IN VITRO ANTIMICROBIAL ACTIVITIES OF *Phyllanthus amarus* EXTRACT, ANTIBIOTICS AND THEIR COMBINATION AGAINST *Leptospira interrogans*

CHE AIN MUNIRAH BINTI ISMAIL

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by

CHE AIN MUNIRAH BINTI ISMAIL

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

ß	Beta
n	Numbers of FIC in the 96-well plate
≤	Less than or equal to
≥	More than or equal to
>	Greater than
<	Less than
=	Equals to
Σ	Sum of
°C	Degree Celsius
µL	Microliter
µg/mL	Microgram per milliliter
µm	Micrometer
%	Percentage
cm	Centimeter
g	Gram
g/L	Gram per liter
mg	Milligram
mL	Milliliter
mL/L	Milliliter per liter
nm	Nanometer
rpm	Revolutions Per Minute
x	Times

LIST OF ABBREVIATIONS

aa-tRNA	Aminoacyl-transfer ribonucleic acid
AE	Aqueous extract
APEC	Avian pathogenic *Escherichia coli*
AST	Antimicrobial susceptibility testing
ATP	Adenosine triphosphate
CEFT	Ceftriaxone
CFU	Colony-forming units
CLSI	Clinical and laboratory standards institute
CSF	Cereberospinal fluid
DNA	Deoxyribonucleic acid
DOX	Doxycycline
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
EGCG	Epigallocatechin-3-gallate
ESBL	Extended spectrum ß-lactamase
EMJH	Ellinghausen McCullough Johnson Harris
ELISA	Enzyme Linked Immuno Sorbent Assay
FICi	Fractional inhibitory concentration index
FIC A	Fractional inhibitory concentration of drug A
FIC B	Fractional inhibitory concentration of drug B
FRIM	Forest Research Institute Malaysia
HAS	Hospital Sultanah Aminah
ICU	Intensive Care Unit
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IV	Intravenous
LAMP	Loop-mediated isothermal amplification method
MAT	Microscopic agglutination test
MBC	Minimum inhibitory concentration
MDR	Multi-drug resistant
ME	Methanol extract

MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NMR	Nuclear Magnetic Resonance
PBPs	Penicillin-binding proteins
pH	Power of hydrogen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEN	Penicillin
RNA	Ribonucleic acid
tRNA	Transfer ribonucleic acid
UTI	Urinary tract infection
WHO	World Health Organization
5-FU	5-fluorouracil

LIST OF APPENDICES

AKTIVITI *IN VITRO* PERENCATAN BAKTERIA OLEH EKSTRAK *Phyllanthus amarus*, ANTIBIOTIK DAN KOMBINASI TERHADAP *Leptospira interrogans*

ABSTRAK

Pokok *P. amarus* telah digunakan untuk merawat pelbagai penyakit secara tradisional, termasuk penyakit berjangkit. Kajian terdahulu telah menunjukkan aktiviti perencatan bakteria oleh *P. amarus* ke atas pelbagai jenis mikroorganisma termasuk spesis *Leptospira*, namun masih tidak mencukupi. Aktiviti perencatan *Leptospira* oleh ekstrak *P. amarus* (air dan metanol) dan antibiotik dikaji terhadap serovar *Leptospira interrogans*: Australis, Bataviae, Canicola dan Javanica. Kepekatan perencat minimum (MIC) daripada ekstrak air dan metanol ditentukan menggunakan kaedah Ellinghausen McCullough Johnson Harris (EMJH) mikrodilusi *broth* di dalam plat 96-well, dengan menentukan nilai penyerapan oleh pembaca plat ELISA pada 420 nm dan dilihat di bawah mikroskop medan gelap selepas inkubasi selama 7 hari, pada 30 °C dan 40 rpm. Kepekatan bakterisid minimum (MBC) ditentukan dengan melakukan subkultur inokulum *Leptospira* daripada ujian MIC di atas media pepejal EMJH selama dua hingga tiga minggu. Ujian sinergi terhadap kombinasi ekstrak *P. amarus* bersama antibiotik ditentukan dengan menggunakan kaedah ujian papan semak dalam nilai indeks kepekatan pecahan (FICi) bersama tafsirannya. Kombinasi yang mempunyai nilai FICi paling rendah diteruskan dengan analisis mikroskop elektron pengimbas (SEM) berdasarkan nilai MIC masing-masing; ekstrak *P. amarus* dan antibiotik terhadap kultur *Leptospira* secara tunggal dan gabungan selepas 18 jam rawatan pada suhu 30 °C dan 40 rpm. Kedua-dua ekstrak air dan metanol menunjukkan aktiviti perecatan *Leptospira* terhadap semua

serovar-serovar yang dikaji. Kesemua antibiotik yang dikaji; ceftriaxone, doxycycline dan penicillin G, mempamerkan aktiviti perencatan *Leptospira* dengan nilai MICs dan MBCs masing-masing bermula dari 0.05 hingga 0.78 µg/ml, dan 0.05 hingga 0.78 µg/ml, 0.39 hingga 3.13 µg/ml dan 12.5 hingga 25.0 µg/ml, dan <0.01 hingga 0.78 µg/ml dan <0.01 hingga 3.13 µg/ml. Kombinasi ekstrak air bersama antibiotik mempamerkan efek indiferen ke atas serovar-serovar *Leptospira* dengan nilai FICi bermula dari 2.24 hingga 2.40 untuk ceftriaxone, 1.59 hingga 3.09 untuk doxycycline dan 2.42 hingga 3.41 untuk penicillin G. Kombinasi ekstrak metanol bersama antibiotik mempamerkan efek indiferen ke atas serovar-serovar *Leptospira* dengan nilai FICi bermula dari 2.51 hingga 3.22 untuk ceftriaxone, 2.82 hingga 3.58 untuk doxycycline dan 2.95 hingga 3.38 untuk penicillin G. Namun, efek antagonis ekstrak metanol dipamerkan oleh kombinasi bersama doxycycline ke atas *L. interrogans* serovar Bataviae dengan nilai FICi 4.22, dan penicillin G ke atas *L. interrogans* serovar Canicola dengan nilai FICi 4.87. Mikrograf-mikrograf SEM menunjukkan perubahan yang ketara ke atas sel-sel *Leptospira* yang dirawat. Kombinasi ekstrak air bersama doxycycline menyebabkan kerosakan teruk pada sel-sel *Leptospira* berbanding dengan efek ekstrak dan antibiotik sendirian. Kecacatan-kecacatan yang dilihat adalah pengurangan struktur menggelung, penipisan, pemendekan, permukaan yang tidak sekata dan bleb. Kesimpulannya, *P. amarus* menunujukkan sifat perencatan bakteria yang baik terhadap spesis *Leptospira* dengan ektrak air memberikan hasil yang lebih baik daripada ektrak metanol. Ujian sinergi menunjukkan kesan yang sederhana walaupun analisis SEM mempamerkan penemuan yang baik. Justeru itu, penambahbaikan di dalam kajian ini adalah diperlukan. Kajian ke atas pokok sebagai ejen perencatan bakteria terhadap spesis *Leptospira* adalah terhad, maka penyelidikan yang lebih lanjut amat diperlukan.

