ISOLATION OF *Leptospira* spp. AND SEROLOGICAL DIAGNOSES IN PATIENTS WITH ACUTE FEBRILE ILLNESS IN HOSPITAL UNIVERSITI SAINS MALAYSIA

AMIRA WAHIDA BINTI MOHAMAD SAFIEE

UNIVERSITI SAINS MALAYSIA

2018

ISOLATION OF *Leptospira* spp. AND SEROLOGICAL DIAGNOSES IN PATIENTS WITH ACUTE FEBRILE ILLNESS IN HOSPITAL UNIVERSITI SAINS MALAYSIA

by

AMIRA WAHIDA BINTI MOHAMAD SAFIEE

Thesis submitted in fulfilment of the requirements

for the Degree of

Master of Science

September 2018

ACKNOWLEDGEMENTS

Bismilliahirahmanirrahim.

Alhamdulillah, all the praise due to Allah. The research project that I have worked tirelessly on has come to completion with His mercy and guidance. First and foremost, I am grateful to Dr. Nabilah Ismail, my project supervisor. I am extremely thankful and indebted to her for sharing expertise, sincere and valuable guidance, advice and encouragement extended to me. My appreciation also goes to my first co-supervisor, Assoc. Prof. Dr. Chan Yean Yean for endless support and advices on improving the results. I'm also grateful to my second co-supervisor, Dr. Hashairi Fauzi from Emergency Department, Hospital Universiti Sains Malaysia (HUSM) for assist me in collecting the samples in Emergency Department. All the sample collection related works would not be smooth without the kind assistant from him.

I take this opportunity to express gratitude to the Department of Medical Microbiology & Parasitology and Emergency Department members and staff for their help, support and for providing equipment and services along my master project. Besides, I wish to thanks all the staff at MKA Perol for providing me the training and some materials for my research.

I wish to express my sincere thanks to my parents Mohamad Safiee bin Selamat and Fariza binti Ali@Fauzi and family who always give encouragement, moral support, financial support and attention to me every time. A million thanks you for the supports and loves. Not forgotten to my siblings for always give me the inspirations to move forward and making possible for us to move together, against wind and tide, until the end.

In addition, my sincere thank you to all the lab seniors and colleagues in the lab, especially to Ridhuan, Nik Zuraina, Nurul Najian, Iman, Yasmin, Ain, Adila, Izati, Eafifah, Ilia, Jalilah, Afiqah, Foo, Lily, Yuszrin, Azhar, Nik Hafiza, Che Ain, Siti and Amirah. Thank you for helping me in my research, giving me a kind guidance, patience and supporting me when I was in troubles and having problems with my research. A thousand thanks for sharing valuable information and providing excellent teamwork to me in all the times.

Most importantly, I would like to take this opportunity to acknowledge the financial support received from various parties. This study was supported by long term grants 203/PPSP/6770004.

I also place on record, my sense of gratitude to one and all, who directly or indirectly, have lent their hand in my research project.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
µg	microgram
µm	micromole
µM	microMolar
µl	microliter
µg/ µl	microgram per microliter
µg/ml	microgram per millilitre
>	more than
<	less than
≥	more than equal to
≤	less than equal to
°C	degree celcius
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
bp	base pair
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Haris
et al.	*ET alii*
g	gram
HUSM	Hospital Universiti Sains Malaysia
kb	kilo base pair
ligA	*Leptospira* immunoglobulin-like A
ligB	*Leptospira* immunoglobulin-like B
ligC	*Leptospira* immunoglobulin-like C
lipL21	lipoprotein 21
lipL32	lipoprotein 32
lipL41	lipoprotein 41
mg	milligram
mg/ml	milligram per millilitre
min	minute
ml	milliliter
MOH	Ministry of Health
NCBI	National Centre for Biotechnology Information
nm	nanometer
OmpL1	Outermembrane protein L1
pH	exponential of the concentration of hydrogen ion
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
sec	second
Taq	*Thermos aquaticus*
TBE	Tris Borate EDTA
Vol	Volume
v/v	volume over volume
w/v	weight over volume
WHO	World Health Organization
×	times

PEMENCILAN *Leptospira* spp. DAN SEROLOGI DIAGNOSIS DALAM PESAKIT DENGAN PENYAKIT DEMAM AKUT DI HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRAK

Leptospirosis ialah penyakit demam akut dan dikategorikan sebagai penyakit yang muncul semula di seluruh dunia. Kebanyakan insiden berlaku di negara tropikal seperti Malaysia. Leptospirosis disebabkan oleh spesis *Leptospira* patogenik. *Leptospira* kekal di persekitaran kerana bakteria ini berada di dalam perumah takungan yang mengalami jangkitan renal kronik terutama rodensia. Penularan kepada manusia berlaku melalui sentuhan secara langsung atau tidak langsung dengan urin haiwan yang dijangkiti. Oleh itu, kajian ini bertujuan untuk mengesan dan memencilkan *Leptospira* spp. daripada pesakit dengan penyakit demam akut di Hospital Universiti Sains Malaysia (HUSM) dan untuk mengkaji pelbagai cara pengkulturan untuk pemencilan *Leptospira* spp. Kajian in adalah sebuah kajian rentas diskriptif. Seramai 109 pesakit dengan simptom-simptom penyakit demam akut telah direkrut daripada jabatan kecemasan HUSM. Sampel darah telah diambil dan diinokulasikan di dalam medium Ellinghausen McCullough Johnson Harris (EMJH) yang diubah suai dengan penambahan pelbagai kepekatan 5-Fluorouracil. Kultur telah diinkubasi pada 30°C selama 6 bulan dan diperiksa setiap minggu di bawah mikroskop bagi mengesan kehadiran *Leptospira*. Ujian serologi melalui immunochromatografik (ICT) dan aglutinasi mikroskopik (MAT) telah dijalankan bagi menentukan kehadiran antibodi spesifik terhadap *Leptospira* dalam kalangan pesakit yang direkrut. Kultur yang positif telah diamplifikasi dan dikenalpasti melalui PCR dengan menggunakan penjujukan 16S rRNA gen. Kehadiran gen patogenik juga telah ditentukan

