LEPTOSPIRAL PROTEINS INDUCED *IN-VIVO* AND ITS APPLICATION IN THE DEVELOPMENT OF ANTIBODY AND ANTIGEN DETECTION TESTS FOR ACUTE LEPTOSPIROSIS

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-d-[3-ethylbenthiazoline sulfonate]
APS	ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CAI	codon adaptation index
COV	cut-off value
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
e.g.	*Exempli gratia* "for example"
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
g	gravity force
His	histidine
HRP	horseradish peroxidase
i.e.	that is
IgG	immunoglobulin G
IgM	immunoglobulin M
IMR	Institute for Medical Research
IPTG	isopropyl-beta-D-thiogalactopyranoside
IVIAT	*in-vivo* Induced Antigen Technology
Kb	kilo base pair
kDa	kilodalton
LFD	lateral flow dipstick
LL	leptospiral lysates
LPS	lipopolysaccharide
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization-time of flight/time of flight

MOH	Ministry of Health
NC	nitrocellulose membrane
Ni-NTA	nickel-nitrilotriacetic acid
NPV	negative predictive value
OD	optical density
OM	outer membrane
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
pI	isoelectric point
PI	protease inhibitor
PPV	positive predictive value
RC DC™	reducing compatible detergent compatible
rpm	rotation per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
TB	terrific broth
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
UV	ultra violet
V	Volt
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PROTEIN ARUHAN *IN-VIVO Leptospira* DAN APLIKASINYA DALAM PEMBANGUNAN UJIAN PENGESANAN ANTIBODI DAN ANTIGEN UNTUK PENYAKIT LEPTOSPIROSIS AKUT

ABSTRAK

Leptospirosis yang disebabkan oleh *Leptospira spp.* patogenik merupakan ancaman kesihatan sedunia yang muncul semula. Justeru, adalah penting untuk mengenalpasti penanda diagnostik baru dan menyelidik potensi penggunaannya dalam asai pengesanan antibodi dan antigen bagi diagnosis leptospirosis akut. Dengan menggunakan panel sampel serum daripada leptospirosis fasa akut (Kumpulan I) dan campuran (Kumpulan II), kajian ini dimulakan dengan menilai prestasi dua kit diagnostik pantas leptospirosis, iaitu Leptorapide dan VISITECT-LEPTO, yang lazim digunakan di Malaysia. Kedua-dua kit ujian tersebut menunjukkan sensitiviti diagnostik yang rendah (≤34%) terhadap sampel serum fasa akut, tetapi sensitiviti yang lebih baik terhadap sampel fasa campuran. Sampel serum yang terpilih dari Kumpulan I digunakan untuk mengenalpasti penanda diagnostik baru daripada perpustakaan ekspresi DNA genomik *Leptospira* dengan menggunakan teknologi antigen aruhan *in-vivo* (IVIAT). Klon faj S8A1 telah dikenalpasti dan pecahan gen-nya (dinamakan sebagai LepS8A1FL) telah diklonkan ke dalam plasmid rekombinan. Protein tersebut seterusnya diekspres dalam sistem ekspresi protein *Escherichia coli* dan ditulen dengan menggunakan "Immobilized Metal Affinity Chromatography". Dua terbitan terpenggal LepS8A1FL (dinamakan sebagai LepS8A124 and LepS8A134) dan penanda-penanda diagnostik leptospirosis yang pernah dilaporkan (dinamakan sebagai LigA, LipL41, OmpL1 dan LipL32) turut dihasilkan dengan kaedah yang ternyata di atas. Dalam blot western Immunoglobulin M (IgM),

LepS8A1FL menunjukkan prestasi yang memuaskan dengan sensitiviti dan spesifisiti 75%. Ia mengungguli LipL41 dan LigA yang pernah dilapor dalam mengesan leptospirosis akut. Walau bagaimanapun, protein tersebut menunjukkan nilai diagnostik sederhana dalam format asai "Enzyme-Linked Immunosorbent" (ELISA) IgM. Satu asai dipstik aliran sisi (LFD) IgM yang menggunakan LepS8A1FL seterusnya telah dibangunkan, dan masing-masing menunjukkan sensitiviti dan spesifisiti diagnostik sebanyak 65.7% dan 63.2%. Kajian ini diteruskan dengan ujian pengesanan antigen untuk leptospirosis akut. Untuk tujuan ini, antibodi poliklonal arnab terhadap pecahan sel kasar *Leptospira* dan kesemua protein rekombinan leptospiral tersebut telah dihasilkan. Asai LFD yang menggunakan antibodi anti-pecahan leptospiral sebagai kedua-dua antibodi tangkapan dan antibodi berkonjugat emas mengungguli kombinasi antibodi yang lain. Ia menunjukkan limit pengesanan yang tinggi, spesifisiti yang baik serta reaktiviti yang luas. Ujian tersebut menunjukkan sensitiviti dan spesifisiti diagnostik masing-masing 57.4% dan 87.2%. Ujian pengesanan antigen tersebut juga mengesan antigen leptospiral di dalam air kencing manusia. Di samping itu, dengan menggabungkan keputusan ELISA IgM LepS8A1FL dan asai LFD pengesanan antigen tersebut, sensitiviti diagnostik 86.2% dan spesifisiti diagnostik 73.7% tercapai. Secara kesimpulannya, kedua-dua ujian pengesanan antibodi dan antigen dalam kajian ini patut dibangunkan selanjutnya untuk digunakan dalam diagnosis leptospirosis akut pada manusia.

LEPTOSPIRAL PROTEINS INDUCED *IN-VIVO* AND ITS APPLICATION IN THE DEVELOPMENT OF ANTIBODY AND ANTIGEN DETECTION TESTS FOR ACUTE LEPTOSPIROSIS

ABSTRACT

Leptospirosis, caused by pathogenic *Leptospira spp*., is a re-emerging global health threat. Therefore, it is pertinent to identify novel diagnostic marker and investigate its potential use in antibody and antigen detection assays for detection of acute leptospirosis. Using panels of serum samples from acute (Group I) and mixed (Group II) phase leptospirosis, the present study was initiated by evaluating performance of two leptospirosis rapid diagnostic kits, namely Leptorapide and VISITECT-LEPTO, that were commonly used in Malaysia. Both test kits showed low diagnostic sensitivity (≤34%) with the acute phase serum samples, but better sensitivity with the mixed phase samples. Selected serum samples from Group I were used to identify novel diagnostic marker(s) from *Leptospira* genomic DNA expression library using *In-vivo* Induced Antigen Technology. A phage clone, S8A1, was identified and its gene fragment (named as LepS8A1FL) was cloned into recombinant plasmids. The protein was then expressed in *Escherichia coli* protein expression system and purified using Immobilized Metal Affinity Chromatography. Two truncated derivatives of the LepS8A1FL (namely LepS8A124 and LepS8A134) and previously reported diagnostic markers for leptospirosis (namely LigA, LipL41, OmpL1 and LipL32) were produced with the methods described above. In Immunoglobulin M (IgM) western blot, LepS8A1FL showed a satisfactory performance with 75.0% sensitivity and specificity. It outperformed the reported LipL41 and LigA in detecting acute leptospirosis. In an IgM Enzyme-Linked

Immunosorbent Assay (ELISA) format, the protein, however, has moderate diagnostic value. An IgM lateral flow dipstick assay (LFD) using LepS8A1FL was subsequently developed and demonstrated diagnostic sensitivity and specificity of 65.7% and 63.2%, respectively. The present study also pursued an antigen detection test for acute leptospirosis. For this purpose, rabbit polyclonal antibodies against *Leptospira* crude cell lysate and all of the above leptospiral recombinant proteins were produced. The LFD assay which used anti-leptospiral lysate antibody as both immobilized and gold-labelled antibody was superior to other antibody combinations. It showed high limit of detection, good specificity and broad reactivity. The test demonstrated diagnostic sensitivity and specificity of 57.4% and 87.2%, respectively. The antigen detection test also detected leptospiral antigen in human urine. Meanwhile, by combining the results of LepS8A1FL IgM ELISA and the antigen detection LFD test, a diagnostic sensitivity of 86.2% and diagnostic specificity of 73.7% was achieved. In conclusion, both antibody and antigen detection tests in this study merit further development for use in diagnosing acute leptospirosis in human.