IN VITRO* ANTIMICROBIAL ACTIVITIES OF *Phyllanthus amarus* EXTRACT, ANTIBIOTICS AND THEIR COMBINATION AGAINST *Leptospira interrogans

ABSTRACT

P. amarus had been traditionally used in treating ailments including infectious diseases. Previous researches proved its antibacterial properties towards groups of microorganisms including *Leptospira* spp., but scanted. In vitro antimicrobial activity of *P. amarus* extracts (aqueous and methanol) and antibiotics were studied against *Leptospira interrogans* serovars Australis, Batavie, Canicola and Javanica. Minimal inhibitory concentrations (MIC) of aqueous and methanol extracts were determined using Ellinghausen-McCullough-Johnson-Harris (EMJH) broth microdilution method in 96-well plates, by determining the absorbance values using ELISA tray reader at 420 nm and viewed under dark field microscope after incubation of 7 days , at 30 °C and 40 rpm. Minimal bactericidal concentrations (MBC) were determined by sub-culturing *Leptospira* inoculum from MIC test onto EMJH solid media for two to three weeks. Synergistic testing of *P. amarus* extracts in combination with antibiotics were determined using checkerboard assays in the form of fractional inhibitory concentration index (FICi) values with their interpretations. The combinations with the lowest FICI values are preceded with scanning electron microscopic analysis (SEM) based on their MIC values; *P. amarus* extract and antibiotic against the *Leptospira* culture, both in singly and in combination after 18 hours of treatment at 30 °C and 40 rpm. Both aqueous and methanol extracts showed antimicrobial activity towards all tested *L. interrogans* serovars. MICs and MBCs ranges of aqueous extract were 100 to 400 µg/ml, and

methanol extract ranging from 400 to 800 µg/ml. All tested antibiotics; ceftriaxone, doxycycline and penicillin G, exhibited antimicrobial activities with MICs and MBCs values ranging from 0.05 to 0.78 µg/ml and 0.05 to 0.78 µg/ml, 0.39 to 3.13 µg/ml and 12.5 to 25.0 µg/ml, and <0.01 to 0.78 µg/ml and <0.01 to 3.13 µg/ml, respectively. Combination of aqueous extract and antibiotic presented with indifferent effects towards *Leptospira* serovars with FICi values ranging from 2.24 to 2.40 for ceftriaxone, 1.59 to 3.09 for doxycycline and 2.42 to 3.41 for penicillin G. Combination of methanol extracts and antibiotics presented with indifferent effects towards *Leptospira* serovars with FICi values ranging from 2.51 to 3.22 for ceftriaxone, 2.82 to 3.58 for doxycycline and 2.95 to 3.38 for penicillin G. However, antagonistic effects of methanol extracts were observed in combination with doxycycline on serovar Bataviae with FICi of 4.22, and penicillin G on serovar Canicola with FICi of 4.87. SEM micrographs revealed notable changes towards the treated *Leptospira* cells. The combination of aqueous extract and doxycycline caused severe damages as compared to extract and antibiotic alone. Deformities observed include less coiling, thinning, shortening, less smooth with irregular surfaces, and blebbing appearances. In conclusion, *P. amarus* showed promising antimicrobial properties on the *Leptospira* spp. with aqueous extract yielded better results than methanol extract. Synergy testing revealed moderate effects even though SEM analysis presented with good findings, indicating a need for improvement in the study. As studies on plants as antimicrobial agents towards *Leptospira* spp. are limited, further research in this area is required.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Leptospira spp.

Leptospirosis is caused by *Leptospira* genus, known as spirochaetes which belongs in family *Leptospiraceae*. *Leptospira* appears to be cocksrew-shaped and has distinctive hooked ends at both sides, making it differs from the other Spirochaetes. Leptospires are helical, thin and motile organisms with length ranging from 10 to more than 20 µm, and 0.1 µm in diameter, but in occasional cultures leptospires may appear much longer (Levett, 2001). Leptospires can be observed under the dark field microscopy (Adler and Faine, 2006) (Figure 1.1A) or under scanning electron micrograph (Figure 1.1B) (Saito et al., 2013). They also can be observed through phase contrast microscopy (Mohammed et al., 2011). However, due to the technical limitations of phase contrast microscopy on its thick suspensions and optical characteristics, therefore its usage in the availability of dark-field microscopy has no practical purpose.

Leptospires are obligate aerobes which need the temperature of 28 to 30ºC for optimum growth and cannot resist drought or hypertonicity. However, leptospires can survive in alkaline environment up to pH 7.8 for 152 days (Mohammed et al., 2011; Thibeaux et al., 2017) and pathogenic leptospires were present in soil after several infecting event. *Leptospira* widely cultured in the Ellinghausen McCullogh Johnson Harris (EMJH) media (Adler and de la Pena Moctezuma, 2010; Fraga et al., 2015), with the addition of 5-Fluorouracil, nalidixic acid, rifampicin or gentamicin for inhibitions of contaminants from the clinical samples. The growth of leptospires is often slow, especially on the primary isolation. Therefore, *Leptospira* cultures need to be preserved for about 13 weeks before discarding (Adler and de la Pena

Moctezuma, 2010). *Leptospira* growth reaches its maximum density in a zone beneath the surface of the semisolid media in relation to the optimum oxygen tension in the area, which increases its turbidity as the incubation precedes, producing a Dinger's ring or disk (Figure 1.2). Solid media have been used in *Leptospira* isolation (Figure 1.3), separating the mixed cultures of leptospires (Levett, 2001).