berdasarkan sembilan jenis gen patogenik iaitu *lfb1, flaB, OmpL1, ligA, ligB, ligC, lipL21, lipL32* dan *lipL41*. Keseluruhan sampel yang berjumlah 109 dikumpul daripada pesakit yang memerlukan rawatan di jabatan kecemasan HUSM. Berdasarkan pemerhatian mikroskopik, 1.85% (n= 2/109) sampel didapati positif dengan pemencilan *Leptospira* dan dilabel sebagai B004 dan B208. Sebanyak 2.75% (n= 3/109) sampel didapati positif melalui ujian immunokromatografik manakala semua sampel didapati negatif melalui ujian aglutinasi mikroskop. Tambahan pula, sampel positif kultur (B004) negative bagi ujian ICT tetapi sampel B208 intermediate dengan ICT dan negative dengan MAT. Dua isolat positif tersebut dikenal pasti sebagai *Leptospira interrogans* dan *Leptospira weilli* dengan menggunakan 16S rRNA. Kedua-duanya diklasifikasi di bawah kumpulan *Leptospira* patogenik dan masing-masing telah ditentukan dengan kehadiran sembilan dan lima gen patogenik. Pokok filogenetik telah dibina untuk menentukan hubungkait genetik antara dua spesis berbeza tetapi berada di bawah kumpulan patogenik. Kaedah optimisasi bahan tambahan kultur ke dalam EMJH media dijalankan dengan menggunakan pelbagai jenis bahan tambahan kultur dan jenis sampel dengan menggunakan pelbagai jenis serovar bagi penambahbaikan pemencilan. Kajian in menunjukkan EMJH dengan tambahan darah penuh dan tanpa sebarang bahan tambahan merupakan medium yang terbaik berbanding EMJH dengan tambahan serum manusia atau serum arnab. Kesimpulannya, dua isolat *Leptospira* patogenik berjaya dikultur daripada pesakit dengan demam akut di HUSM dan pencirian kedua isolat telah ditentukan dengan sembilan gen patogenik. Kajian lanjutan secara pendekatan komprehensif perlu dijalankan untuk penambahbaikan kadar pemencilan dan kajian molekular perlu lebih diterokai.

ISOLATION OF *Leptospira* spp. AND SEROLOGICAL DIAGNOSES IN PATIENTS WITH ACUTE FEBRILE ILLNESS IN HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRACT

Leptospirosis is an acute febrile illness and re-emerging disease that occurs worldwide and most incidence in tropical countries such as Malaysia. Leptospirosis is caused by the pathogenic *Leptospira* species. The disease is maintained in the nature by chronic renal infection of reservoir host particularly rodents and human transmission occurs through indirect or direct contact with the urine of infected animals. Leptospirosis is difficult to diagnose because of the unspecific symptoms and serological tests results that need to be interpreted carefully. There is much overlap in the clinical presentation of undifferentiated febrile illnesses, which includes leptospirosis, malaria, rickettsioses, and arboviral diseases, it is not possible to reliably predict the pathogen based on clinical signs and symptoms. Therefore, the aim of this study is to isolate the *Leptospira* spp. and to perform serological diagonoses from patients with acute febrile illness in Hospital Universiti Sains Malaysia (HUSM). This is a cross sectional descriptive study. All patients (n= 109) were recruited from the emergency department of HUSM with the symptoms of acute febrile illness. The blood samples were taken and inoculated in the modified Ellinghausen McCullough Johnson Harris (EMJH) media with addition of different concentration of 5-Fluorouracil. The cultures were incubated in incubator shaker at 30°C for 6 months and examined weekly under dark-field microscopy for presence of *Leptospira*. Serology tests which were immunochromatography test (ICT) and microscopic agglutination test (MAT) were carried out to determine the presence of

specific antibodies against *Leptospira* in the recruited patients. The positive cultures were amplified and identified by PCR on 16S rRNA gene by sequencing. The presence of the pathogenic genes also was determined by using nine pathogenic genes which are *lfb1, flaB, OmpL1, ligA, ligB, ligC, lipL21, lipL32* and *lipL4.* A total of 109 samples from patients whose seek treatments at emergency department of HUSM were collected. Based on microscopic observation under dark field microscope, 1.85% (n= 2/109) of the samples were positive with *Leptospira* isolation which were labelled as B004 and B208. Only 2.75% (n=3/109) were positive when tested with ICT. All samples with positive and intermediate ICT tested with MAT were all negative. In addition, sample with positive culture (B004) was tested negative for ICT meanwhile, B208 was tested intermediate with ICT and negative with MAT. Isolates B004 and B208 were identified by 16S rRNA as *Leptospira interrogans* and *Leptospira weilli* respectively. Both of the isolates were classified under pathogenic *Leptospira* and were determined by the presence of nine and five pathogenic genes respectively. The constructed phylogenetic tree confirms the genetic relationships between the two species which arised from different species under pathogenic group. The optimization of different culture supplementation and type of samples were conducted by using different type of serovars for isolation improvements. The results showed whole blood and EMJH without addition of others supplement were the best among others which were human serum and rabbit serum. In conclusion, two pathogenic *Leptospira* isolates were successfully cultivated from patients with acute febrile illness in HUSM and both were characterized by nine pathogenic genes. Further study with comprehensive approaches need to be conducted to improve the isolation rate and molecular study could be more explored.

CHAPTER 1

INTRODUCTION

1.1 Taxanomy & Classification

Leptospira belong to the order of Spirochaetales, family *Leptospira*ceae, genus *Leptospira* (Faine *et al.*, 1999). Historically, *Leptospira* were divided into two species which are *L. interrogans* and *L. biflexa*, pathogenic and non-pathogenic respectively. *Leptospira* is divided into several species and subspecies which are called serogropus and serovars. To date, more than 24 serogroups and 250 serovars of pathogenic *Leptospira* have been described (Galloway & Levett, 2010). Within each species, large number of serovars were differentiated (Mohammed *et al.*, 2011). Genus *Leptospira* is divided into 23 species classified into saprophytic, intermediate and pathogenic groups as shown in Table 1.1 (Puche *et al.*, 2018). There are two ways to classify the *Leptospira* which are by serological and genotypic classification. The precise identification and classification of *Leptospira* spp. is vital for epidemiological and public health surveillance.