CHAPTER ONE

INTRODUCTION

Leptospirosis is a bacterial infection caused by pathogenic species of *Leptospira*. The disease is most prevalent in tropical regions because of the warm and high humidity atmosphere (Picardeau, 2013). The pathogen may potentially infect all mammals, including human. Depending on the animals and the infecting serovars, the disease may cause a wide range of manifestation to animals, including asymptomatic, acute, chronic symptoms and carrier stage (Jobbins and Alexander, 2015; Adler, 2014). Upon acquiring the infection, the chronic-infected or carrier animals can further contaminate the surrounding environment by passing urine containing the bacteria into soil and fresh water streams. Thus, other potential hosts who live in the epidemic area may be exposed to the bacteria and further propagate the disease (Subharat *et al.*, 2011).

Human is an accidental host for the pathogen. Leptospirosis cases are estimated to be approximately one million and 58,900 deaths per annum (Costa *et al.*, 2015). This represents 14.77 cases and 0.84 deaths per 100,000 population, respectively. Human-to-human transmission is practically non-existent. The disease is transmitted to human upon contact with water contaminated by animal hosts as described above. The bacteria enters human body via the breached skins and mucous membranes (Picardeau, 2017). Four major risk factors expose human to pathogenic *Leptospira*: i) recreational water activities, ii) watery-associated occupations *e.g.* paddy and poultry farmers as well as workers in slaughterhouse, iii) post-natural disasters *e.g.* flood and typhoon, and iv) poor hygiene.

Leptospirosis is a biphasic disease (Gasem *et al.*, 2009). Following an incubation period of 5-14 days, a patient is in acute (leptospiraemic) phase and

develops symptoms such as high fever, headache and myalgia, which are similar to other febrile illnesses such as dengue and malaria. If left untreated, the pathogen may infect vital organs during convalescent phase and cause mortality to a patient. Nevertheless, the disease can be treated effectively by administrating appropriate antibiotic regimen (Phimda *et al.*, 2007). Hence, a good clinical management highly depends on correct identification of the disease. Due to unspecific clinical symptoms of acute leptospirosis, laboratory diagnosis become a critical tool to detect the disease and further support clinical decision of a suspected patient (McBride *et al.*, 2007). Many diagnostic tools to detect anti-leptospiral antibody are readily available in the market. However, there is a big room to improve leptospirosis diagnosis because performance of most kits are poor in detecting acute phase of leptospirosis due to the absence or low anti-leptospiral antibody in the early disease stage (Bajani *et al.*, 2003).

Currently, most of the serological tests for leptospirosis used crude cell proteins from the non-pathogenic *L. biflexa* as the antigens. The strategy is relatively straightforward and convenient for manufacturers. However, one of its major drawback is its inconsistency of diagnostic performance in different countries. This is because the predominant *Leptospira* serovar varies between countries. As a result, the anti-leptospiral antibody developed by a patient, which is serovar specific, may not be efficiently detected by those serological tests (Blacksell *et al.*, 2006). To overcome this limitation, it is necessary to identify diagnostic marker(s) that is conserved across different *Leptospira* serovars. To date, several protein antigens, such as LipL21, LipL32 and LipL41 have been identified (Natarajaseenivasan *et al.*, 2008; Boonyod *et al.*, 2005; Cullen *et al.*, 2003). Yet, their performance differs particularly in detecting the IgM antibody in acute leptospirosis sera (Toyokawa *et al.*, 2011). As such, new approaches should be implemented to identify novel antigenic markers for

serodiagnosis of leptospirosis. It is known that bacterial proteins which are highly expressed during infection event (also known as *in vivo* induced proteins) may be incorporated into diagnostic tool as antigens to detect the antibodies raised in a patient (Cao *et al.*, 2004). *In Vivo* Induced Antigen Technology (IVIAT) is a method to identify these diagnostic markers by immunoscreening genomic or complementary DNA library of a particular microorganism without the need to use an animal model (Hu *et al.*, 2014). The technology facilitates identification of immunogenic leptospiral protein antigens that are expressed during infection event. Previously, IVIAT has been used to discover novel diagnostic markers from several pathogens such as *Mycobacterium tuberculosis*, *Vibrio cholera* and *Bacillus anthracis* (Kumar *et al.*, 2011; Rollins *et al.*, 2008; Hang *et al.*, 2003). Based on the results of the above studies, IVIAT-identified protein(s) may be useful for IgM detection of leptospirosis in human.

On the other hand, it is known that leptospiral antigens are circulating in a leptospirosis patient during acute phase (Picardeau, 2013). Thence, detection of the circulating antigens demonstrates direct evidence of acute leptospirosis. Unlike IgM detection tests, antigen detection test is not in the market. As a result, the current study pursues this approach by using antibodies to selected leptospiral antigens, such as LigA, LipL32, LipL41 and OmpL1, since these proteins are reported to be abundant in the bacteria (Malmstrom *et al.*, 2009). In addition to that, this study was also designed to detect whole cell antigens of *Leptospira spp.* as an approach to include undefined circulating leptospiral antigens. This antigen detection strategy may complement with antibody detection test described above for improved diagnosis of acute leptospirosis in human.

CHAPTER TWO

LITERATURE REVIEW

2.1 World Epidemiology

Due to *Leptospira spp.* preference to proliferate in hot and wet atmosphere, majority of leptospirosis incidence occurred in tropical countries located between tropic of Capricorn and Cancer (**Figure 2.1**). In tropical countries, its annual incidence rate exceeds 10 cases/100,000 population which was higher than the reported 0.1-1 case/100,000 population in temperate climates (Picardeau, 2013). Highest morbidity rate was reported at Oceania (150.68/100,000 population), followed by South East Asia (55.54/100,000 population), Caribbean (50.68/100,000) and East Sub-Saharan Africa region (25.65/100,000) (Costa *et al.*, 2015). Of note, small tropical countries or islands in these regions are highly endemic for leptospirosis. A work by Pappas *et al.* (2008) revealed Seychelles (43.2/100,000 population) to be the country in the world with highest incidence rate, followed by Trinidad and Tobago (12.0/100,000 population) and Barbados (10.0/100,000 population). However, the actual prevalence might be more serious than those reported because data from developing countries are normally under-estimated and less reliable (Pappas *et al.*, 2008). Considering low socioeconomic status of the regions, the disease might be under-recognized as a potential public health threat (Schneider *et al.*, 2013). In addition to that, long heavy rainfall season in tropical climate further promotes the disease incidence (Ko *et al.*, 1999).