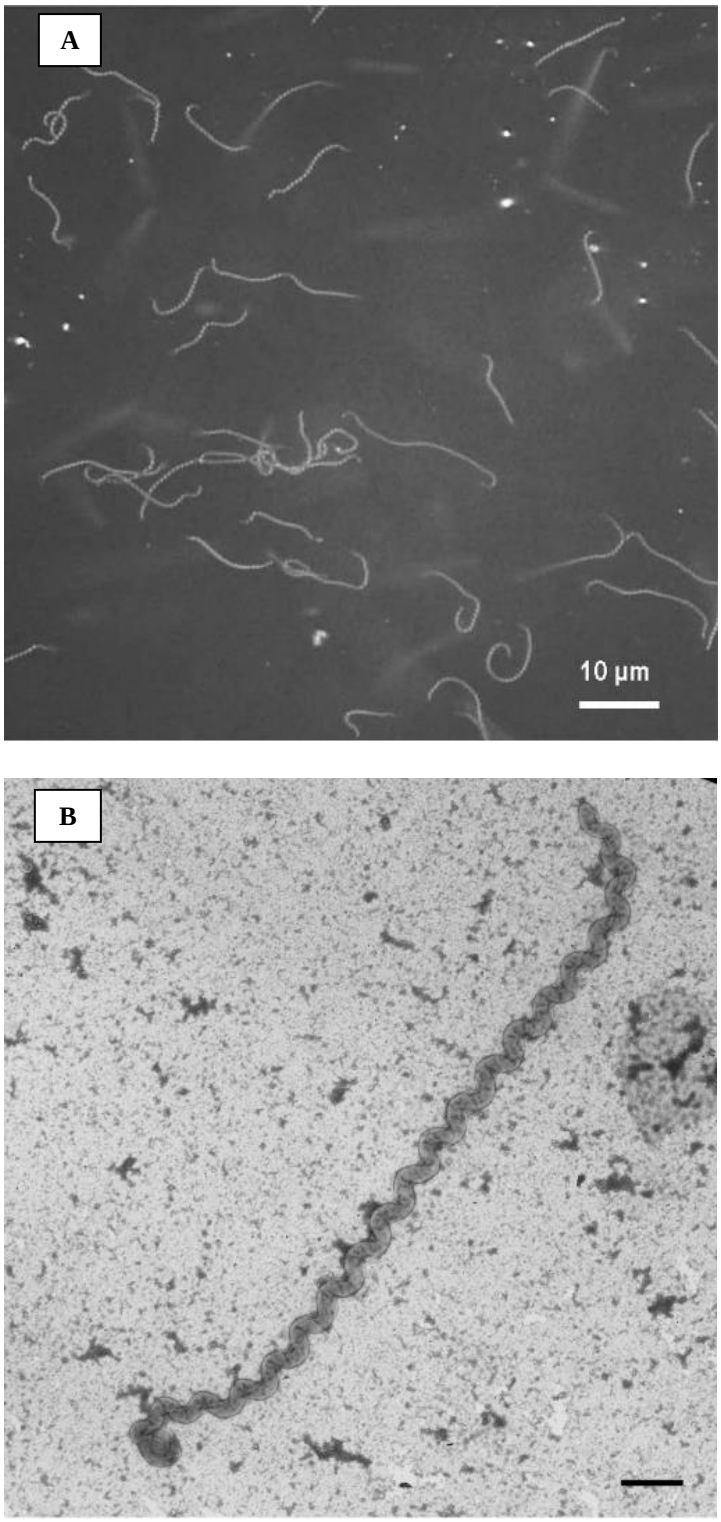

Figure 1.1 The microscopic view of *Leptospira biflexa* strain Patoc 1 under dark field microscope, bars 10 µm **(**A). The electron micrograph of *L. biflexa* strain Patoc 1, bars 1 µm (B). (Reproduced from Saito *et al.*,2013)

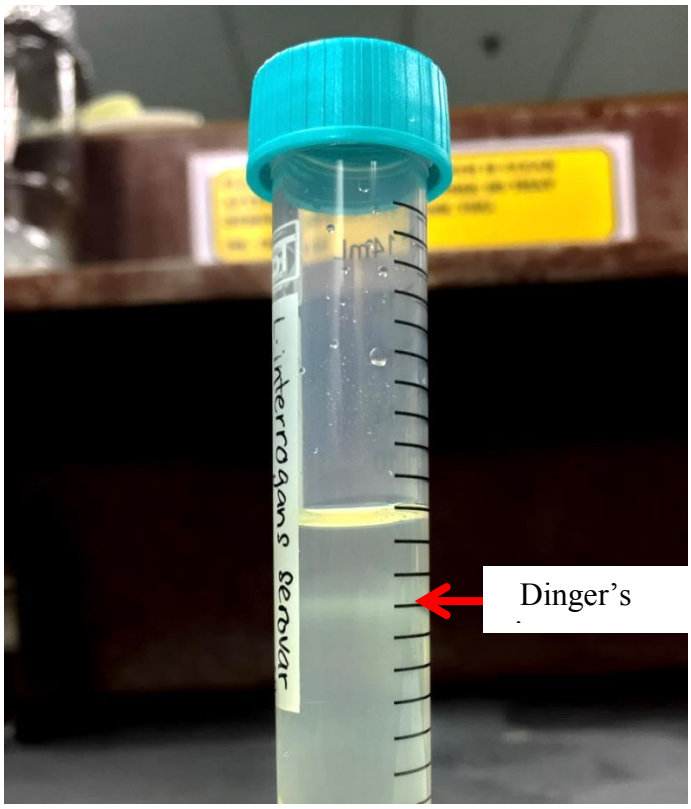

Figure 1.2 The appearance of Dinger's ring in the *Leptospira* culture using EMJH semisolid media, a week after incubation at 30ºC.

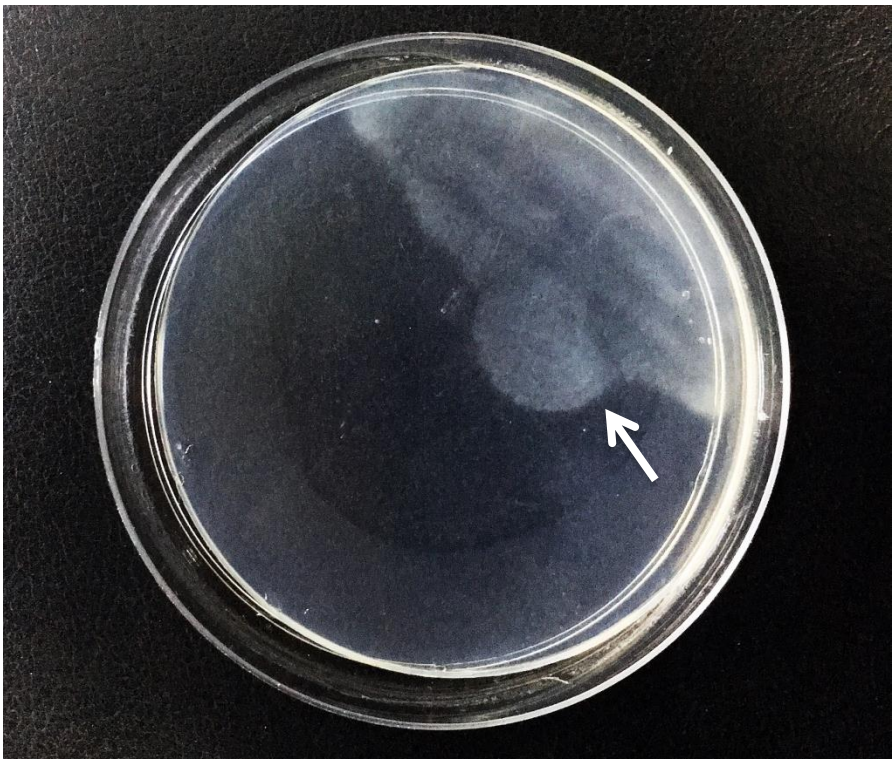

Figure 1.3 Arrow shows the *Leptospira* growth observed in the EMJH solid media.

