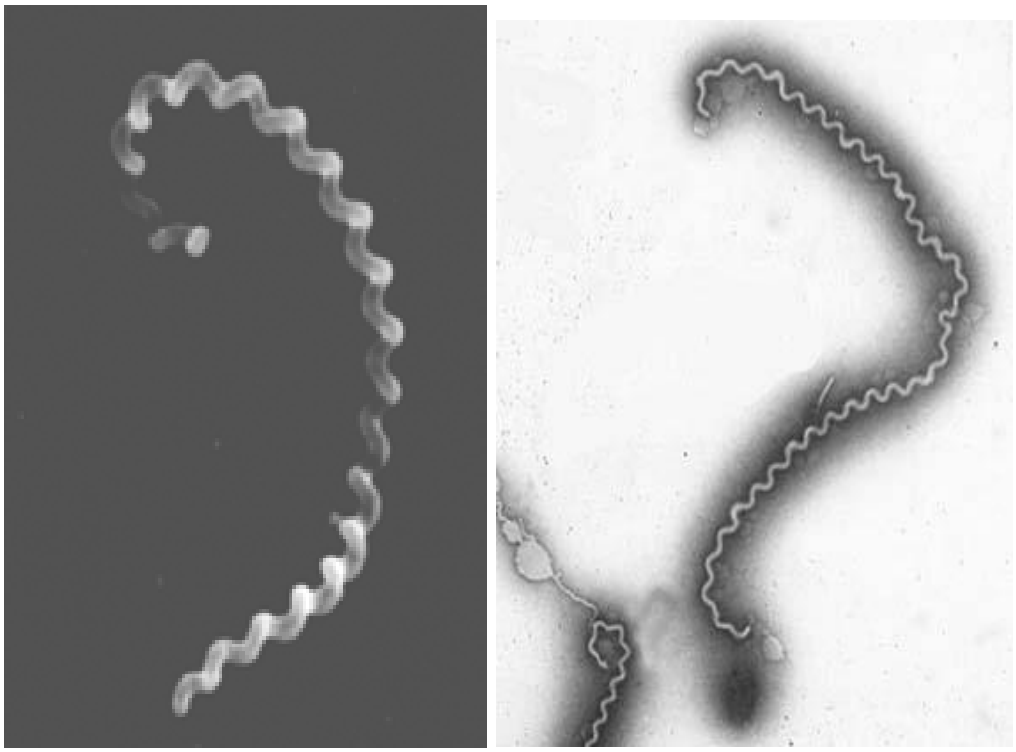

Figure 1.1 Scanning electron micrograph of *Leptospira* spp. showing the corkscrew appearance of the bacterium. (www.cherokeeanimalclinic.com)

1.3.2 Taxonomy and Classification

The order Spirochaetales has three families namely Brachyspiraceae, Spirospiraceae and Leptospiraceae. Within Leptospiraceae there are only three genera i.e. *Leptonema, Turneria* and *Leptospira*. Prior to 1989, the genus *Leptospira* was divided into two species based on antigenic classification namely pathogenic *Leptospira interrogans* and saprophytic *Leptospira biflexa* (Faine, 1982). Both *L. interrogans* and *L. biflexa* are further divided into numerous serovars that is the basic taxon used to classify these bacteria and is defined by agglutination after cross-absorption with homologous antigens (Dikken *et al.,* 1978; Faine, 1984; Kmety, 1993; Gustavo, M., & Mathieu, P., 2009). Over 60 serovars of *L. biflexa* have been recorded **(**Faine, 1984**)**. Serovars having antigenic similarities are formed into serogroups, and there are over 230 pathogenic *L. interrogans* serovars which are divided into 25 serogroups (Table 1.1). Different strains with small antigenic differences can sometimes be found within certain serovars. Identification and classification of *Leptospira* species is important because some species have host preferences.