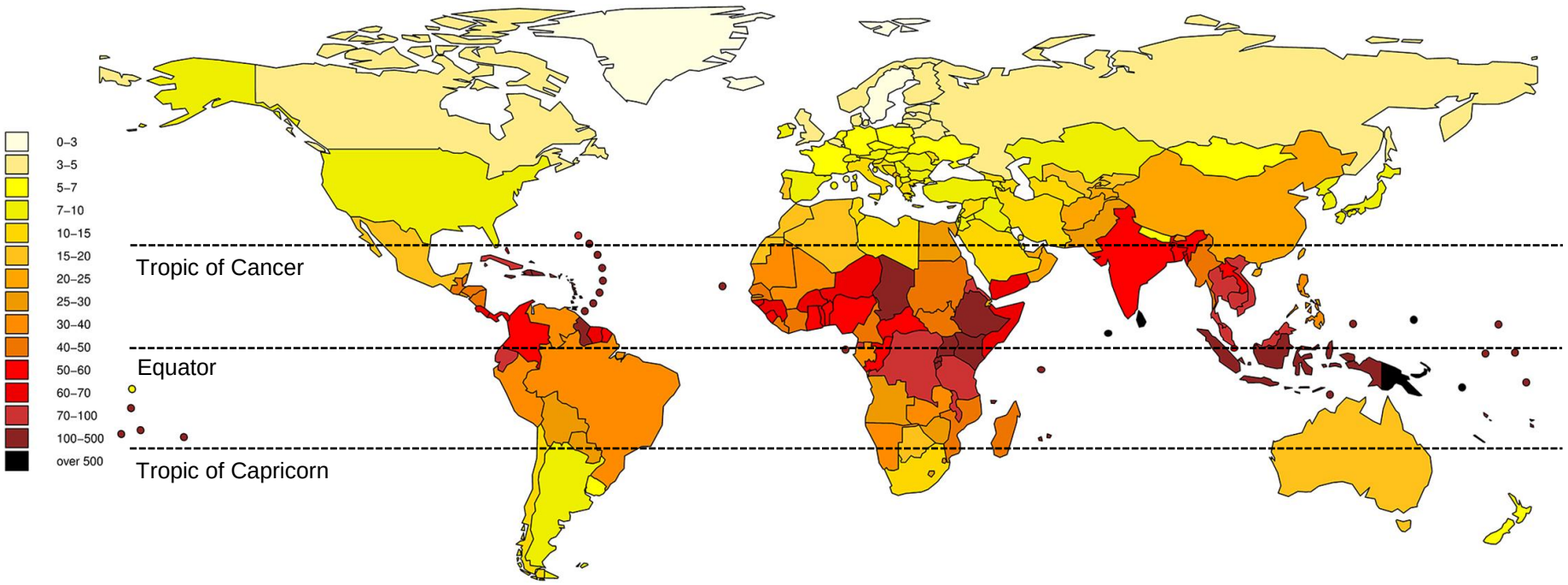


Figure 2.1 Distribution and leptospirosis burden illustrated in Disability Adjusted Life Years (DALYs)/100,000 population per year. One DALYs unit represents one year which a healthy individual lost due to a circumstance, *e.g.* disease and disability. Reprinted from Torgerson, P. R., *et al.* (2015).

2.1.1 Leptospirosis in Malaysia

Leptospirosis is an endemic disease in Malaysia. The first recorded human leptospirosis study in Malaysia was performed on rural inhabitants and rubber plantation workers by Fletcher (1928). As an endemic country for leptospirosis, the average morbidity rate was estimated to range between 1-10/100,000 population (Lim, 2011). **Figure 2.2** illustrates trends of leptospirosis incidence and death cases for the past 11 years. A nine-year study (2004-2012) conducted by Benacer *et al.* (2016) demonstrated an average annual incidence rate of 4.83/100,000 population. However, the annual incidence rate markedly increased year to year, noting from 0.97 (2004) to 12.47 (2012) cases/100,000 population throughout the study. Abdul Wahab (2015) reported that the highest leptospirosis incidence rate (25.5 cases/100,000 populations) and mortality cases (92 cases) in Malaysia occurred in 2014. A spiked increase over 60% of cases reported compared to its preceding year has been linked to heavy rainfall and flood that happened in many states of Malaysia during the year (Garba *et al.*, 2017). Continuous increase in reported leptospirosis cases was correlated to the fact that leptospirosis was instated as a national notifiable disease since 9th December 2010 under Prevention and Control of Infectious Disease Act 1988. Following year, an official guideline by Ministry of Health (MOH) Malaysia regarding diagnosis, management, prevention and control of the disease was published. These approaches commit better surveillance and monitoring by health institution and government. Particularly, it raises awareness of the disease among clinical practitioners, which contributed to better prognosis and early treatment initiation to the suspected patients.

In Malaysia, male gender constituted 78.7% of total leptospirosis cases in between 2004-2012 (Benacer *et al.*, 2016). This contributed a male-to-female ratio of 3.69:1. The phenomenon of male outnumbered female patients is common in

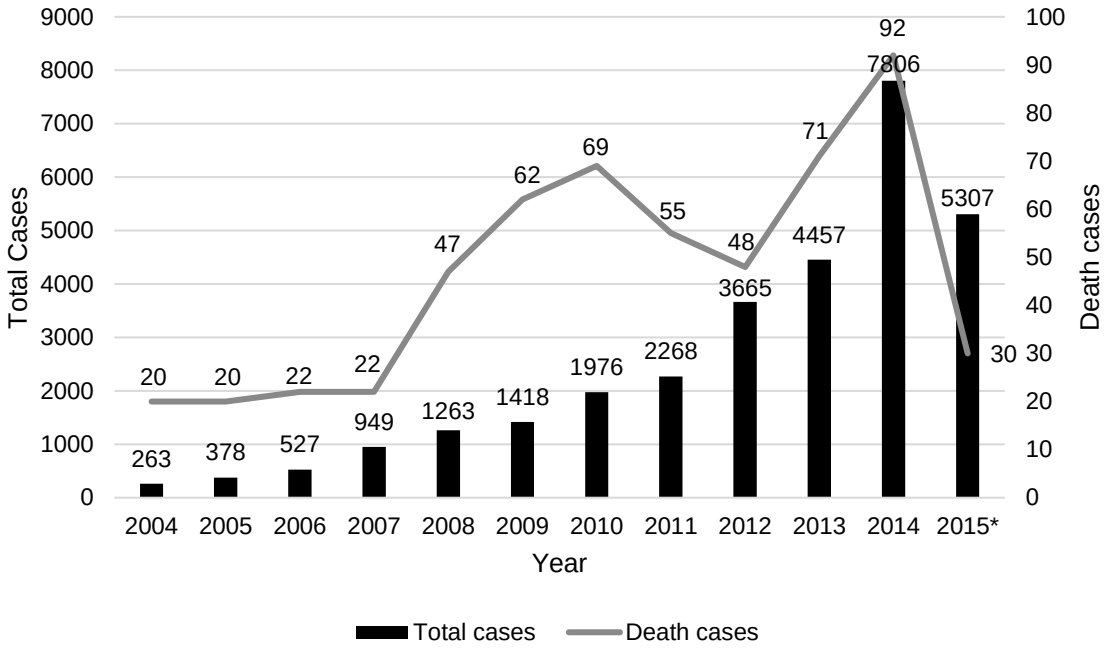


Figure 2.2 Trends on leptospirosis incidence and death cases in Malaysia between 2004 to July 2015. Data adapted from Abdul Wahab (2015).