1.1.1 Motility of *Leptospira*

Leptospires appear as rapid and fastidious bacteria under the dark field microscope. The motility of leptospires is one of the crucial factors on its virulency upon the infected hosts (Goldstein and Charon, 1988; Lambert et al., 2012; Picardeau et al., 2001). *Leptospiraceae* exhibit with short and single periplasmic flagellum, attached subterminally, extending towards the cell's center at both ends without overlapping one another (Bromley and Charon, 1979; Kan and Wolgemuth, 2007).

Leptospires exhibit rapid translational motility which travel approximately 20 µm in 2 to 3 seconds in the ordinary media (Cameron, 2015). Three different types of morphological swimming forms of leptospires have been observed. Nontranslating cells have either spiral-shaped (Figure 1.4B) or hook-shaped at both ends (Figure

1.4A) (Charon et al., 1984; Goldstein and Charon, 1988; Jarosch, 1967; Kan and Wolgemuth, 2007) meanwhile translating cells have spiral-shaped at anterior end and hook-shaped at posterior end (Figure 1.4C) (Charon et al., 1984; Goldstein and Charon, 1988; Inada et al., 1916; Jarosch, 1967; Noguchi, 1918). The cells were able to change from one form to another and can reverse its direction of swimming rapidly, occurs in relation with the change from spiral-hook to hook-spiral end shaped (Charon et al., 1984; Goldstein and Charon, 1988) due to the pattern of rotation by the flagellum. The counterclockwise rotation generated by the flagellum results in the creation of spiral-shaped end and the clockwise rotation creating hook-shaped end (Wolgemuth et al., 2006). Hence, the translating leptospires which rotate their flagella at anterior and posterior ends in the opposite directions will either result in the formation of hook-spiral shaped or spiral-hook shaped end, and they move in the direction of the spiral end as illustrated in the Figure 1.4. Meanwhile, both of the flagella of nontranslating leptospires rotate in the same directions producing either hook-hook shaped or spiral-spiral shaped end (Cameron, 2015; Charon and Goldstein, 2002; Li et al., 2000; Wolgemuth et al., 2006). Therefore, the structure of *Leptospira* plays the vital role in its motility (Bromley and Charon, 1979) either in the environment or the hosts.

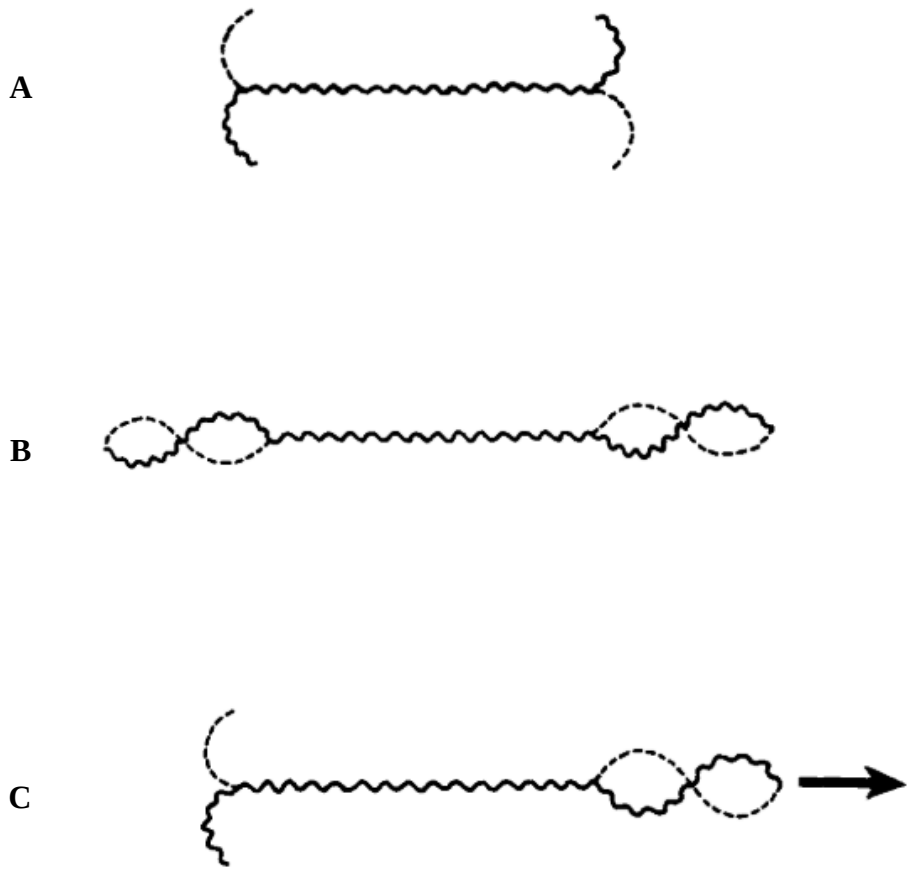

Figure 1.4 Types of *Leptospira* movements in the liquid media. Figure A and B are representing the nontranslational forms of cell, referred as hook-hook (A) or spiral-spiral shaped (B) at both ends. Figure C is translational cell forms, referred as hook-spiral ends or spiral-hook shaped ends. The arrow directs towards the direction of swimming motion by leptospires (Reproduced from Goldstein & Charon, 1988; Charon *et al.,* 1984).

1.1.2 Taxonomy and classification

The family of *Leptospiraceae* consists of three genera, which are *Leptospira, Leptonema* and *Turneriella.* The genus *Leptospira* comprises of three clades with overall 23 species, according to DNA-relatedness and phylogenetic analyses: ten pathogenic, six intermediate and seven nonpathogenic (saprophytic) *Leptospira* as shown in the Table 1.1 (Grassmann et al., 2017; Puche et al., 2017). The groups were furthered divided into serovars and antigenically clustered into serogroups (Levett, 2015; Ricaldi et al., 2012). More than 300 serovars have been identified, including 200 pathogenic serovars (Mohammed et al., 2011).