During recent years the taxonomy of leptospires has undergone a state of transition from an antigenic to a genetic classification. The genetic classification is based on DNA-DNA hybridization which has revealed considerable heterogeneity in pathogenic species. This method has been the basis of the division of leptospires into genomospecies (Yasuda *et*

al., 1987; Ramadass *et al*., 1990; Ramadass *et al*., 1992; Perolat *et al*., 1998; Brenner *et al*., 1999; Romero *et al*., 2006). Based on the genetic classification as determined by DNA-DNA hybridization, there are currently 20 species of *Leptospira*. These species can be further divided into pathogenic, non-pathogenic and opportunistic/ possibly pathogenic. Pathogenic Leptospira species include: *L. interrogans, L. kirschneri, L. santarosai, L. weilii, L. alexanderi, L. borgpetersenii, L. alstonii* and *L. noguchii*. Non pathogenic Leptospira include: *L. biflexa, L. meyeri, L. kmetyi, L. vanthielii, L. terpstrae, L. wolbachii,* and *L. yanagawae*. Opportunistic/intermediate Leptospira include: *L. broomi, L. fainei, L. inadai, L. licerasiae,* and *L. wolffii* (Faine et al., 1999; Hawrami and Breuer., 1999; Nogva and Lillehaug., 1999; Levett et al., 2001; Slack et al., 2006; Gustova & Mathieu., 2009).

Although antigenic classification has been replaced by a genetic one, the former is still widely accepted since serovar is the basis for taxonomy at the subspecies level (Ellis, 1991).

Table 1.1 Serogroups and some serovars of *Leptospira interrogans* sensu lato (Levett, 2001).

Serogroup	**Serovar(s)**
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai, Zimbabwe
Hebdomadis	Hebdomadis, Jules, Kremastos
Autumnalis	Autumnalis, Fortbragg, Bim, weerasinghe
Pyrogenes	Pyrogenes
Bataviae	Bataviae
Grippotyphosa	Grippotyphosa, Canalzonae, Ratnapura
Canicola	Canicola
Australis	Australis, Bratislava, Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe, Saxkoebing, Hardjo
Panama	Panama, Mangus
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini, georgia
Tarassovi	Tarassovi
Ballum	Ballum, aroborea
Celledoni	Celledoni
Louisiana	Louisiana, Lanka
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

1.3.3 Growth and metabolic activities

All leptospires are chemoorganotrophs, growing in aerobic or microaerophilic environments and using oxygen as the final electron receptor. Cytochrome c, catalase, and oxidase are present (Green *et al.*, 1967). The only major source of carbon and energy is long-chain fatty acids using beta oxidation metabolic process, and these are essential to trigger growth (Henneberry, 1970). In addition, CO_2 is also required for growth.

The growth curve of leptospires follow the same stages as seen in other bacteria, except that the time scale is longer. A typical generation time for pathogenic leptospires is about 6-8 hours. However saprophytic leptospires grow faster in about 2-3 days as compared to the pathogenic ones that require about 4-7 days. They are aerobic, so oxygen is required and cultures benefit from gentle aeration or shaking. The yield from cultures may be increased as much as one log by aeration.

Saprophytic leptospires will grow at 11-13ºC, although optimum development occurs at 28-30ºC (Johnson & Harris, 1967). The optimum growth of pathogenic leptospires is also 28-30ºC. However they will not grow at 13ºC. Other than differentiating the two species by growth at 13 ºC, the two species can be also differentiated by the fact that, unlike *L. interrogans*, the saprophytic *L. biflexa* can grow in the presence of 8-azaguanine and its failure to form spherical cells in 1M NaCl.

A primary concern for cultivating samples is concentration-induced lysis where the lipases produced by the bacteria begin to outweigh the absorptive ability of the culture, and the formation of toxic lipids. This can be very rapid either during the last portion of the log curve or the stationary phase, and can cause damage to the cultures if they are not sub-cultured and diluted often enough (Adler Adler & de la Pena Moctezuma *et al.*, 2010).

1.3.4 Culture methods

Leptospires are the only member of spirochete family which can be cultured on an artificial medium enriched with animal serum, long-chain fatty acids and vitamins. Growth characteristics for *Leptospira* differ from those of many other bacteria, for example, the optimal temperature for growth *in vitro* is 28–30°C, even though they are pathogenic for mammals with higher body temperatures (Faine *et al.*, 1999).

Leptospires are obligate aerobes that grow well in liquid and semisolid medium. They are slow growers with long generation interval of 6–16 hours, requiring 7–10 days for a normal culture to grow in full (Faine, 1999). Growth of leptospires is also slow in a patient's primary sample, and cultures need to be retained for up to 13 weeks before reported as negative. A fully grown culture (approximately $5x10^{7}$ -10^{8} leptospire/ml) in liquid media appears turbid to the naked eye. Leptospires do not settle

well on standing in liquid medium due to low cell mass and rapid motility of leptospires.