Table 1.1: List of *Leptospira* species (adapted Puche *et al.*, 2018).

Group	Species
Pathogenic	*Leptospira interrogans*
	Leptospira kirschneri
	Leptospira noguchii
	Leptospira borgpetersenii
	Leptospira alexanderi
	Leptospira weilii
	Leptospira santorasai
	Leptospira kmetyi
	Leptospira alstoni
	Leptospira mayottensis
Intermediate	*Leptospira licerasiae*
	Leptospira wolffii
	Leptospira fainei
	Leptospira broomii
	Leptospira inadai
	Leptospira venezuelensis
Saprophytic	*Leptospira idonii*
	Leptospira meyeri
	Leptospira terpstrae
	Leptospira biflexa
	Leptospira vanthielii
	Leptospira yanagawae
	Leptospira wolbachii

1.2 Biology of *Leptospira* spp.

1.2.1 Microbiology

Leptospira species are Gram negative and aerobic bacteria with a hook-like end, very thin, spiral and motile which rapidly rotate on their longitudinal axis (Smith & Self, 1955). *Leptospira* are motile and small in diameter requiring dark field microscope or phase contrast for observation. In addition, *Leptospira* are bacteria which can be either pathogenic or saprophytic. The saprophytic *Leptospira* is a free living and normally not to cause disease to human (Mohammed *et al.,* 2011). Saprophytic *Leptospira* can be found in many types of wet or humid environment, which varies from surface water and moist soil to tap water. In contrast, the pathogenic *Leptospira* have the possibility to cause disease in humans and animals (Faine *et al.,* 1999; Issazadeh *et al.,* 2008; Victoriano *et al.,* 2009).

1.2.2 Morphology

Leptospira spp. are spirochetes bacteria with corkscrew-shape but different from other spirochetes for the presence of a hook-end like with 0.1 μm width and tightly coiled with length of 6-20 μm (Figure 1.1, picture A). The cells have pointed ends, one or both end is usually bent into a characteristics hook. They are obligate aerobic, do not persist in drought or hypertonicity, however they support alkaline environment to pH 7.8 (Mohammed *et al.,* 2011). Meanwhile, *Leptospira* is very thin, it cannot be seen under light microscopy. They also cannot be stained by aniline dye and were stained faintly by Geimsa stain. The best stain for *Leptospira* is silver impregnation techniques (World

Health Organisation, 2010) or artificial thickening by immunoperoxidase or immunofluorescence (Andre-Fontaine *et al.,* 1992).

Under dark field microscopy, they appear as actively motile spirochetes. The responsible motility for these bacteria are two periplasmic flagella with polar insertions and located at the periplasmic space. Under electron microscope, flagella B showed that it mutant to be deficient in endoflagella and non-motile. Besides, *Leptospira* have a typical double membrane structure in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane. The main antigen for the *Leptospira* is lipopolysaccharides (LPS) and it is located within the outer membrane. It is similar in structure and immunology with the LPS from Gram negative organisms (Andre-Fontaine *et al.,* 1992). All of *Leptospira* look alike with only minor differences, so the morphology does not help to distinguish between pathogenic and saprophytic *Leptospira* or between the various pathogenic *Leptospira* (World Health Organisation, 2003).

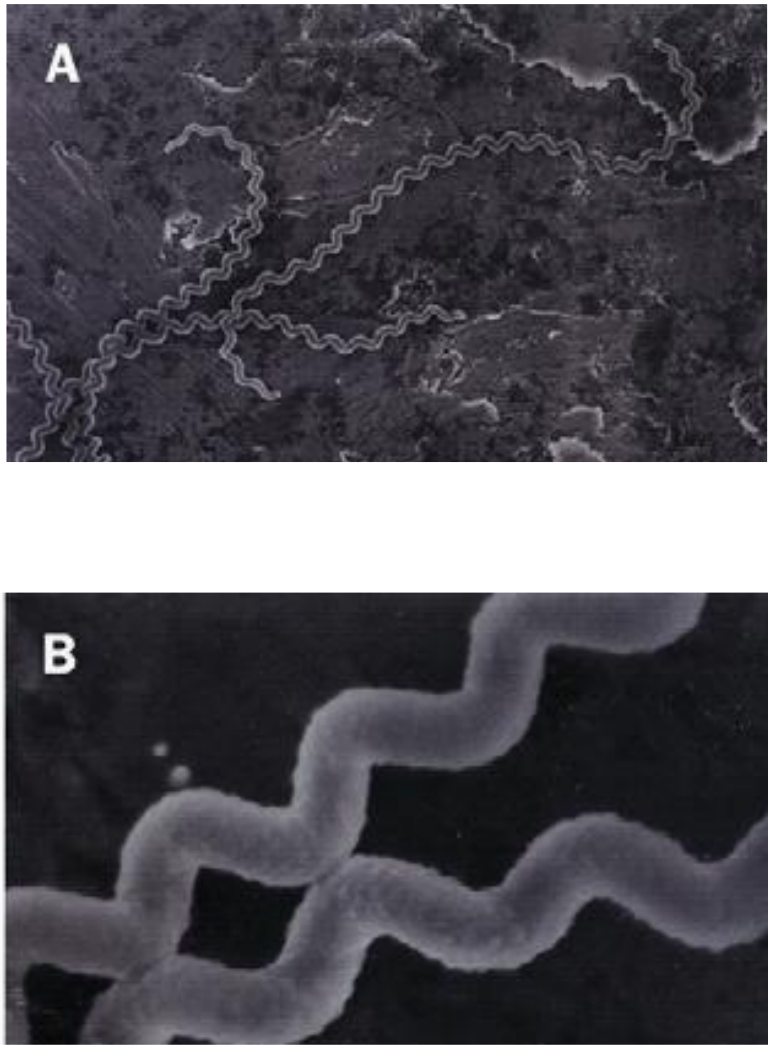

Figure 1.1: High-resolution scanning electron micrograph of *Leptospira interrogans* serovar Copenhageni (adopted Bharti *et al.*, 2003).