Pathogenic *Leptospira* species cause severe leptospirosis on humans. However, the pathogenicity of two species of *L. kmetyi* and *L.alstonii* is still not known and in on-going research (Slack et al., 2009; Smythe et al., 2013; Voronina et al., 2014). Intermediate *Leptospira* species are reported causing mild leptospirosis on humans. However, there are lacks of studies regarding the intermediate *Leptospira* species even though it also can cause leptospirosis outbreaks in the community, as there are many challenges encountered in confirming their virulence (Thayaparan et al., 2015). Saprophytic *Leptospira* species are nonpathogenic, not causing any disease on humans (Cerqueira and Picardeau, 2009). They can be found freely in wet or humid environment, either in surface waters, moist soil or even in the tap water. Saprophytic salt-loving (halophilic) leptospires are found in the seawater. However, all leptospires look alike when observed under dark field microscopy, with only minor differences between them. Therefore, morphology does not help in characterizing and differentiating pathogenic and saprophytic leptospires (Terpstra, 2003).

Table 1.1 Classification of *Leptospira* species (Adopted from Grassmann *et al*.,. 2017; Puche *et al*., 2017)

Group	Species	Reference
Pathogenic	*L. alexanderi*	(Brenner et al., 1999)
	L. alstoni	(Smythe et al., 2013)
	L. noguchii	(Yasuda et al., 1987)
	L. borgpetersenii	(Yasuda et al., 1987)
	L. santorasai	(Yasuda et al., 1987)
	L. weilii	(Yasuda et al., 1987)
	L. interrogans	(Faine and Stallman, 1982)
	L. kmetyi	(Slack et al., 2009)
	L. kirschneri	(Ramadass et al., 1992)
	L. mayottensis	(Bourhy et al., 2014)
Intermediate	*L. broomi*	(Levett et al., 2006)
	L. inadai	(Yasuda et al., 1987)
	L. fainei	(Perolat et al., 1998)
	L. licerasiae	(Matthias et al., 2008)
	L. venezuelensis	(Puche et al., 2017)
	L. wolffii	(Slack et al., 2009)
Saprophytic	*L. biflexa*	(Faine and Stallman, 1982)
	L. idonii	(Saito et al., 2013)
	L. meyeri	(Yasuda et al., 1987)
	L. vanthielii	(Yasuda et al., 1987)
	L. wolbachii	(Yasuda et al., 1987)
	L. yanagawae	(Smythe et al., 2013)
	L. terpstrae	(Smythe et al., 2013)

1.1.3 Host, carriers and transmission of *Leptospira*

Leptospirosis is one of the most common zoonotic diseases occurring worldwide, infecting both human and animals. Leptospires can be associated with the animal host or found as free-living in the environment (Adler and Faine, 2006). Leptospires were maintained in the nature involving a wide range of animal hosts (Vijayachari et al., 2008), according to its serovars (Bharti et al., 2003) as shown in the Table 1.2. Pathogenic leptospires *L. interrogans* are able to survive outside the hosts in the environment, such as surface waters for a longer period of time, unlike *L. borgpetersenii* (Bulach et al., 2006; Trueba et al., 2004; Xue et al., 2009).

Leptospires are recorded able to survive in the fresh water up to 152 days (Wynwood et al., 2014).

Table 1.2 Reservoirs of different *Leptospira* serovars in the wide range of infected animals (Adopted from Mohammed *et al*., 2011)

***Leptospira* serovars**	**Usual host**
ballum	Mice
icterohaemorrhagiae (Terpstra, 2003) and *canicola*	Dogs
grippotyphosa and *hardjo*	Dairy cattles
icterohaemorrhagiae and *ballum*	Rats
pomona and *hardjo*	Sheep
pomona and *tarassovi*	Pigs

Maintenance and accidental hosts can be found among human and animals. Natural maintenance host of leptospires involves in the generation of commensal relationship between the species with the pathogenic leptospires in their kidneys. However, the leptospires either produce little or no detectable harm towards the hosts. During the pregnancy, leptospires are transferred to the host's offspring either via *in utero* or during neonatal period which therefore maintaining the infection among (Terpstra, 2003) the ecosystems. Most maintenance hosts commonly show the evidence of leptospirosis infection during the pregnancy, exhibiting the appearance of reproductive failure which includes infertility, still-births, abortions or the birth of weak offspring (Zuerner, 2015). Nevertheless, if the maintenance host of a particular *Leptospira* is infected with another serovar, symptoms and signs of leptospirosis may emerge. The accidental or incidental host is defined as a non-natural maintenance host which was infected with a serovar and it often develops the leptospirosis. However, the dynamic interaction between the pathogenic leptospires and animal host species along with the possibility of leptospires in adapting to new animal host species makes the distinction between the natural maintenance host and accidental hosts not be clearly defined (Terpstra, 2003).

The most common *Leptospira* hosts are mammalian species (Adler and de la Pena Moctezuma, 2010), including wild and domestic animals. Rats and rodents are the first recognized carriers of leptospirosis which act as the main carrier of the bacterial-causing leptospirosis in the ecosystems and becomes the primary source of infection to humans (World Health Organization, 2009). The livestock farming plays a vital role in contributing to occupational risk factor in human leptospirosis, besides farmed deer (Mohammed et al., 2011). Common reservoirs of leptospires include cattle, buffaloes, sheep, dogs, pigs, goat and horses (World Health Organization, 2009). However, the main role of the wildlife animals becoming the source of leptospirosis infections towards livestock and human, and its reservoir is still undergoing research and remains unclear (Adler and de la Pena Moctezuma, 2010; Petrakovsky et al., 2014; Vieira et al., 2018).

The pathogenic *Leptospira* spp. are shed from its natural habitats, which are proximal renal tubules (Adler and de la Pena Moctezuma, 2010) and genital tracts of the carrier animals (World Health Organization, 2009). Leptospires later passed to the environment through the excretion of urine, becoming the main reservoir of the leptospires transmissions. The animals that excrete leptospires in their urine are called shedders (Terpstra, 2003). Leptospires persist well in the water, mud and soil, may due to the contributor factors: cell aggressions with other bacteria (Trueba et al., 2004) and biofilm formations (Ristow et al., 2008). Heavy rainfall and flooding are associated with the outbreaks of leptospirosis. The condition involves the passing of leptospires from urine-contaminated soil, where it is washed off and collected in the rivers and puddles. Pigs and cattles can act as important sources of leptospirosis infection as they can excrete enormous amount of leptospires in their carrier state,

during chronic leptospiral colonization of their renal tubules (World Health Organization, 2009).