leptospirosis worldwide and has been associated with high risk occupation and recreational activities predominated by male (Costa *et al.*, 2014; Felzemburgh *et al.*, 2014). Malaysian patient demography reported the mean (±SD) age of leptospirosis patients was 33.79 (±17.55) and median of 31 years old (Benacer *et al.*, 2016). Malaysian within age group of 30-39 years old has the highest morbidity rate (16.21 cases/100,000 population). Constitution of age group 30-39 years old as the major group for leptospirosis was similarly observed in different epidemiological settings (Costa *et al.*, 2015; Goris *et al.*, 2013a). Compared to school children and adolescent, it was predicted that middle age adult possessed more mobility which lead to higher exposure risk to the disease (Benacer *et al.*, 2016).

Leptospirosis risk is frequently present during rainfall and flood season in developing countries, whereas water recreational activities are more relevant in developed countries (Mwachui *et al.*, 2015). In Malaysia, annual reported leptospirosis cases similarly follow the seasonality pattern. A spiked case number was reported in October-March in peninsular Malaysia and in October-February in east Malaysia (Benacer *et al.*, 2016). This is in concordance with wet season in the country. Weinberger *et al.* (2014) claimed that rainfall flush the *Leptospira* spp. bacteria which typically survive in wet soils into water bodies. As such, human at watery grounds is more prone to the infection. However, excessive volume of rainfall provides dilution effect to the bacteria load, which inversely present as a protective factor to the flood victim (Suwanpakdee *et al.*, 2015).

Thayaparan *et al.* (2015) demonstrated a seroprevalence of 35.9% among villagers (n=198) in periurban area of Kuching, Sarawak. Villagers who work around forest and involve in national service exhibit seroprevalence of more than 50%. Meanwhile, plantation workers in Johor and Melaka recorded a seroprevalence of

28.6% (Janudin *et al.*, 2016). *Leptospira* serovar Lepto 175 Sarawak was the predominant serovar in these oil palm plantations, contributing to 62% among the seropositive workers (Janudin *et al.*, 2016). A cross-sectional study of leptospirosis seroprevalence among 999 febrile patients in ten Kelantan government hospitals showed higher leptospirosis seroprevalence among patients from high-risk occupational group, *e.g.* outdoor worker, agriculture worker and military (Rafizah *et al.*, 2013a). In addition to that, Rafizah *et al.* (2013b) reported that patient with exposure to recreational activity has 2.4 times higher risk for leptospirosis.

2.1.2 Outbreaks and Case Reports in Malaysia

As an endemic country, leptospirosis outbreaks happen intermittently in Malaysia. The most recent scientific literature about leptospirosis outbreak in Malaysia was dated 2012. Leptospirosis outbreak was reported in Pauh, Perlis among family members after fisheries activity at a swamp in Kampung Padang Telela (Baharudin *et al.*, 2012). The swamp was an abandoned paddy field and has been neglected for long time. After approximately two weeks, eight out of 28 of the participants involved showing common febrile symptoms such as fever, headache, vomiting and muscle pain. Serology test for the presence of leptospiral IgM using immunochromatographic test (VISITECT-LEPTO, Omega-Diagnostic, UK) demonstrated six samples to be positive. Most of the samples were then further confirmed to be positive by MAT and/or Polymerase Chain Reaction (PCR) at Institute for Medical Research (IMR) Malaysia. Pathogenic *Leptospira* spp. DNA was identified in seven out of eight water samples from the swamp. Hence, the incident was categorized as a point-source outbreak.

On 26th June 2010, a search and rescue operation on a drowned young man was conducted in Lubuk Yu, Pahang. Lubuk Yu is a recreational forest with river and waterfall. Among 153 people who participated the rescue, 21 of them developed fever ≥ 38ºC seven days post-operation (IQR 1-13 days) (Sapian *et al.*, 2012). Ten serum samples of the patients were cultured-positive for *Burkholderia pseudomallei*. Out of which four serum samples were positive for leptospirosis by PCR, suggesting co-infection with *Leptospira spp.* High fatality rate of 38% (8/21) was reported in this outbreak. In detail, fatality rate for melioidosis alone and melioidosis-leptospirosis co-infection were 66.7% (4 out of 6) and 75% (3 out of 4), respectively. All water samples (n=6) and two out of four soil samples collected on site of operation showed positive for *Leptospira spp*. This has been linked to heavy rainfall during first two days of the operation which might flush the bacteria onto the soil surface and into the river.

The first international leptospirosis outbreak happened in Sabah (Sejvar *et al.*, 2003). During 21st August to 1st September 2000, a number of 304 athletes participated in Eco-Challenge Sabah multisport endurance race. Fifteen days (range one to 24 days) after the race, 80 out of 189 athletes showed common febrile symptoms, including fever, headache, chills, diarrhea and muscle aches. In addition to that, 40 athletes showed conjunctival suffusion which is a representative symptom for leptospirosis. A total of 29 case-patients were admitted to hospital. All the patients recovered and no death case was reported. Serological test showed positive reaction in 68% (26/38) of the serum samples. Multivariate stepwise logistic regression demonstrated that swimming in Segama River is the main risk factor contributed to this point-source outbreak. Abrasions and cuts on the athletes in jungle trekking preceding to swimming in the river increased the risk of exposure to *Leptospira spp*.

2.2 Classification and Typing of *Leptospira*

2.2.1 Taxonomy

Leptospires belong to family Leptospiraceae in the phylum of Spirochetes. The phylum members consisted of mammalian pathogens which cause a vast array of serious diseases, particularly in human. Besides leptospirosis, some notorious human diseases caused by the spirochetes members are syphilis (*Treponema pallidum*), Lyme disease (*B. burgdorferi*), relapsing fever (*Borrelia spp.*), yaws (*T. pallidum* subsp. Pertenue), pinta (*T. carateum*) and periodontal disease (*Treponema spp*.). In general, spiral shapes and endoflagella motility are the hallmarks of spirochetes. They demonstrated morphology of long, thin bacteria with flat-waves, helices or irregular shape under the microscope (Wolgemuth, 2015). These special modes of propulsion and morphology indeed represents virulence factors of spirochetes. However, in year 2012 a novel genus, namely Sphaerochaeta, was included as a member of phylum Spirochaetes (Caro-Quintero *et al.*, 2012). It implies an exception to the morphology hallmark of spirochetes as the species are non-motile and sphere in shape.

The Leptospiraceae family was defined in 1979 to initially cover genera Leptonema as well as *Leptospira*. A decade ago, Levette *et al.* (2005) transferred *Leptospira parva* to genus *Turneriella* as *Turneriella parva*, contributing three genera under the family. The three genera were characterized by divergences in GC content %, 16S rRNA gene sequences and DNA-DNA relatedness (Adler, 2015). GC content% within members of Leptospiraceae ranged from 35 to 54 mol%. *Leptospira* demonstrated the lowest ratio of 35-41 mol% (Ko *et al.*, 2009). *Leptonema* has a GC content% of 54 mol%, while *Turneriella* has the largest ratio of 53.6% mol%.