(A) Characteristic hooked ends.

(B) At high magnification the surface of the spirochete seems ruffled and beaded.

1.2.3 Physiology, metabolism and growth of *Leptospira*

Leptospira spp. are able to live in alkaline sludge, soil, streams, rivers, swamps, tissue and organ of live or deceased animals. They need particular condition for their growth. These are several factors that contribute to the survivability of the pathogenic *Leptospira* which are pH, temperature and the presence of the inhibitory compound. Basically, they are susceptible to the acid, basic disinfectants, heat and dryness (Faine *et al.,* 1999). In the environment, they need high humidity for survival and can be killed by dehydration or temperatures higher than 50 °C. They can stay alive up to a few months in contaminated soil and several weeks in livestock slurry. Under laboratory condition, they can survive for several months in water but do not survive in river water under natural conditions.

According to World Health Organisation, 2010 *Leptospira* are aerobic and can consume a long chain of fatty acids as their carbon and energy sources and which are metabolized by β-oxidation. Besides the long chain of fatty acids, they also require Vitamin B1, Vitamin B12 and ammonium salts for their growth rates. *Leptospira* are also resistant to the antibacterial activity of pyrimidine analogue 5-flurouracil because they utilize purine bases but not pyrimidine bases (Faine *et al.*, 1999a).

The growth of the *Leptospira* is often slow on the primary isolation and it has to be maintained until 13 weeks before discarded. The most widely used medium to culture the *Leptospira* is oleic-albumin medium Ellinghausen-McCullough-Johnson-Harris (EMJH) (Levett, 2001). To reach a maximum growth, agar may be added at a low concentration of approximately 0.1%-0.2%. They can reach the maximum density in such semisolid media. They can grow well in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds (Mohammed *et al.,* 2011).

Besides, the growth is also related to the optimum oxygen tension which is known as Dinger's ring or disk as shown in Figure 1.2. For a long term storage, to yield good result and to maintain the virulence, it can be stored in liquid nitrogen (Mohammed *et al.,* 2011). *Leptospira* can also grow on solidified media (Girons *et al.,* 2000; Turner, 1970) which has been used to isolate the bacteria or to separate mixed cultures of *Leptospira*. It is also used for detection of hemolysin production by *Leptospira* (Sonrier *et al.,* 2000). The colony of the bacteria depends on the concentration of the agar and the type of serovars (Tripathy *et al.,* 1980).

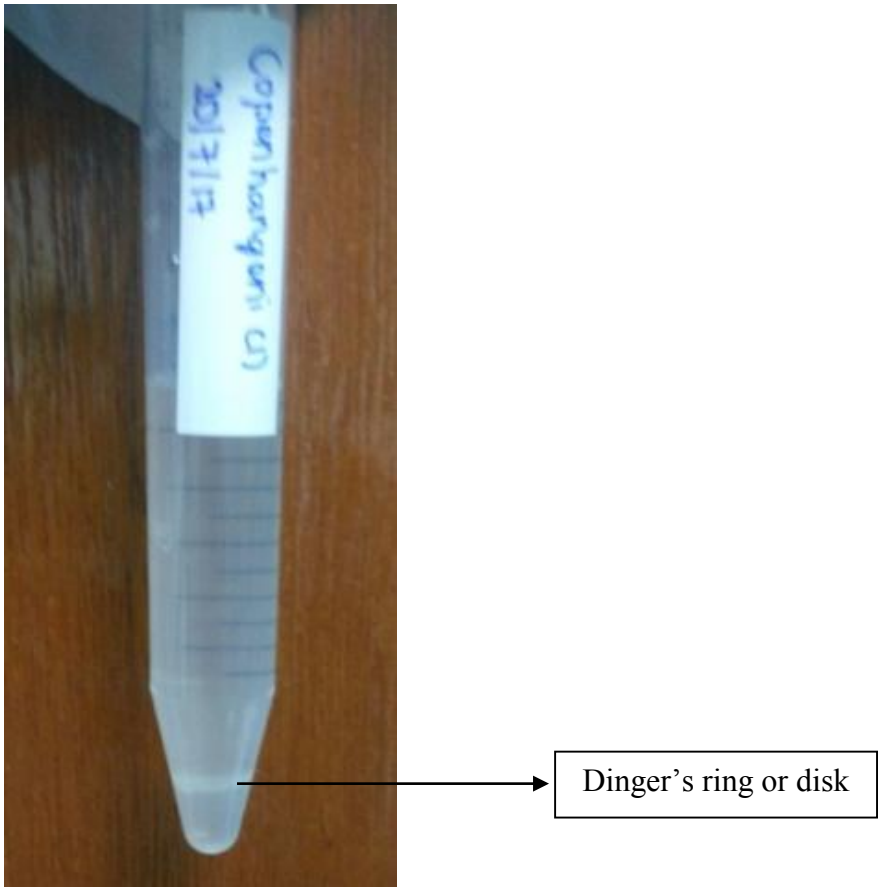

Figure 1.2: The growth of *Leptospira* forms Dinger's ring or disk

1.2.4 Distribution in soil and water

The saprophytic *Leptospira* is a free living bacteria and normally not to cause disease in human. It can be found in many types of humid and wet environment (Mohammed *et al.,* 2011). However pathogenic *Leptospira* is excreted to the environment from the urine of host animals. The bacteria can survive up to weeks or months in suitable fresh water and soil (Levett, 2001; Plank & Dean, 2000). In tropical climates, the movement of flood water can also carry the bacteria from place to place, distributing the contaminated sites all over the community (Maciel *et al.*, 2008; Reis *et al.,* 2008). The prevalence of the environment associated with the infection of leptospirosis increased, most probably because the contact rate of the susceptible hosts and the contaminated environment is high (Desvars *et al.*, 2011; Ko *et al.*, 1999).