Human acts as accidental host in transmission of leptospires in the ecosystems however, the shed leptospires are not sufficient to make human as a reservoir of the leptospires transmission (Ko et al., 2009)**.** Human leptospirosis is caused by indirect transmission of pathogenic *Leptospira*, through contacts of food, soil and water which were contaminated with urine of the infected animals (maintenance hosts) (Levett, 2004), or direct exposure through a direct contact with the urine or any body fluids (blood, organs and tissues) except saliva, of the infected animals itself (Seguro and Andrade, 2013). One can get infected through inhalation of water or aerosols containing leptospires, infecting the hosts via mucous membrane of the respiratory tract. In rare situations, the hosts may get infected through animal bites (Levett, 2004). Human to human infections are rare, but happen occasionally through sexual intercourse, lactation and transplacentally from a mother to the fetus. Urine and blood of an infected patient suffering from leptospirosis are considered as infectious. Leptospires can be cultured from the blood, therefore it should be considered as infectious before the symptoms to onset, and during the first 7 to 10 days of the illness (Terpstra, 2003). The transmissions of leptospires in the ecosystems are as visualized in the Figure 1.5.

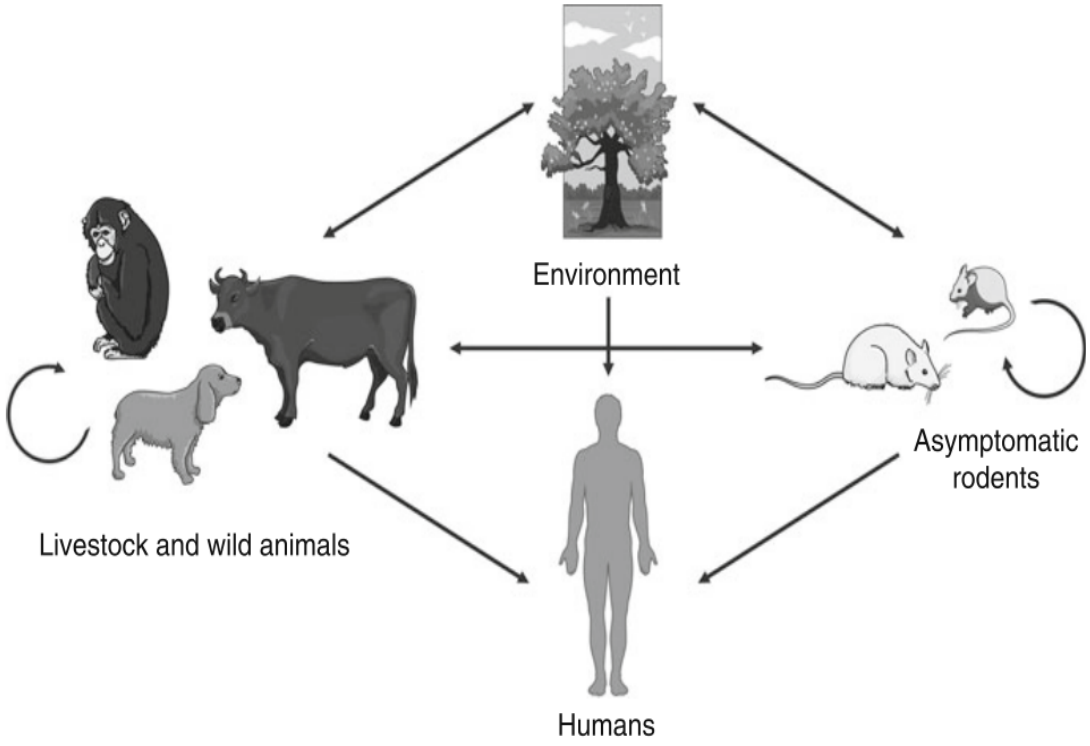

Figure 1.5 The transmission of *Leptospira* in the ecosystems, involving wide ranges of animals and human as hosts (Reproduced from Grassmann *et al.*, 2017).

1.1.4 Risk of exposures

The risk factors of leptospirosis are classified into main groups of animal, environmental and human factors (Porta et al., 2008; Sakundarno et al., 2014) strike the most vulnerable populations with high risk factor of exposure involving the recreational activities (swimming, kayaking, wading and rafting in contaminated rivers and lakes), occupational exposures (veterinarians, mine workers, animal caretakers, slaughterhouse workers, fish workers, laboratory workers, farmers, sewer workers, abattoir workers, ranchers and loggers), household exposures (infestations of infected rodents, rainwater catchment systems and domesticated livestock) and skin lesions (Southall et al., 2002). Ones also can be exposed by living under poor hygienic environment (Ricaldi et al., 2012; World Health Organization, 2010) where

infestations of infected rodents were active in the area (poor housing and slum dwellings areas). An increase event of rainfall and flooding along with inadequate floodwater drainage can act as one of the risk factors in leptospirosis exposure (World Health Organization, 2010).

1.2 Leptospirosis

Leptospirosis or known as rodent-borne disease (Meerburg et al., 2009) is an emerging infectious disease occurring worldwide (Lim et al., 2011), found in most of the countries except for polar climates areas such as Antarctica. Leptospirosis cases are endemic in tropical and subtropical regions, and become epidemic in monsoon seasons and flooding (Haake and Levett, 2015). The situations may be due to the spreading of leptospires in the urine of animals to the environment, which were eventually washed away and collected into the water catchment areas. The incidence is significantly high in countries with warm climates compared to the temperate regions (Levett, 2001) causing the long survival rate of leptospires in the warm and humid conditions.

Leptospirosis is seasonal where the incidence falls in the temperate regions and at its peak in summer regions (Levett, 2001), proving the temperature as one of the vital role on the survival rate of leptospires in the environment. The case is gradually increasing over the years (Southall et al., 2002), and the cycle of transmission remains maintained as people are continuously exposed with an inadequate awareness, surveillance and control measures (Abela-Ridder et al., 2010). According to World Health Organization (2009), leptospirosis cases are underdiagnosed due to difficulty of diagnosis in confirming the infection (probability to be confused with other disease, the infection itself may be mild and not being investigated in the laboratory, and the non-availability of the laboratory test or if available) and the tests have low sensitivity in detecting the infection during early phase of the disease as they only detect antibodies of the patient reacting towards the infection (World Health Organization, 2009).

1.2.1 Leptospirosis cases in Malaysia

The disease burden of leptospirosis was difficult to be assessed due to lack of reliable epidemiological data. However, known high-risk areas have been recognized including China, India, Malaysia, Pacific Islands, Seychelles, Sri Lanka, Thailand, the Caribbean, Brazil, and Vietnam (Lau et al., 2010). According to World Health Organization (2009), it was reported that leptospirosis cases are commonly occurred in the countries of South-East Asia regions and their magnitude is differs between the countries, depending on attitude and awareness of the public health care decision makers (World Health Organization, 2009). It is estimated that 1.03 million annual cases with 58, 999 deaths occurring worldwide due to leptospirosis (Costa et al., 2015). The incidence of leptospirosis able to reach up to 50 per 100, 000 during outbreaks and in the high-exposure risk groups (World Health Organization, 2009). The reasons for an increase of leptospirosis cases around the globe are due to increasing rats' population and flooding seasons, causing a spike in leptospirosis cases among the community (Seguro and Andrade, 2013).