The type genus is defined as *Leptospira* Noguchi in the honor of Noguchi (1917) who proposed the genus name after studying the pathogen isolates from USA,

Japan and Europe. The type species is *L. interrogans* (Stimson, 1907) Wenyon 1926. Since 1980, the type strain has been designated as serovar icterohaemorrhagiae RGA^T strain (ATCC 43642) as per enlisted in Approved Lists of Bacterial Names (Skerman *et al.*, 1980).

2.2.2 Classification of *Leptospira* Species And Subspecies

Three distinct classifications were used for *Leptospira* species, namely serology, genetic and phylogeny classification. While these classifications methods have little relatedness between each other, they present advantages and disadvantages that appropriately suit the aim of a particular study.

Since 1914, rapid isolation of the bacteria happened throughout different location of the world. Following proposal of "*Leptospira*" as the genus name, a number of species names have been assigned based on serological typing, such as the outcome of cross-agglutinin absorption test and difference in antigenicity of the bacteria. Examples of *Leptospira* species defined by the serological typings are *L. canicola*, *L. hebdomadis*, *L. icterohaemorrhagiae* and *L. biflexa* (Robinson, 1948). Considering inappropriate assignation of species name based on serological classification, Wolff and Broom (1954) proposed to use "serovar" for naming of serologically distinct strains. Following that, the bacteria strains were divided into two *Leptospira* species, namely *L. icterohaemorrhagiae* and *L. biflexa*, comprising all pathogenic and saprophytic strains, respectively. The name for pathogenic strain was then amended to *L. interrogans* (Wolff and Turner, 1963). To date, there are more than 250 serovars have been identified for *Leptospira spp.*, which were grouped into 24 serogroups (Levett, 2001). Classification based on *Leptospira* serovars is widely used in

epidemiological and clinical setting. Yet, it has no relevance to the bacteria taxonomy. In Malaysia, the three most common pathogenic *Leptospira* serovars are *L. interrogans* serovar Australis, Birkini and *L. borgpetersenii* serovar Javanica (Fairuz Amran, personal communication).

Methods above classifies pathogenic and non-pathogenic *Leptospira* into two species, namely *L. interrogans* and *L. biflexa*, respectively. With advance in genomic study, the members in the two *Leptospira* species were found to have low DNA homology, suggesting there are more species that laid within *L. interrogans* and *L. biflexa* categorized using the above methods (Haapala *et al.*, 1969). Thus, DNA-DNA hybridization technique has been widely used in many studies to characterize species within genus *Leptospira*. A total of 21 species have been identified to date. With the advance in molecular and analytical technique, several methods which are faster and more reliable have been prompted for species identification. For instance, MALDI-TOF has been adopted to identify *Leptospira* species in a recent study (Rettinger *et al.*, 2012). Multilocus sequence typing has also been demonstrated to be able to identify the species similar to that of DNA-DNA hybridization (Boonsilp *et al.*, 2013). It is expected that next generation sequencing will be the future trend for species identification.

By analyzing 16S rRNA and housekeeping genes such as *gyrB* and *rrs*, the *Leptospira* species mentioned above can be further categorized in a phylogenetic tree based on its pathogenicity (Picardeau, 2017; Morey *et al.*, 2006; Slack *et al.*, 2006). As illustrated in **Figure 2.3**, a phylogenetic tree can be constructed into three major clades, following pathogenic, intermediate and saprophytic characteristic of the bacteria. The pathogenic *Leptospira* comprised species that have been characterized

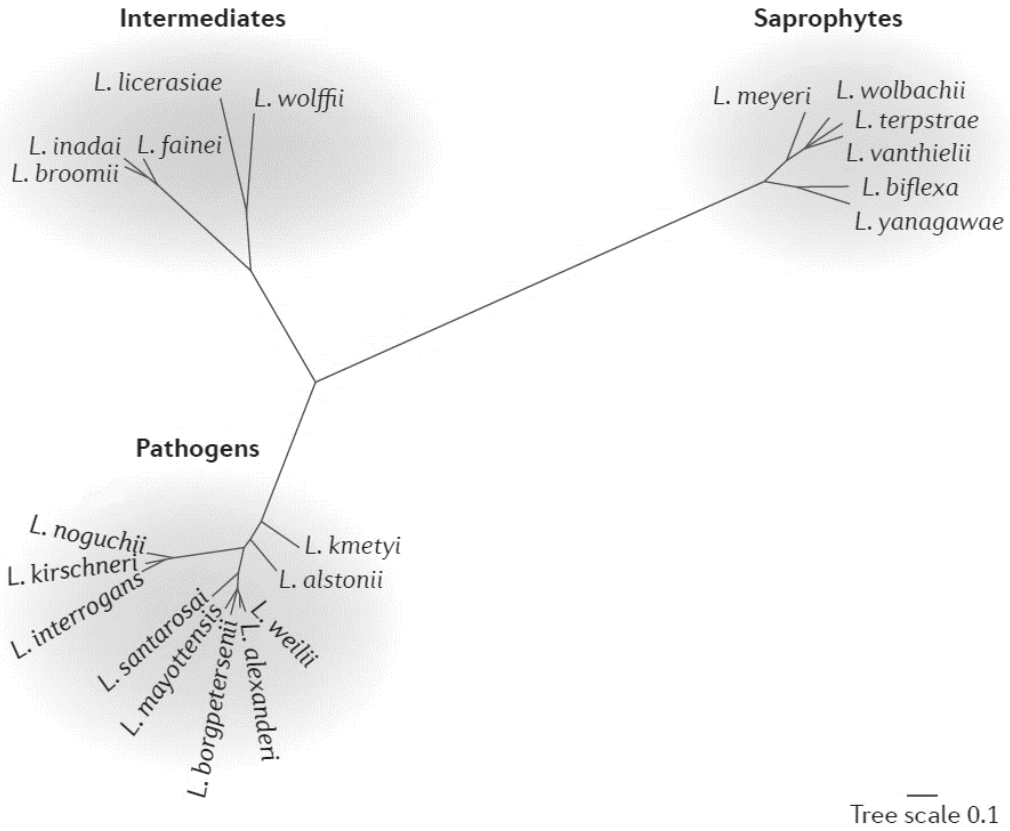


Figure 2.3 Phylogenetic analysis of *Leptospira spp.* 16S rRNA and classification of *Leptospira* based on its pathogenicity. Reprinted from Picardeau (2017) with permission from Springer Nature.

to cause human and animal leptospirosis. On the contrary, species in saprophytic clade are free-living and have never demonstrated evidences in infecting a host. The intermediate *Leptospira* may occasionally cause leptospirosis with mild symptoms in human and animal host.

2.3 Animal Reservoir for Pathogenic *Leptospira spp.*

Leptospirosis has been claimed as the most common zoonotic disease. All mammals, including bats and pinnipeds, can virtually be hosts for the pathogenic bacteria (Picardeau, 2017). Unlike human who is an accidental host, leptospirosis in animal is either in asymptomatic, acute, chronic or acute-to-chronic stage with persistent bacteria carriage and excretion into the environment for a duration varies between species. As a consequence, this amplifies the infection within the ecosystem and engenders leptospirosis risk to human and animal surrounding.