1.2.5 Animal carriers

Leptospirosis is recognized as a zoonotic disease. Rodent or small mammals are known as reservoirs or maintenance host for the disease. Usually rodent mice are maintenance host for the serogroup Ballum and rats are maintenance host for serogroup Icterohaemorrhagiea and Ballum. Household animals such as pig may harbor Pomona, Tarassovi or Bratislava, dogs may harbor Canicola, sheep may harbor Pomona and Hardjo, dairy cattle may harbor Grippotyphosa, Pomona and Hardjo are also know as maintenance hosts for the infecting bacteria (Bolin, 2000). The incidence of the leptospirosis reveals a complex relation between animal hosts, human and environment. The bacteria colonize the kidney of reservoir hosts, allowing a persistent discharge of the *Leptospira* into the environment throughout their life time. Since rodents not experience

with the mortality and morbidity caused by *Leptospira*. (Mason *et al.*, 2015; Somrongthong *et al.*, 2012).

1.3 History of leptospirosis

According to World Health Organization 2010, leptospirosis was described as a disease entity by Adolf Weil in Heidelberg, 1886 and it is known as Weil's disease. Nowadays, all the *Leptospira* infections are called leptospirosis regardless of clinical symptoms and signs. The symptom that was described by Weil is a syndrome of severe multisystem disease, presenting with profound jaundice and renal function impairment (Faine *et al.*, 1999). However between severe icteric leptospirosis and yellow fever continued to be diagnostic confusion, but with prominent researchers such as Stokes and Naguchi were dying in their research to discover the causative agents (Feigin & Anderson, 1975). Almost simultaneously in Japan and Germany, they were first visualized in autopsy specimens from a patient thought to have yellow fever, but were not isolated until several years later (Everard, 1996). Both of them had been detected with spirochetes and specific antibodies in the patients' blood. Independently, in the second half of the twentieth century, Inada and Ida in Japan, and Uhlenhuth and Fromme in Germany had discovered the pathogen responsible for the disease which is *Leptospira*.

In 1917, it has been discovered that rat as a role of source of human infection (Ido *et al.*, 1917). However since the cases of workers in Japan and Germany, *Leptospira* have been isolated from almost all mammalian species such as dogs and canines except for Antarctica (Ben Adler & Pen, 2010). Some years later, leptospirosis in livestock was

recognized (Smith, 1952). It is occurring globally but was most recognized in tropical regions such as Malaysia. Human infection can occur by direct and indirect transmission but most regular is via indirect exposure to the organisms in soil and water (Levett, 2001).

The first leptospirosis case in Malaysia was in 1925 discovered by Flecther in Kuala Lumpur General Hospital (Lim *et al.,* 2011; Benacer *et al.,* 2016). Flecther was not only the earliest to discover and isolate the *Leptospira* from blood, kidney and liver, but he also was able to identify three different serovars which are *Leptospira interrogans* serovar Icterohaemorrhagiae, *L. interrogans* serovar Hebdomadis and *L. interrogans* serovar Pyrogenes. Besides, he also had introduced a medium used to isolate *Leptospira* spp. and still used in many laboratories in Malaysia. After the first cases have been reported, subsequent cases have been recorded with a rising number of cases over the years (Benacer *et al.,* 2016).

1.4 Epidemiology of leptospirosis

The occurrence of the leptospirosis is higher in warm climate countries than in temperate region. Furthermore, most of the tropical countries are developing countries which have greater chances for exposure of human population to the infected animals, domestic pets or wild, livestock and feral animal (Levett, 2001). Thus, most of the developing countries have been reported with an outbreak including Malaysia. After the first reported case in the early 1920s, there are many occurrence of cases in Malaysia have been reported (Sejvar *et al.,* 2003; Koay *et al.,* 2004; Benacer *et al.,* 2016). During the year 1984, outbreak was occurring in Mulu Caves, Sarawak. Leptospirosis was suspected

in sixteen British cave explorers returning to British ill. Later five of the patients had fever with unidentified origin and hepatomegaly without renal failure. After that, the diagnosis of leptospirosis was confirmed by serology test (Waitkins, 1986). The chronology of the leptospirosis outbreaks in Malaysia since 1984 were shown in Table 1.2 below.

Table 1.2: The chronology of the leptospirosis outbreaks in Malaysia since 1984 (adopted Benacer *et al.,* 2016).

Outbreaks	Year	Description
Mulu caves, Sarawak	1984	After exploration of Mulu caves in Gunung Mulu National Park, Sarawak, 16 of British cave explorers return to Britain ill; 5 patients had fever of unknown origin and hepatomegaly without renal failure. Leptospirosis was suspected and later confirmed by serology.
Sarawak	1985	A group of British tourists visited the Sarawak chamber and 2 contracted leptospirosis.
Beaufort, Sabah	1999	After swimming in a creek near an oil palm plantation in Kampung Kebatu, Beaufort, Sabah, 46 locals fell ill. One fatality was reported when a 15-year-old boy died from hemorrhagic shock secondary to pulmonary haemorrhage. Investigations revealed creek water contaminated with urine tainted with leptospirosis of animal origin (cattle, pigs, dogs, rodents, and wild animals), with prior flooding facilitating the spread of the organism.
The EcoChallenge, Segama River, Sabah	2000	Athletes kayaking and swimming in Segama River were diagnosed with leptospirosis. This outbreak was recognized as the first international outbreak associated with outdoor adventure. Experts pinpointed the river water as the source of outbreak. Athletes who took doxycycline prior to the challenge were spared from infection.
Johor	2006-2007	Following floods that affected all 8 districts in Johor between December 2006 and January 2007, 20 cases of leptospirosis, with 2 deaths were reported.
Juru, Penang	2009	There were 26 leptospirosis cases, with 2 deaths, reported at the illegal migrant detention center in Juru, Penang. The 2 who died were Burmese migrants, and drank water contaminated with animal urine, potentially rats, was suspected to be the cause.
Maran, Pahang	2010	A total of 8 deaths were reported among the 83 people involved in the rescue operation of a drowned victim. The investigations disclosed that the river water was contaminated with urine of rats or other animal carriers. The infections occurred while rescuers used river water for their daily chores. Upon outbreak confirmation, the recreational park was temporarily closed to the public.