Leptospirosis is one of the common infectious diseases to be found in Malaysia. The geographical conditions of Malaysia of humid and warm environment assist more towards the growth of leptospires and therefore maintaining the spreading of leptospires and enabling the *Leptospira* to survive for a long period of time in the environment. The tropical rainforest of 250 feet high with forms of the six layers canopy by shorter trees beneath them, resulting in less penetration of sunlight to reach the ground. Therefore, constant moisture can be maintained besides due to the regular movement of the clouds through the forest. The jungle floor is the habitat of mammals which susceptible to the leptospirosis, including jungle rats, and uses the jungle streams as water and food sources, and as routes for travel. Baker tested

the infectious level between the jungle stream and swamps, and found the streams to be far more infectious for occasional periods (during the rainfall and flooding) (Baker, 1965). The monsoon season contributing to the incidence of leptospirosis in Malaysia as there are risks of exposure and contamination of *Leptospira* via rat's urine to the water and soil (Lim et al., 2011; Lim, 2011).

Leptospirosis cases are common in Malaysia since almost hundred years ago. Human case of leptospirosis in Malaysia is recorded to be first discovered in 1925 by Fletcher by chance during the search of isolating the causal organism of tropical typhus by inoculating the blood from the patients with fever of unknown origin into the guinea pigs. The blood of one of the patient causes guinea pigs to develop jaundice, nose hemorrhage, shows rises in temperature on the ninth day and collapses on the twelve day. The guinea pig then died on the thirteenth day after inoculation. Postmortem examination concludes to be leptospirosis. Leptospires were found in the blood, kidney and liver of the guinea pigs (Bahaman and Ibrahim, 1988; Fletcher, 1928). Fletcher isolated leptospires in the blood, kidney and liver of 21 infected patients and identified 3 serovars: *Leptospira interrogans* serovar Icterohaemorrhagie, *L. interrogans* serovar Pyrogenes and *L. interrogans* serovar Hebdomadis in Malaysia. Since then, subsequent leptospirosis outbreaks have been reported, showing an increasing numbers of leptospirosis cases over the years. The chronology of leptospirosis outbreaks in Malaysia since 1984 is recorded in the Table 1.3.

Table 1.3 The chronology of leptospirosis outbreaks reported in Malaysia since 1984 (Adopted from Benacer *et al*., 2016).

Outbreaks	Year	Description	Reference
Mulu caves, Sarawak	1984	After exploration of the Mulu caves in Gunung Mulu National Park, Sarawak, 16 British cave explorers return to Britain ill; 5 patients had fever of unknown origin and hepatomegaly with no renal failure. Leptospirosis was suspected and later confirmed by serology.	(Benacer et al., 2016; Waitkins, 1986)
Sarawak	1985	A group of British tourists visited the Sarawak chamber and 2 contracted leptospirosis.	(Benacer et al., 2016)
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 hemorrhage. Investigations revealed creek water contaminated with urine tainted with leptospirosis of animal origin (cattle, pigs, rodents, and wild animals), with prior flooding facilitating the spread of the organism.	(Koay et al., 2004)
The Eco-Challenge, Segama River, Sabah	2000	Athletes kayaking and swimming in the 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 sprared from infection.	(Sejvar et al., 2003)
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.	(Hisham et al., 2009)

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 drinking water contaminated with animal urine, potentially rats, was suspected to be the cause.	(Benacer et al., 2016; International Detention Coalition, 2009)
Lubuk Yu, 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 chores. On outbreak confirmation, the recreational park was temporarily closed to the public.	(Sapian et al., 2012)
Kedah	2010	Three recreation areas (Puncak Janing, Bukit Wang and Lata Bayu) in Kedah were closed to public due to leptospirosis cases, involving one death and four were positive with leptospirosis.	(The Star Online, 2010)
The Bukit Jugra Royal Malaysia Air Force base, Kuala Langat, Selangor	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.	(Benacer et al., 2016; Malay Mail Online, 2011)
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 leptospiral IgM confirmed that 6 of the 8 men tested positive. Water samples from the swamp were screened and confirmed by PCR as being	(Benacer et al., 2016)

			tainted *Leptospira.*	
Kelantan		2014	A total of 168 cases of leptospirosis involving 6 deaths were recorded since January up to March 2014.	(Bernama, 2014a)
Kedah	Kuala Nerang	2014	Fifteen years old student admitted to intensive care unit (ICU) at Sultanah Bahiyah Hospital, suspected to be leptospirosis after a swim at Sungai Perik, Kuala Nerang. The youth experienced chills, fever with yellow eyes and later confirmed to have been contracted with leptospirosis.	(My Metro, 2014)
	Yan		An eight years old girl had high fever and epilepsy before death while her sister had confirmed positive with leptospirosis which was treated in Sultanah Bahiyah Hospital. The mother and one other daughter were down with fever. The family had gone for a picnic at a waterfall in Yan about a week before.	(Bernama, 2014b, 2014c)
Pahang		2015	Twenty three suspected and seven confirmed leptospirosis cases recorded in Pahang during the flood.	(New Straits Times, 2015a)
Lata Bayu Recreational Park, Kedah		2015	University student had fever and experienced severe vomiting, 2 weeks after a swim at Lata Bayu recreational park. The patient's lung was infected by water-borne disease and on ventilator support. Preliminary tests suggested a contraction of leptospirosis.	(New Straits Times, 2015c)
Kelantan		2015	According to Health Ministry, the number of leptospirosis cases has tripled following massive floods in Kelantan, with 94 cases were reported.	(New Straits Times, 2015b)
Jerai Toi Recreation Park, Negeri Sembilan		2016	A college student died due to leptospirosis after a dip at Jeram Toi Recreation Park while other two infected students received treatments at the hospital which later	(Bernama, 2016a)