The most important advancement in leptospire *in-vitro* culture was the successful growth demonstration by Ellinghausen and McCullough (1965). Leptospires would grow in a medium containing long-chain fatty acids as nutritional source, provided serum albumin was present as a de-toxicant. This medium was modified later by Johnson and Harris (1967) and became known as Ellinghausen-McCullough-Johnson-Harris or EMJH medium that is commonly used for growing leptospires in the laboratory.

1.3.4(a) Semi-solid culture media

Leptospires grow well in tubes of semi-solid media containing 0.1-0.3% agar or gellan gum **(**Rule and Alexander**.** 1986). A drop or two of the leptospiral culture are inoculated onto the surface of the medium or stabbed below the agar surface. Growth in semi-solid medium will appear as a white disc below the agar surface known as Dinger's disc, about 0.2-0.5 mm thickness (Figure 1.2).

1.3.4(b) Liquid culture media

Liquid media is the standard medium for leptospiral culture, and the bacteria seem to prefer it over the other types. Maximum concentrations are smaller than in other bacteria, and often reach only 10^7/ml or 10^8/ml, and the medium will have an obvious turbidity. Leptospires are extremely

small and highly motile, so cultures that are not stirred will not settle out, although there can be precipitation of waste and nutrient material.

1.3.4(c) Solid culture media

On agar plate (1%), leptospires appear as subsurface opaque colonies of variable sizes (Figure 1.3). The medium must be protected against dehydration, and even with the best temperature and gas conditions it can take 3-4 weeks to see the growth with the naked eye.

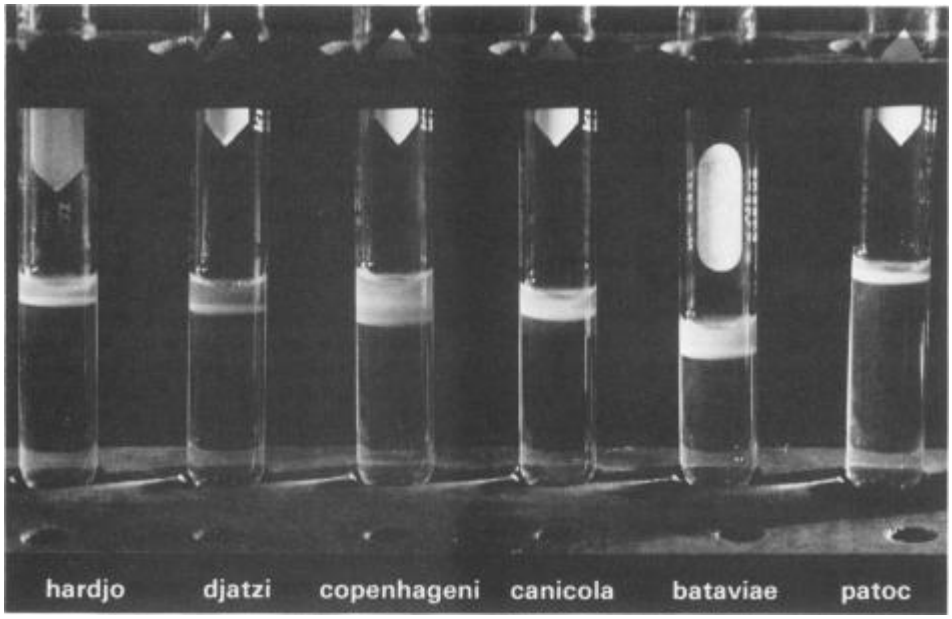

Figure 1.2 Ten-day-old growths of leptospiral strains in EMJH semisolid medium gelled with gellan gum (Rule and Alexander**.** 1986).