Rodent is well known to be the main reservoir for *Leptospira spp.* and plays an important role in transmitting the disease to human and animals. As a natural reservoir, rat did not show any symptom upon infection. Although systemic infection occurs in the early stage, the bacteria is rapidly clear from blood and most organs of rats (Athanazio *et al.*, 2008). The rat acts as a chronic carrier because the bacteria remain colonizes proximal tubules of kidney and being continuously shed into environment via urine. With bacterial load as high as 10^7/mL, an area may be seriously contaminated following repeating urination as the virulent leptospire is highly viable in surface water, stream, river or moist soil for weeks to months (Monahan *et al.*, 2008).

Domestic animals likewise impose leptospirosis risk to human as well as surrounding environment. However, these domestic animals show different degree of symptoms upon infected. Dogs demonstrate clinical presentation most resemble to human leptospirosis. Canine leptospirosis may lead to life-threatening with febrile illness symptoms in addition to vascular, liver and kidney damage (Pijnacker *et al.*, 2016). Stray dogs transmit pathogenic *Leptospira spp.* from wild and natural environment close proximity to human; domestic dogs put human at the risk of contamination by shedding the bacteria in household environment (Hua *et al.*, 2016; Gay *et al.*, 2014).

Staying in the same farm, livestock acquires the disease from infected herd mates and rodents that shed urine into the soil and water (Subharat *et al.*, 2011). Since basic necessity needs (food, water, refuge) are available *ad libitum* in farm, it becomes a habitat for wildlife reservoirs to stay close to livestock (de Oca *et al.*, 2017). The factors above expose workers at livestock farm to occupational-acquired leptospirosis. Besides as a health threat to the workers, leptospirosis is associated with economic impact in livestock sector particularly with goats, sheep, pigs and cattle. In general, the infected livestock may suffer from reproduction disorders (Rizzo *et al.*, 2017; Ramos *et al.*, 2006). Meanwhile, recurrent uveitis, as a result of autoimmune response between ocular tissue and leptospiral membrane proteins, is a hallmark for equine leptospirosis (Verma *et al.*, 2013). Even though the disease is of veterinary significance, Garba *et al.* (2017) claimed a lack in livestock leptospirosis studies. In fact, inter-species transmission of the disease between sheep and cattle has been demonstrated in a herd two decades ago, suggesting presence of leptospirosis among livestock in Malaysia (Bahaman, 1991).

Despite similarity in genetic, *Leptospira* serovars show characteristic preferences to specific animal reservoir. Icterohaemorrhagiae and Ballum serogroup are usually predominated in rats and mice, respectively (Bharti *et al.*, 2003). Although not absolute, serovar Canicola in dogs, Harjo in cattle Pomona in pigs as well as Bratislava in horses have been primarily demonstrated (Schuller *et al.*, 2015; Gamage *et al.*, 2014; Andre-Fontaine, 2006; Grooms, 2006). Ellis (2010) reported that dog is the only maintenance host for serovar Canicola. Molecular background which contributes a serovar to host specificity is unknown. Frequently, these serovars do not cause severe manifestation in their highly-adapted reservoir hosts (Bharti *et al.*, 2003). These serovars, on the other hand, may cause severe clinical outcome to other incidental hosts.

2.4 Anatomy of *Leptospira*

The genus name, *Leptospira*, derives from Greek leptos (thin) and Latin spira (coiled). The bacteria is a thin spirochete with approximate diameter of 0.15 µm and length of 6-20 µm. The organism is easily distinguishable from other bacteria due to their distinctive morphology of thin, right-handed helix coil and is highly motile. Frequently, at least one end of the bacteria bends into hook which resemble a question-mark, thus contributing to the species name interrogans ("interrogate", ask question). Morphology of *Leptospira spp.* is shown in **Figure 2.4**.

2.4.1 Lipopolysaccharide

As illustrated in **Figure 2.5A**, *Leptospira spp.* has a Gram negative-like cell wall. The outer membrane consists of many surface exposed outer membrane (OM) proteins,

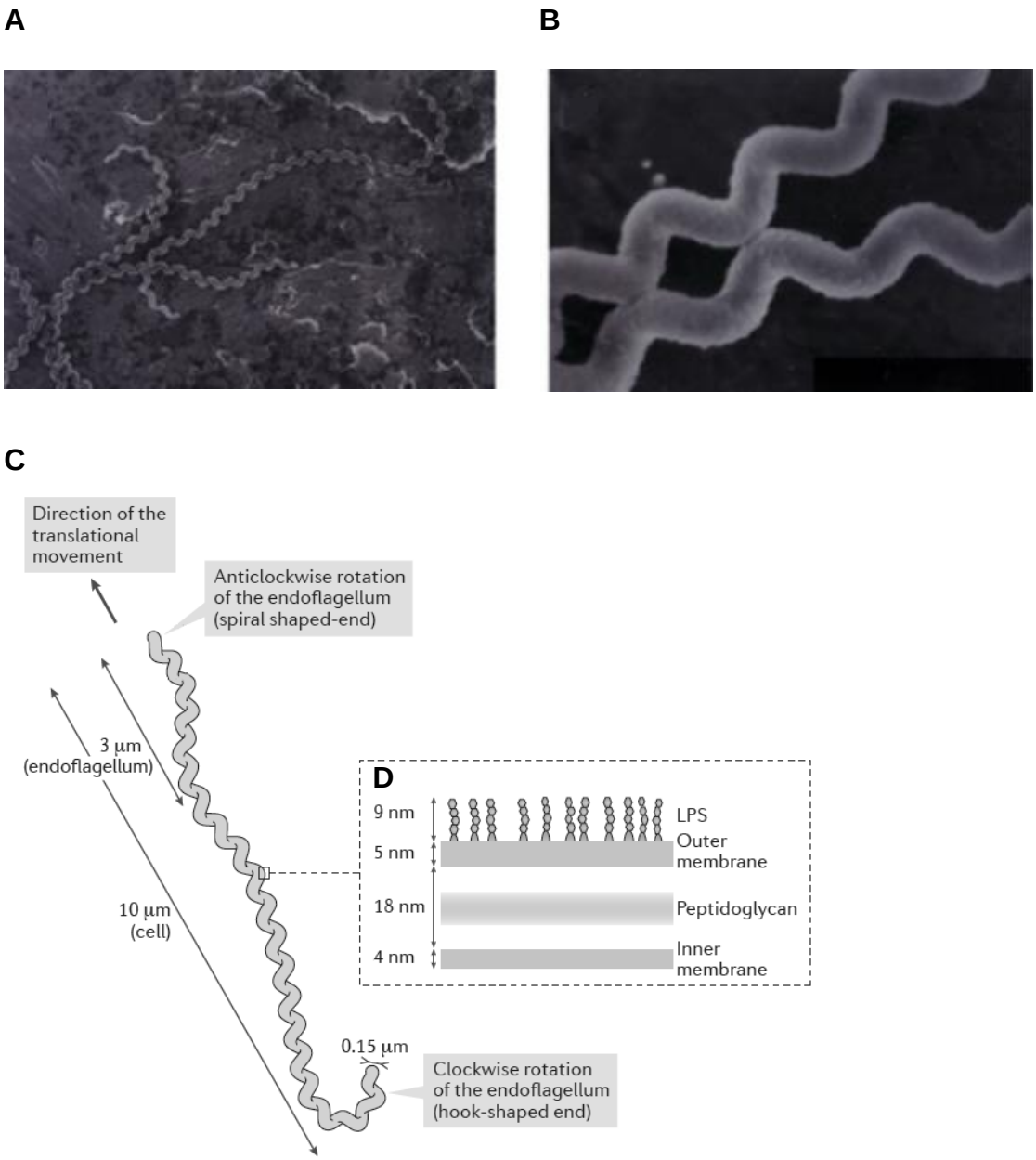
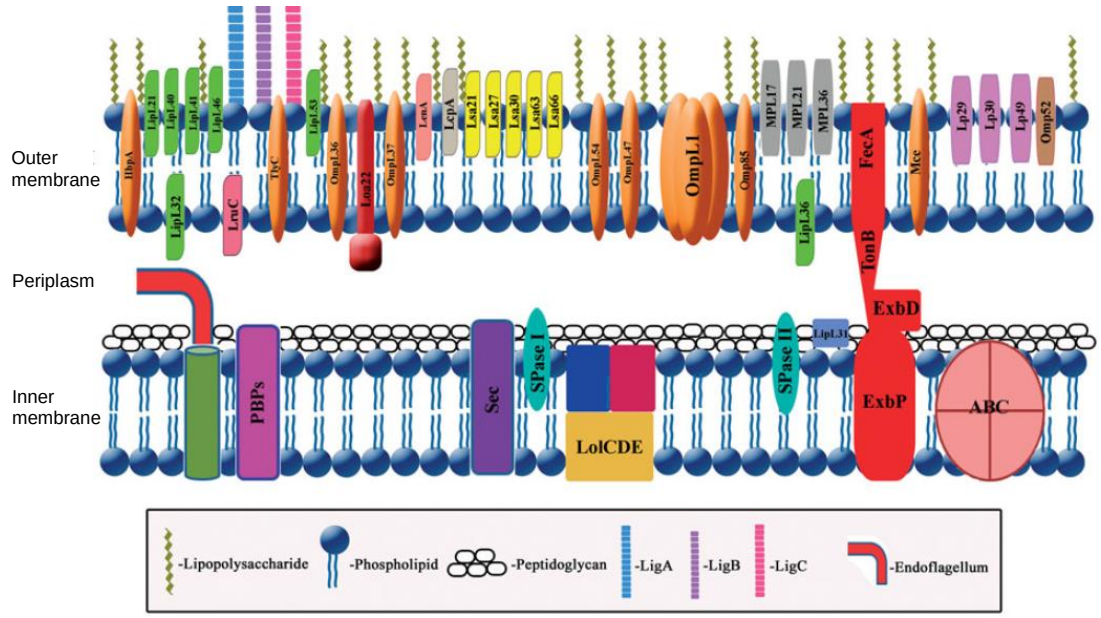