Table 1.2: continued

The Bukit Jugra Royal Malaysian Air Force base	2011	A total of 24 air force trainee commandos were infected; it was confirmed serologically as caused by *Leptospira*. Investigation showed that the infection occurred while training in water contaminated with urine of rats or other animals.
Kangar, Perlis	2012	A family of 8 of 28 men who went fishing at a swamp developed symptoms and were hospitalized in Hospital Tuanku Fauziah, Kangar, Perlis. Serological tests for *Leptospira* IgM confirmed that 6 of the 8 men tested positive. Water samples from the swamp were screened and confirmed by PCR as being tainted with *Leptospira.*

Therefore, Malaysia is an endemic area for leptospirosis (El Jalii & Bahaman, 2004). Moreover, the spread of the *Leptospira* also connect with the incident of severe climate and flooding which washes contaminated soil associate with animal urine into the supply of water for human utilize (Ko *et al.,* 1999; Sanders *et al.,* 1999; Barcellos & Sabroza, 2001; Benacer *et al.,* 2016). Besides that, several outbreaks have been reported that relate leptospirosis with high rainfall. Poor sanitary condition will also draw the attention of rodents to come to the area and contaminate the water and soil which in turn risk an infection to human (Koay *et al.,* 2004; Victoriano *et al.,* 2009).

The annual incidence of leptospirosis is estimated at 0.1 to 10 in every 100,000 people globally and could be higher in the event of flooding and heavy rainfall (F. Costa *et al.,* 2015; Pappas *et al.,* 2008; World Health Organisation, 2003). The incidence of leptospirosis has become serious a public health worldwide and a prominent increasing in number of reported cases and outbreaks have been reported in Southeast Asia including Indonesia, India, Thailand, Malaysia and also South and Central America (Mendoza, 2010; Victoriano *et al.,* 2009).

Parallel to the cases that have been reported in Malaysia, other countries in Asia-Pacific region have also documented several outbreaks in which the annual occurrence in the region ranging from low to moderate to higher incidence with mortality case between 5% to 40% (Lim *et al.,* 2011). In addition, Thailand reported the highest incidence of leptospirosis, which occurred primarily during rainfall season and documented to have a drastic increase with occurrence of 0.3 per 100 000 in 1995, which spiked in 2000 to an occurrence of 23.7 per 100 000 population (World Health Organisation, 2009). *L.*

interrogans serovar Autumnalis was the major serovar circulating in the Thailand population (Thaipadungpanit *et al.,* 2007).

Besides, Philippines has also recorded outbreaks of leptospirosis during typhoon season which from July to October of the year. During the natural disaster of typhoon, Metro Manila reported to have 2121 patient and 178 died in 15 hospitals with suspected symptoms of leptospirosis (Yanagihara *et al.,* 2007; Benacer *et al.,* 2016). The predominant serovar found in Philippines were Bataviae, Grippotyphosa, Manilae, Pyrogenes, Javanica and Pomona, associated with the workers that involve with animals (Victoriano *et al.,* 2009).

Leptospirosis in Indonesia is frequently linked to being clinical apparent due to the lacking in the diagnostic confirmatory test for definitive result or misdiagnosed with other tropical disease such as dengue fever. Regardless, the prevalent serovar that was identified in the country is *L. interrogans* serovar Bataviae (Sakundarno *et al.,* 2014).

Many factors contributed to leptospirosis including population density, the level of contact between accidental hosts and maintenance and also climate. Besides, leptospirosis is also known as an occupation disease, thus occupation associated with the recreational activity, animals, climates and socioeconomic are related to the incidence of leptospirosis (Vke Mbbs, 2011). In addition, there are three patterns of epidemiology of leptospirosis were defined by (Faine, *et al.,* 1999) which are leptospirosis usually occurs in temperate climate, the second is occurs in tropical wet areas and last but not least is rodent-borne infection in the urban area.

1.5 Leptospirosis

Leptospirosis is one of the re-emerging infectious diseases. It is also an acute infectious disease in human caused by pathogenic spirochetes of the genus *Leptospira* and classified as zoonosis. Leptospirosis is an important disease and can be a severe public health concern in tropical and subtropical countries with high rainfall such as Malaysia. Leptospirosis is a serious public issue due to its epidemic potential, its global distribution, its presence in animals or natural environment and its high potential for human mortality if left untreated (Picardeau *et al.,* 2014).

1.5.1 Pathogenesis

Leptospira penetrates into the body through cuts and abrasions, mucous membrane or conjunctivae, aerosol inhalation of microscopic droplets, genital track or breaches of the surface integument (Mohammed *et al.,* 2011). A case study reported a large leptospirosis outbreak happened in the 1998 Springfiled Illnois Triathlon is by ingestion of the lake water by the participants A case control study of a large leptospirosis outbreak in the 1998 Springfiled Illnois Triathlon is by ingestion of the lake water by the participants (Prescott *et al.,* 2002). Therefore, the most crucial way of entry is by oral mucosa after the ingestion. This requires chemotaxis mechanisms for adhesion and transmembrane passages. In order to go through the host body, they need to win the vascular compartment. Then, they will retain in renal tubules and only discarded in the urine for a period of few weeks to several months and intermittently even longer. After that, *Leptospira* will cause lesion due to the exploit of the undefined *Leptospira* toxin(s)

or toxic cellular components and consequent symptoms will appear. Endotoxin activity has been reported in several serovars (Mohammed *et al.*, 2011).