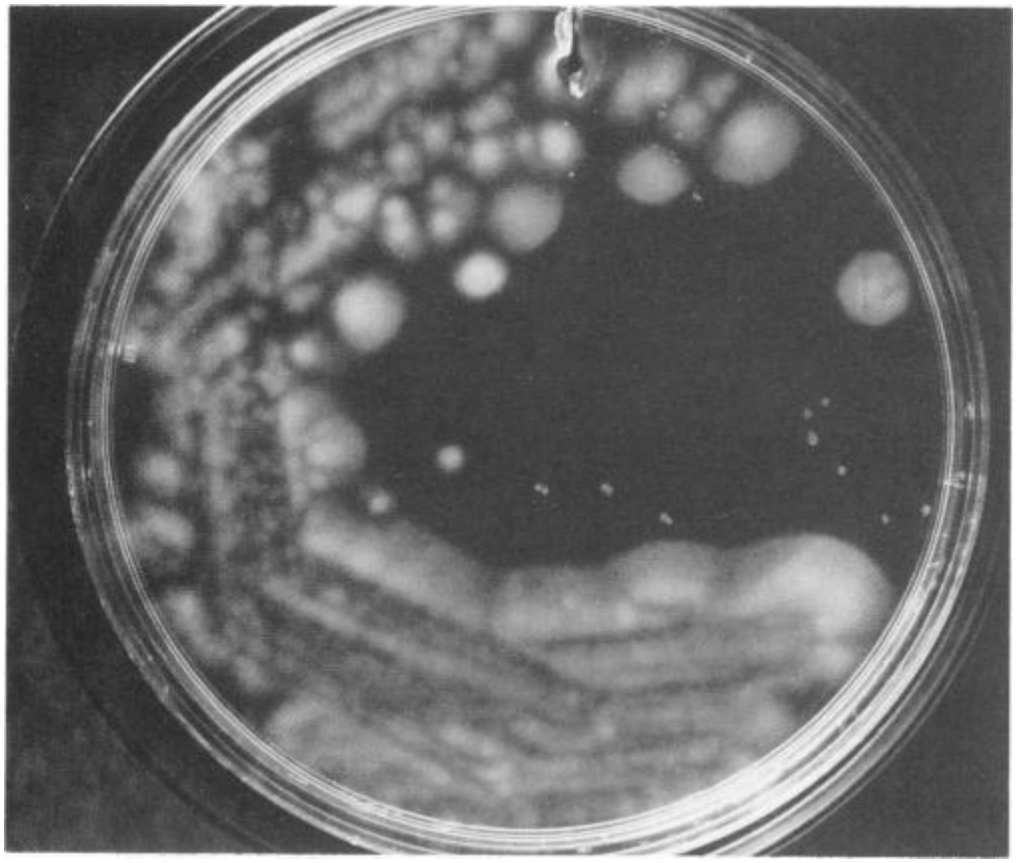

Figure 1.3 Twenty-one-day-old culture of *L. interrogans* serovar *hardjo* grown in EMJH medium gelled with gellan gum **(**Rule and Alexander**.** 1986).

1.3.5 Molecular biology aspects of *Leptospira*

Molecular biology has been widely used in areas such as genetic taxonomy, molecular epidemiology, and molecular diagnosis. Leptospires are phylogenetically related to other spirochetes (Paster *et al*, 1991**)**. Genetic taxonomy involves DNA-based classification using DNA/DNA hybridization and the G+C percentage content of DNA. The use of quantitative DNA-DNA hybridization to measure DNA-relatedness among leptospiral strains is the reference method for allocating strains to species. The sequence of *rrs* gene, coding for 16S rRNA, is the most commonly used and accepted for phylogenetic studies (Perolat *et al.*, 1998). Yasuda *et al.* (1987) proposed a new classification of the genus *leptospira* based on a DNA homology study on 46 pathogenic and non-pathogenic serovars. These authors proposed seven new genospecies i.e. *L. borgpetersoni, L. inadai, L. noguchii, L. santarosai, L. weilii, L. meyeri,* and *L. wolbachii.* Ramadass *et al.* (1992) analyzed 66 serovars of potentially pathogenic leptospires by DNA hybridization, and obtained data in close agreement with that of Yasuda *et al.* (1987). In addition, *L. kirschneri* was proposed as a new genospecies. DNA homology methods have the disadvantage of being complicated for routine use, hence a number of other DNA fingerprinting techniques have been developed that are simpler such as restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE).

In RFLP analysis, the DNA sample is broken into pieces by restriction enzymes and the resulting restriction fragments are separated according to their lengths by electrophoresis. However, in PFGE is a technique used for the separation of large DNA molecules by applying an electrical field that periodically changes direction to a gel matrix. The PFGE method is commonly considered a gold standard in epidemiological studies of pathogenic organisms.