			reported to be stable. The victims had visited Jeram Toi in separate groups.	
Sungai Ketil, Kedah		2016	A teacher died due to leptospirosis after a trip to Sungai Ketil in Baling for sports about 2 weeks before came down with fever and vomiting. The condition worsens as it involves his kidneys, liver, heart, lungs and bleeding in the brain. The blood test confirmed positive for leptospirosis.	(Nation, 2016; The Star Online, 2016)
Kelantan	Pasir Puteh	2016	A student suffered shortness of breath, fever, chills and vomited blood before his death after a swim at Jeram Mengaji, Selising, Pasir Puteh. The laboratory test confirmed positive with leptospirosis.	(Bernama, 2016b)
	Tanah Merah	2017	Forty family members from Tanah Merah have been quarantined after 59-year old man suffered from a cough and difficulty in breathing before his death from unidentified virus. 11 family members developed symptoms of respiratory infections, with 3 people shows positive results of leptospirosis among the 6 family members who have been admitted to the Tanah Merah hospital on suspicion of having mysterious viral disease, while other five receiving outpatient treatment. The family reported to be recently returned from family gathering at Kajang, Selangor. The incubation period indicates the family might have contracted the leptospirosis in their hometown Tanah Merah due to recent floods in the area, before heading to Kajang.	(MIMS Today, 2017)
Mount Pulai waterfall, Johor		2017	An 18 years old died of leptospirosis and organ failure	(The Star, 2017)

		after admitted to ICU at Sultanah Aminah Hospital (HAS) for 3 days. The youth had gone swimming at Mount Pulai waterfall before complaining of stomachache, general weakness, cold and fever for 3 days. The area was closed for 3 months and the water samples tested positive for leptospirosis.	
Mount Belumut Recreational Forest Area, Johor	2018	Gunung Berlumut recreational forest in Kluang had been temporarily closed after two children (aged 5 and 7) were infected with leptospirosis after a 3 day family trip at the area from February 16 to 18. The children experienced diarrhea and vomiting on February 18 and warded at Kulai Hospital on February 23, treated as acute gastroenteritis and discharged the next day. However, the symptoms persisted leading to second blood test on March 13, showing the presence of leptospires.	(The Star, 2018; World of Buzz, 2018)
Lubuk Sungai Kerawat, Jerantut, Pahang	2020	On 15th June, twenty-nine years old man died of suspected leptospirosis while receiving treatment at ICU, while three others aged between 30 and 32 were in stable condition and treated at Sultanah Nur Zahirah Hospital (HSNZ), Terengganu. All of them showed similar symptoms of leptospirosis disease with fever, arthralgia, muscle pain, diarrhoea and vomiting. Investigations revealed all of them to have engaged in recreational activities in Lubuk Sungai Kerawat, Jerantut in Pahang from 1 to June 4 along with nine other individuals. Rapid test showed only one of the	(Berita Harian, 2020; MyMetro, 2020)

	four presented with positive result.

The cases of leptospirosis in Malaysia were increased progressively from 2004 to 2015, portraying 263 cases with 20 deaths in the year of 2004, and 8,291 cases with 78 deaths in the year of 2015, the highest case recorded over the years (Figure 1.6) (Kementerian Kesihatan Malaysia, 2018; Ministry of Health Malaysia, 2015, 2016, 2019; Wahab, 2015). Floods in many parts of Malaysia, especially in year 2014 contributes to the increase cases of leptospirosis among the residents (Wahab, 2015). This was supported in the research by Radi *et al.*, (2018) on spatial and temporal distribution of leptospirosis in relation to the environmental factors after major flood event in Kelantan, Malaysia, which reports a correlation between the two factors by portraying an increase incidence of leptospirosis after 2-3 weeks of heavy rainfall and flooding, confirming the incubation period of leptospirosis upon exposure (Radi et al., 2018). On June 2020, Director General of Health, Datuk Dr Noor Hisham Abdullah stated in the media that 1,484 cases of leptospirosis were recorded. The decline in the number of zoonotic-related cases was said due to the high standards of hygiene and the implementation of the Movement Control Order since March 2020 (The Malaysian Insight, 2020), after the outbreaks of Coronavirus disease in Malaysia starting January 2020 (Elengoe, 2020).

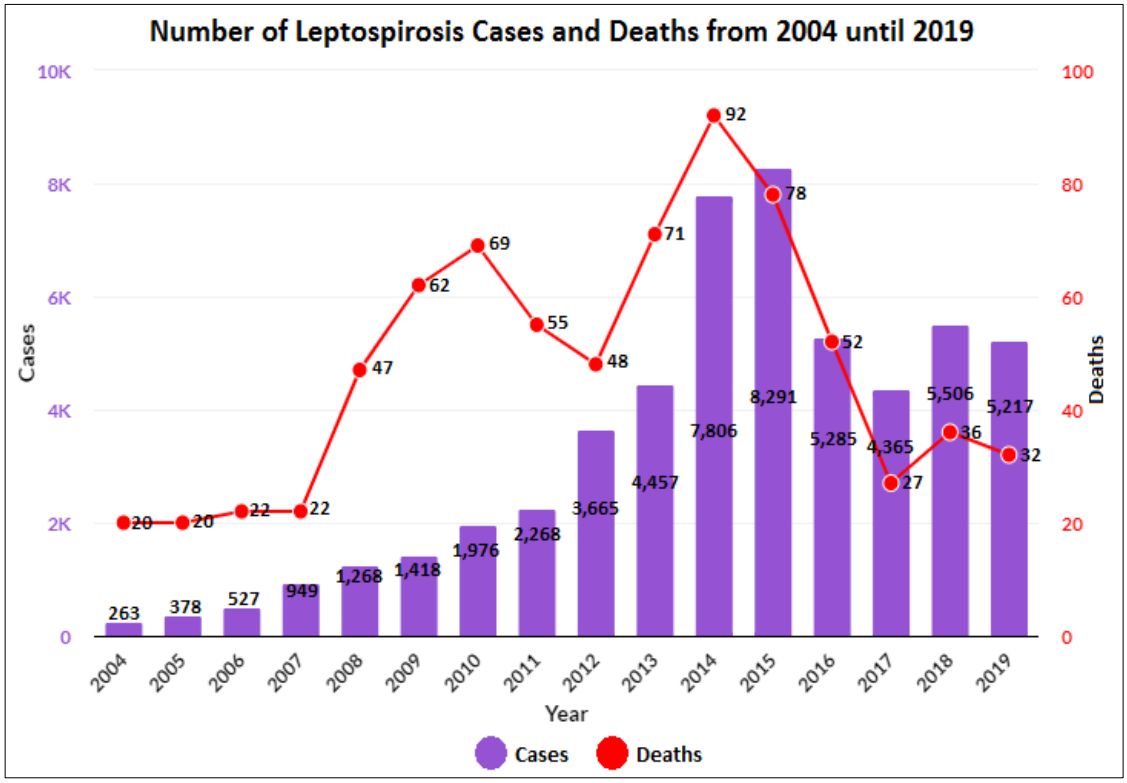

Figure 1.6 Number of leptospirosis cases and deaths in Malaysia from 2004 to 2019