In addition, lipopolysaccharides (LPS) inside the *Leptospira* exhibit the biological assays for endotoxin similar to other gram negative bacteria. Human susceptibility to leptospirosis may be related to poor recognition of *Leptospira* LPS by the innate immune system (Werts *et al.*, 2001). Human toll-like receptor (TLR) 4, responds to extremely low concentrations of gram negative LPS (endotoxin), appears to be unable to bind *Leptospira* LPS (Nahori *et al.*, 2005; Werts *et al.*, 2001) perhaps because of the unique methylated phosphate residue of its Lipid A (Que-Gewirth *et al.*, 2004). Moreover, production of haemolytic toxins which act as sphingomyelinases, phospholipases or pore-forming proteins can cause tissue damage directly (Smythe *et al.*, 2002). Hemolysins have also been suggested to be phospholipases that acts on erythrocytes (Thompson & Manktelow, 1986) and other cell membranes which contain substrate phospholipid and lead to the cytolysis (Smythe *et al.*, 2002).

Furthermore, the incubation period for them to invade the immune system in human bodies depends on growth rate of organisms, immunity, infective dose and their toxicity. The mechanisms whereby *Leptospira* cause the disease are not clearly understood. There are many potential virulence factors such as immune mechanisms, toxin production, adhesins and other surface proteins. In liable host such as human, systemic infection can produce severe multi-organ manifestations. Pathogenicity of the leptospirosis appears complex although the pathogenic mechanisms of *Leptospira* are not clearly defined but potential virulence factors include lipopolysaccharide (LPS), outer membrane proteins (OMPs) and adhesion molecule genes presence in the pathogenic *Leptospira* may help in

pathogenicity mechanisms (Shang *et al.,* 1996; Matsunaga *et al.,* 2003). Differentiation between the pathogenic and non-pathogenic strains is also crucial to classify the pathogenic status for epidemiological and taxonomical study.

1.5.2 Transmission

Leptospira can be transmitted directly or indirectly (Sejvar *et al.,* 2005; Victoriano *et al.,* 2009; Vinetz *et al.,* 1996) from animals to humans. In addition, *Leptospira* retain and infect the host renal tubules of reservoir hosts such as rodents, cattle and also horses. They are excreted into the surroundings via urination, in which they can survive in the moist soil and surface water up to several months (Smith & Self, 1955; Trueba *et al.,* 2004). Then, the animals' urine will contaminate the environment such as water and soil (World Health Organisation, 2010). Human will get infected upon the exposure to the contaminated environment (Waitkins, 1986). Pathogenic *Leptospira* can survive for many days up to several months in wet soil and fresh water with neutral or slightly alkaline pH which can be a vital channel in their transmission (Faine *et al.,* 1999).

Human has high chances to get infected by *Leptospira* through occupational, recreational or domestic contact with the urine of the carrier animals. Furthermore, this disease also associated with occupation especially in developed countries, with agricultural and animal production (Ben Adler & Pen, 2010). Leptospirosis in human can be different according to the serovars that infecting the patient, the age, health and immunological competence of the patient.

Leptospira may be contaminated to humans directly by contact with infected urine or indirectly via contaminated soil or water, particularly in times of flood. Human leptospirosis composes a dead-end infection and human to human transmission is virtually unidentified. The Figure 1.1 showed the cycle of *Leptospira* infection in human population (Victoriano *et al.*, 2009).

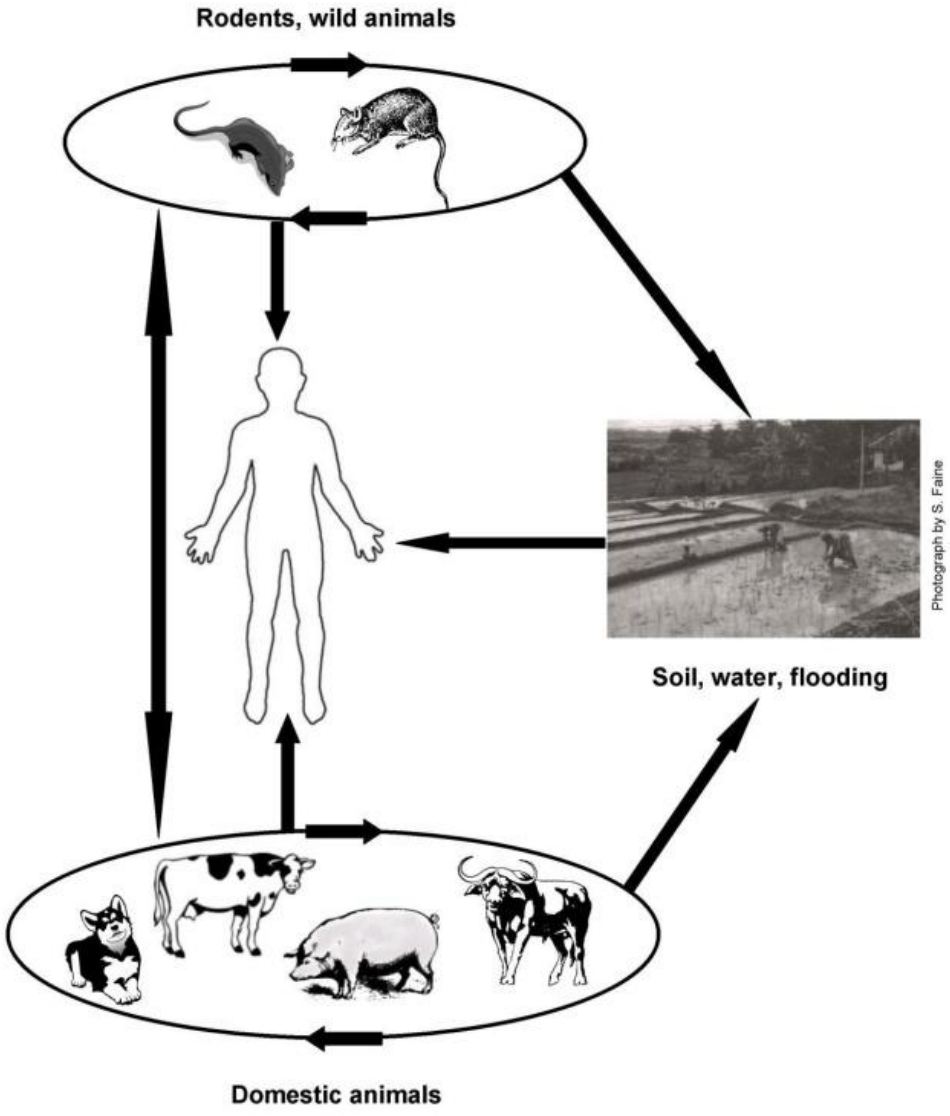

Figure 1.3: Transmission of *Leptospira* in the environment (adopted Victoriano *et al.*, 2009).