Genome sequencing of *L. interrogans* serovar Lai and Copenhegeni have been completed which can facilitate understanding of molecular mechanisms of leptospiral pathogenesis and also to help to identify novel vaccine and diagnostic marker candidates. The leptospiral genome consists of two circular chromosomes of approximately 5,000 kb in total size (Baril *et al.,* 1990; Zuerner, 1991; Boursaux-Eude *et al.,* 1998; and Picardeau *et al.,* 2008). The genome is larger compared with the genomes of other spirochetes such as *Treponema* spp and *Borrelia* spp which indicates the ability of *Leptospira* spp to live within diverse environments (Fraser and Fraser *et al.,* 1997). The genome comprises of a 4,400-kb chromosome and a smaller 350-kb chromosome (Zuerner, 1991). Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene and the rRNA genes are widely spaced (Fukunaga *et al.,* 1989; Baril *et al.,* 1992).

The study of leptospiral genetics is hampered by the lack of a transformation system (Zuerner *et al.*, 1993; Kalambaheti *et al.*, 1999). Little is known about genetic exchange among *Leptospira*, although lateral transfer has been suggested (Pena-Moctezuma, 1999). Recently, a shuttle vector was developed using the temperate bacteriophage LE1 from *L. biflexa* (Saint Girons *et al.*, 2000). This might provide a better understanding of *Leptospira* at the molecular level. Another limitation to the genetic analysis of these bacteria is the lack of accessory genetic replicons (linear or circular plasmids). Therefore, much of the research on the molecular biology of *Leptospira* has used molecular cloning and heterologous expression of genes in suitable bacterial hosts.

A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis, rRNA, ribosomal proteins, RNA polymerase, heat shock proteins, outer membrane proteins, flagellar proteins and lipopolysaccharide (LPS) synthesis (Dohert *et al.*, 1989; Fukunaga, and Mifuchi 1989; Ding and Yelton, 1993; Shang *et al.*, 1996; Lin *et al.*, 1999 and 1997; Pena-Moctezuma *et al.*, 1999; Zuerner *et al.*, 2000; Renesto *et al.*, 2000; Bulach *et al.*, 2000).

The use of PCR technique in epidemiological studies provides a novel basis for typing leptospires using primers from species or strain-specific sequences (Gravekamp *et al.*, 1993; Murgia *et al.*, 1997).

1.4 Epidemiology

Leptospirosis has a worldwide distribution due to a wide range of animal reservoirs (Faine *et al.*, 1999). Figure 1.4 shows the sources and cycles of leptospiral infection. The pathogenic leptospires shed in the urine of the carrier animals contaminate the environment and cause human and animal infections throughout the world. Rats are the main reservoir host but other wild and domestic animals are also known to perform this function. Reservoir hosts can cross-infect each other *via* urine or congenitally, and sexual transmission is known to occur in many species. Humans are accidental host and are not capable of becoming carriers.

Leptospires are naturally aquatic organisms and are found in fresh water, damp soil, vegetation, and mud. They survive in these environments for months or even years, thus allowing re-infection among the animal hosts. Flooding after heavy rainfall may spread the organism because, as water saturates the soil, leptospires pass directly onto surface waters. The organisms die when dry, or in acid conditions (pH <7.0). The sources of non-occupational leptospirosis in temperate climates are mainly rodents and dogs and are spread through leisure activities and travel (Faine, 1999). Leptospirosis is considered an occupational hazard in farmers and veterinarians as well as dairy, slaughterhouse and sewer workers, miners, rice and sugarcane field workers, and military personnel.