Figure 2.4 Morphology of *Leptospira interrogans*. (A) Demonstration of characteristic hook end of the bacteria. (B) Visualization wave body shape of the bacteria by scanning electron microscopy at 3000x. (C) Measurement of the bacterial cell and relative length of its endoflagellum to the cell length. (D) Schematic diagram of the bacterial cell wall. Reprinted from Bharti *et al.* (2003) and Picardeau (2017) with permission from Elsevier and Nature Publishing Group, respectively.

A



B

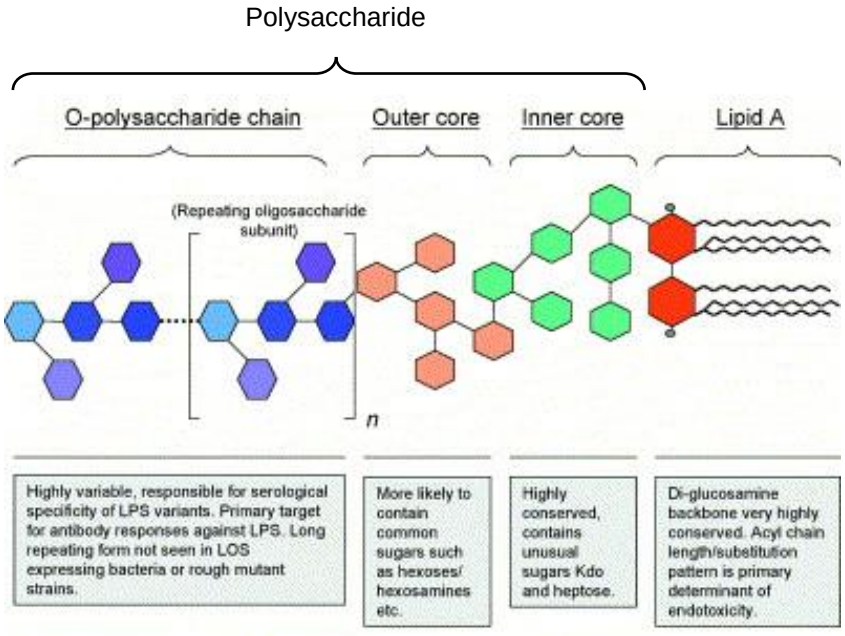


Figure 2.5 Schematic diagram of *Leptospira spp.* cell wall. (A) Illustration of the bacterial cell wall architecture. It consists of outer and inner membrane, spaced by periplasm with contains endoflagellum. Lipopolysaccharide, lipoprotein, outer- and transmembrane proteins are abundantly located at the outer membrane, while several transport systems are located within inner membrane layer. (B) Schematic structure of lipopolysaccharide. It consists of repetitive O-antigen and Lipid A, linked by a core oligosaccharide region. Reprinted from Raja and Natarajaseenivasan (2015) and Erridge *et al.* (2002) with permission from Elsevier.

lipoproteins as well as lipopolysaccharide (LPS). While LPS constitutes the major component of *Leptospira spp.* OM, it is absent from other spirochetal pathogens such as *B. burgdorferi* and *T. pallidum* (Ren *et al.*, 2003). The LPS of *Leptospira spp.* consists of three components which are associated to each other via covalent linkage (**Figure 2.5B**): i) hydrophobic lipid A that protrudes from the bacteria OM, ii) O antigen side chain that highly exposes to the environment and iii) oligosaccharide macromolecules that join the lipid A and O antigen together (Patra *et al.*, 2015). It is generally known that LPS oligosaccharide composition and orientation are different among *Leptospira*. As a consequence, this characteristic contributes to classification of *Leptospira* serovars and serogroups (Adler, 2015). Patra *et al.* (2015) reported that the pathogenic serovar LPS is much more complex and possessed molecular mass higher than the intermediate serovar.

The LPS plays a different role in triggering innate immune response between human and murine. It activates TLR1 and TLR2 receptor in human but TLR2 and TLR4 in murine (Vaure and Liu, 2014). Inability of human TLR4 to recognize the leptospiral LPS could be an escape mechanism of *Leptospira* from being recognized by the human innate immune system. This lead to a delayed immune response in human, resulting an overwhelming and probably lethal infection by the pathogenic *Leptospira spp* (Nahori *et al.*, 2005). Besides that, due to variation of LPS structure in different *Leptospira* serovars, monovalent LPS vaccine tends to provide protection against homologous infection, but partial or absence of protection against heterologous infection in a vaccinated animal (Wang *et al.*, 2007; Bulach *et al.*, 2000; Sonrier *et al.*, 2000). In the course of leptospirosis, the LPS appears to be the predominant antigen in triggering agglutination antibodies of a patient during convalescent phase of infection (Adler, 2015; Guerreiro *et al.*, 2001).