1.5.3 Clinical presentations

Leptospirosis is acknowledged as a great mimicker because of the enormously wide variety symptoms ranging from subclinical disease such as a flu-like illness to severe syndrome of multiorgan infection with high mortality. The symptoms can imitate those of infections such as influenza, hepatitis, meningitis, viral haemorrhagic fever and dengue. One study reported that 38% of the leptospirosis cases were misdiagnosed as haemorrhagic fever or dengue fever due to the similar clinical appearance and imitation to other tropical disease (Rafizah *et al.,* 2012). The history of exposure and risk factors compatible with leptospirosis should alert the clinician to a possible diagnosis (Forbes *et al.,* 2012).

Acute febrile illness is defined as fever more than 38°C lasting for less than 2 weeks (Kashinkunti & Gundikeri, 2013). Acute febrile illness is a common symptoms for patients to seek a treatment at the emergency department or hospital care (Parker *et al.,* 2007; Kashinkunti & Gundikeri, 2013; Tun *et al.,* 2016). However in the tropical or developing countries, symptoms of acute febrile illness is undifferentiated in many diseases for instance hepatitis, meningitis, dengue, malaria, leptospirosis, influenza, influenza or viral hemorrhagic fever, interic fever and rikettsiosis (Ismail *et al.,* 2006; Kashinkunti & Gundikeri, 2013). Many studies have been performed to observe the undifferentiated acute febrile illness patients with the burden of leptospirosis and other tropical diseases. Leptospirosis contributed to 1.1% to 29.5% of the patients with acute febrile illness patients in the studies (Leelarasamee *et al.,* 2004; Manocha *et al.,* 2004; Ismail *et al.,* 2006; Suttinont *et al.,* 2006; Parker *et al.,* 2007; Kashinkunti & Gundikeri, 2013; Tun *et al.,* 2016).

Leptospirosis is mainly presented as biphasic illness. The first stage of the illness is known as septicemia or leptospiremia (Forbes *et al.,* 2012). Usually, the early symptoms are known to be chills, headache (severe and persistent), fever, diarrhea or a rash, myalgia, malaise, retro-orbital pain, prostration, conjunctival suffusion, lung involvement, muscle tenderness and headache. It appears quite abruptly after an incubation period of about 10 days in the range of 4 to 19 days. The headache is regularly severe and has been described as a bitemporal, frontal throbbing headache accompanied by retro-orbital pain and photophobia. Then, muscle tenderness is also familiar and typically involves the calves and lower back. A report showed the conjunctival suffusion is the way to categorize leptospirosis (dilatation of conjunctival vessels without purulent exudate), which happen commonly in leptospirosis, but is uncommon in other infectious diseases (Haake & Levett, 2015).

However, a large number of infected patients by *Leptospira* have asymptomatic infection particularly patients from endemic areas. Mild leptospirosis is the utmost common form of the disease with percentage of 90% of the cases (Forbes *et al.*, 2012). Acute leptospirosis constantly presented with chills, headache, fever, conjunctival suffusion, vomiting, severe myalgia, nausea, anorexia and malaise (Mansour-Ghanaei *et al.*, 2005). Second stage referred to the immune stage or leptospiruric stage of the illness. This is when IgM antibodies are produced and *Leptospira* are prominent features at outset are fever, myalgia and headache (Forbes *et al.*, 2012).

Leptospirosis can be a more severe disease, commonly known as Weil's disease or icteric leptospirosis. The disease frequently present late in the course of disease. Icteric leptospirosis contributes to high mortality rate, which ranging between 5 and 15%.

Pulmonary haemorrhage and acute kidney injury are the main reasons for death in leptospirosis (Costa *et al.,* 2001; Forbes *et al.,* 2012; Katz *et al.,* 2001). One study has reported lung and kidney as the most involved organs in 87% of patients with leptospirosis. The pulmonary involvement has appeared as a serious life threatening event, and becoming the main cause of death due to leptospirosis in some countries (Dolhnikoff at al., 2007). Massive pulmonary haemorrhage was seen in 77% of the patients in the study (Salkade *et al.,* 2005). In addition, renal involvement in leptospirosis was characterized by acute interstitial nephritis that may be connected with acute tubular necrosis. Predisposition to hypokalemia is another particular constituent of renal involvement in this disease (Abdulkader *et al.,* 1996).

Besides, Weil's disease was also characterized by dysfunction of several organs including kidneys, liver, brain and lung. Mortality rate could reach 50% in fulminant Weil's disease which resulted in cardiovascular collapse and pulmonary haemorrhagic pneumonitis (Chawla *et al.,* 2004; Marotto *et al.,* 1999; McBride *et al.,* 2005b).

1.5.4 Pathology

Pathology of the leptospirosis is characterized by the growth of endothelial damage, vasculitis and inflammatory infiltrate composed of plasma cells, neutrophils, histiocytes and moncytic cells (Arean, 1962), In addition, organs are frequently discolored due to the level of icterus (Levett, 2001). Acute and chronic leptospirosis often engage the organ system, thus their components or *Leptospira* can be visualized through various organs for instance lungs, kidney, brain, liver, spleen or genical tract (Schreier *et al.,* 2013). In