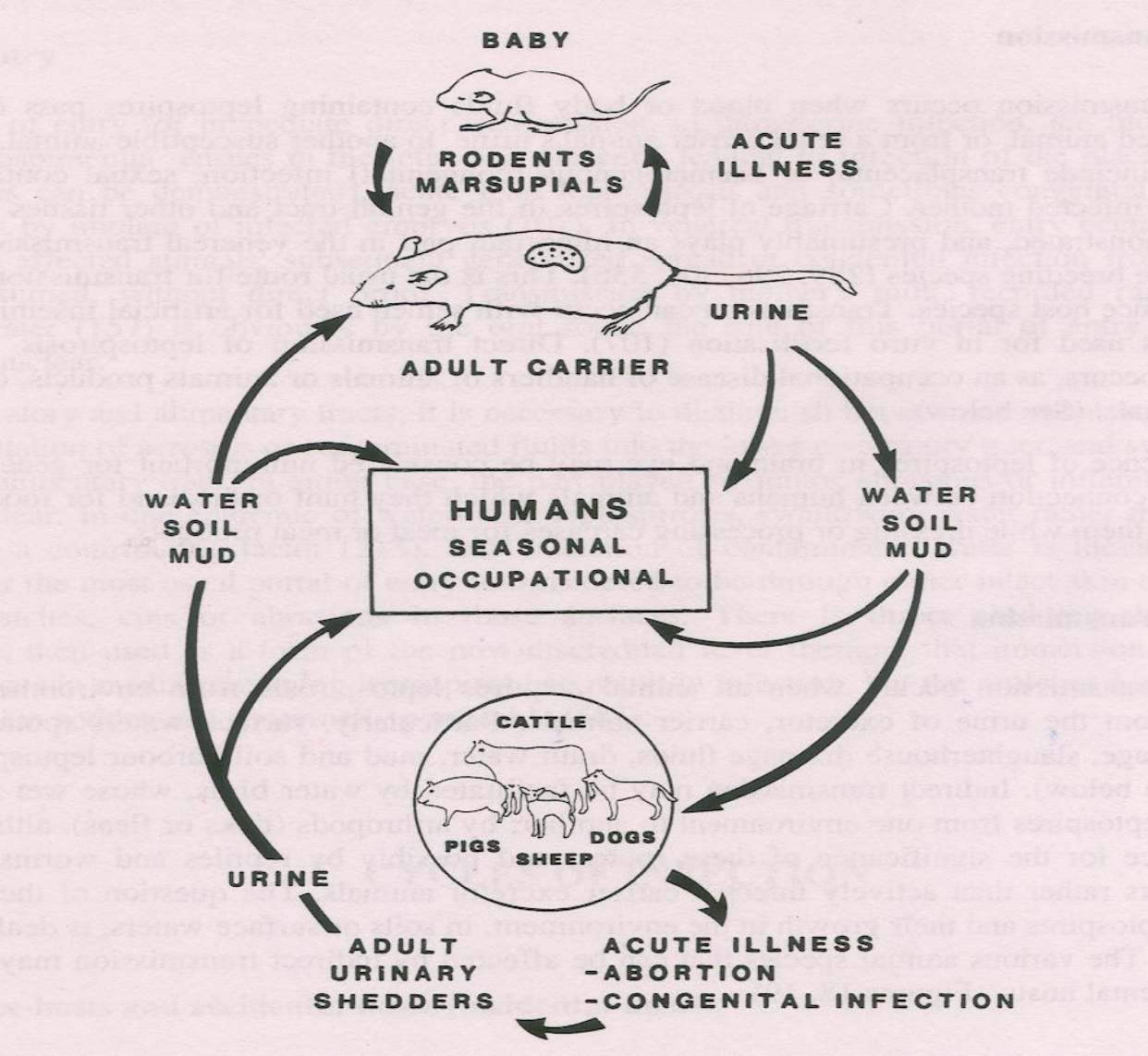

Figure 1.4 Source and cycles of leptospiral infection (Faine *et al.*, 1999)

Leptospires enter a host *via* portals such as damaged skin, certain mucous membranes, the lungs and conjunctiva membranes. They are not thought to be capable of penetrating undamaged skin except where it has been exposed to water for some times and has swollen significantly (www.leptospirosis.org/medical). The disease is not transmitted by ingestion of food or by inhalation of airborne particles (Faine *et al.*, 1999). The source of human infection is usually through either direct or indirect contact with the urine of an infected animal. The incidence is significantly higher in warm climate countries than in temperate regions (Everard and Everard, 1993); this is mainly due to the longer survival of leptospires in the warm, humid environment.