2.4.2 Outer Membrane Proteins

Right beneath leptospiral LPS is the outer membrane layer consisting of abundant OM proteins (**Figure 2.5**). Contrary to LPS, the leptospiral OM proteins are principally conserved across all species members of *Leptospira* (Cullen *et al.*, 2005). For this reason, OM proteins are thought to be good antigen target for diagnosis of leptospirosis as it overcomes serovar-specificity disadvantage of LPS. Lipoproteins are proteins with N-terminal signal peptide which undergo post-translational modification with fatty acid moiety after the signal peptide was removed. In *Leptospira spp.*, LipL32 protein is the most abundant OM protein with ~38,000 copies of molecules per cell occupying ~20% of leptospiral OM inner surface (Malmstrom *et al.*, 2009). This is followed by peptidoglycan associated cytoplasmic membrane protein (30,389 copies/cell), LipL36 (14,100 copies/cell) and LipL41 (10,531 copies/cell) (Malmstrom *et al.*, 2009).

The LipL32 is only conserved in pathogenic and intermediate species of *Leptospira*, but no evidence showed that it is important for OM integrity nor infection (Kumaran *et al.*, 2017). The lipoprotein may interact with host blood proteins such as fibronectin, plasminogen, collagen XX and laminin A5 (Chaemchuen *et al.*, 2011; Vieira *et al.*, 2010). Collectively, the host proteins serve as a masking layer to protect the bacteria cell surface assessable by immune proteins.

Even though LipL36 is conserved in infectious *Leptospira spp.*, evidences demonstrated that LipL36 is down-regulated *in vivo* (Barnett *et al.*, 1999). This downregulation could be related to change in environment as *lipL36* gene of *L. interrogans* serovar Copenhageni was shown to be downregulated at 37^{o}C and in the presence of sodium chloride at physiologic osmolarity (Matsunaga *et al.*, 2007; Nally *et al.*, 2001b).

The third abundant lipoprotein, LipL41, however, demonstrated opposite characteristic. While it is conserved among pathogenic *Leptospira spp.*, its level remained constant throughout different osmolarity and temperature studied to date, nor it is essential for acute leptospirosis event and trigger inflammation (King *et al.*, 2013; Matsunaga *et al.*, 2007; Yang *et al.*, 2006; Nally *et al.*, 2001b). For this reason, the lipoprotein has been used as a loading control in immunoblot application (Matsunaga *et al.*, 2013). Recently, LipL41 has been shown to form a 36-mer macromolecule with heme-binding ability, suggesting it to be involved in iron acquisition and metabolism (Lin *et al.*, 2013).

2.4.3 Periplasm

Periplasm of *Leptospira spp.* contains a thin peptidoglycan layer, a glycosaminopeptide polymer that assemble into exoskeleton and determine helical shape of the bacteria. As shown in **Figure 2.5A**, the peptidoglycan of *Leptospira* is unique as it is closely associated with the inner (cytoplasmic) membrane raher than the OM as in the cases for most of Gram-negative bacteria (Raddi *et al.*, 2012). The distinctive characteristic contributes low stability and high fluidity to OM of *Leptospira* (Tang *et al.*, 2014). On the other hand, Slamti *et al.* (2011) reported helical shape in the purified *Leptospira* peptidoglycan saccule. This leads to a conclusion that the peptidoglycan layer, together with other cytoskeleton proteins, play primary role in determining helical morphology of the bacteria.

2.4.4 Endoflagellum

The leptospiral endoflagellum (also known as periplasmic flagellum) presents as a vital component for corkscrew mobility of *Leptospira*. Similar to the other spirochetes, the leptospiral endoflagellum is located in periplasm. One tightly-coiled endoflagellum is situated near each termini of *Leptospira* and extends towards center of the cells without overlapping with the endoflagellum from the other ends (Wolgemuth, 2015).

Motility of *Leptospira* has been related to rotation direction of the endoflagellum (Wolgemuth *et al.*, 2006). Clockwise and anti-clockwise rotation of the endoflagellum leads to formation of hook- and spiral-shaped ends, respectively (Raddi *et al.*, 2012). As illustrated in **Figure 2.6**, translational motility occurs when spiral-shaped end is formed at the anterior while hook-shaped end is present at the posterior. Such asymmetric shape generates forward thrust that facilitates the bacteria to "swim" (Wolgemuth, 2015). On the contrary, a leptospiral cell rotating on the ground has hook- or spiral-shape at both ends (Wolgemuth, 2015). Interestingly, swimming direction of the bacteria has been observed to be transited as fast as hundred miliseconds facilitated by rapid reversal in the motor rotation direction (Kan and Wolgemuth, 2007; Goldstein and Charon, 1990).

2.5 Pathogenesis

Leptospirosis is a zoonotic disease transmitted to human via direct or indirect contact with pathogenic *Leptospira spp.* Human-to-human transmission are practically non-existence. Frequently, the bacteria gain entrance into the victim body via mucous membrane or cuts on the skin when exposed to leptospiral-contaminated medium such

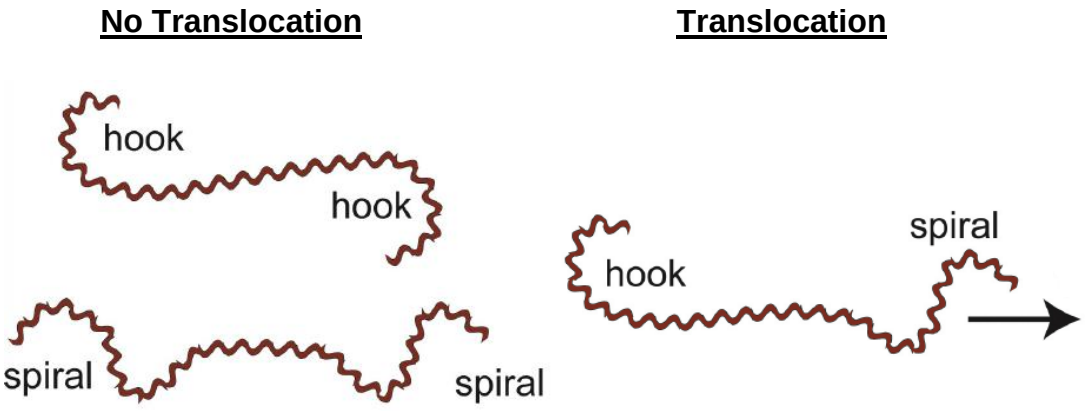


Figure 2.6 Illustration of leptospiral motility. The bacteria rapidly change its ends according to the need for translocation due to rotation of the endoflagellum. When one end is in spiral and the other end is in hook shape, the bacteria gain motility towards the direction of spiral shape. Nonetheless, the bacteria does not translocate when both end of the cell are in the same shape. Reprinted from Wolgemuth (2015) with permission from Elsevier.