In Malaysia leptospirosis is endemic mainly due to the warm and humid climate. Rodents in Malaysia have been reported as the major maintenance hosts for various leptospiral serovars (Bahaman& Ibrahim, 1988). They shed leptospires in their urine, thus contaminating the environment. The incidence of leptospirosis has not been well documented because it has not been a notifiable disease until very recently. Following Fletcher, many investigations on human leptospirosis in Malaysia disclosed a high prevalence of the infection (Tan, 1970a; Supramaniam, 1979; El Jalii, 2002). Seroprevalence for human infection as reported by Supramaniam in 1979 was between 12% - 22% of the population. In another study, prevalence of leptospiral antibody in normal population was reported to be at 12% (Tan, 1970). According to

Tan (1964) rubber estate workers were the most highly infected group followed by workers handling sewage, drains, town cleaning, forestry and anti-malaria workers.

In another study by Tan (1964) a total of 584 cases of pyrexia of unknown origin (PUO) were examined over a period of 4.5 years from different states in Malaysia. The results indicated that 29.6% of those cases were positive for leptospiral infection which means that nearly one out of three PUO cases in Malaysia could possibly be due to leptospirosis.

Tan (1970a) carried out a study on 1993 suspected cases of leptospirosis in rural West Malaysia and reported 559(28%) were confirmed positive. The highest number of cases occurred among Indian males, aged 20-40 years old. Tan *et al* (1986a) examined 36 cases of acute renal failure admitted to Kuala Lumpur General Hospital during 1980 – 1983. She reported that 16 (44.4%) of those cases were due to leptospirosis. The infecting serovars were either serovar Celledoni or Pomona. Important serovars isolated from human cases were Canicola, Icterohemorrhagiae, Pyrogenes, Hebdomadis, and Autumnalis (Bahaman & Ibrahim 1987). Thus far 37 leptospiral serovars from 13 serogroups have been bacteriologically identified in this country i.e. Australis, Autumnalis, Bataviae, Canicola, Celledoni, Grippotyphosa, Hepdomadis,

Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe and Tarassovi (Bahaman & Ibrahim, 1988).

Seasonal flooding and other recent drastic climate changes have also contributed to the spread of the disease, particularly those resulting with more severe symptoms. The two fatalities in the recent flooding in the state of Johor, Malaysia were caused by leptospirosis (Badrul Hisham *et al.*, 2009). In addition, the report of fatality among students participating in national service was also alarming for Malaysian health authorities (www.reuters.com).

1.5 Clinical manifestations

Clinical presentations of leptospirosis initially are often similar to those of other febrile illnesses thus making clinical diagnosis difficult. As a result, leptospirosis is often misdiagnosed as dengue and other haemorrhagic fever, this is particularly common in the tropics. Even though the disease may also appear asymptomatic but very often present flu-like symptoms, with a severe headache, chills, muscle aches, and vomiting. These symptoms are associated with leptospiraemic phase of the disease whereby leptospires are actively present in the blood circulation. The symptoms persist for approximately seven days during which leptospires can be isolated from the patient blood. Conjunctival suffusion, especially in the pericornea, caused by vascular dilatation may occur in some leptospirosis patients during this early stage. With the

appearance of anti-leptospiral antibodies, the acute septicemic phase subsides and the immune phase occurs following a relatively short period of improvement in symptoms. During this phase, organisms disappear from the blood and move to immunologically safe havens such as the kidneys, brain, liver and respiratory system. At this stage the organism can be recovered from the aqueous humor, cerebral spinal fluid, kidney, and urine for many months (McClain, *et al.*, 1984; Van Crevel *et al.*, 1994). In some cases, immune phase may be preceded by a return of fever, jaundice, red eyes, abdominal pain, diarrhoae, or rash. In more severe cases (Weil's syndrome), there may be no period of improvement between the septicemic and immune phase. In addition to those listed above, symptoms of Weil's syndrome include decrease or no urine output, hypotension anemia, shock, and severe mental status changes (Clerke *et al.*, 2002).

Leptospirosis clinical features may also be divided into two categories: anicteric and icteric leptospirosis. The anicteric syndrome is associated with a mild form of the disease whereas icteric syndrome is more severe and the clinical course is often rapidly progressive. Compounded by hemolysis, jaundice presented in icteric syndrome is primarily a product of liver disorganization rather than the hepatocyte death (Murphy, 1980). Late sequelae include chronic fatigue and other neuropsychiatric symptoms such as headache, paresis, paralysis, mood swings and depression. In some cases, uveitis and iridocyclitis may be a late