ISOLATION AND IDENTIFICATION OF PATHOGENIC *Leptospira* spp FROM ENVIRONMENTAL SAMPLES DURING AND AFTER 2017 FLOOD IN KELANTAN

By

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LIST OF SYMBOL AND ABBREVIATION

Bp	**Base pair**
DNA	**Deoxyribonucleic acid**
g	**Gravity**
g	**Gram**
L	**Liter**
M	**Molar**
Min	**Minute**
mL	**Mililiter**
mM	**Milimolar**
µL	**Microliter**
mm	**Millimeter**
nm	**Nanometer**
ng	**Nanogram**
PCR	**Polymerase chain reaction**
Sec	**Second**
U	**Unit**

V	**Volt**
%	**Percent**
°C	**Degree celcius**
DFM	**Dark field microscope**
EMJH	**Ellinghausen-McCullough-Johnson-Harris**
spp	**Species**
XG	**Relative centrifugal force**

Abstract

Leptospirosis is a zoonosis found worldwide, the main reservoir is the rat. Human infection generally results from exposure to contaminated river or lake or animals. The outbreak of leptospirosis around the world has been associated with heavy rainfall and flooding. These extreme weather events increase the risk of leptospirosis to those who live within *Leptospira*-contaminated environments and also the magnitude of outbreaks. The study aimed to isolate and identify the presence of pathogenic *Leptospira* spp in an environmental sample during the flood (52 samples) and after the flood (52 samples) from three districts which were Tumpat, Pasir Mas and Kota Bharu. The sample was cultured and enriched in liquid EMJH, a selective media for *Leptospira* followed by dark-field microscope (DFM) observation to determine the positive morphology of *Leptospira*. PCR targeting *LipL32* were performed, which is present only in pathogenic *Leptospira* spp. Findings revealed 35 positive samples (67 %) during the flood for *Leptospira* under DFM: 19 from Tumpat, 10 from Pasir Mas and 6 from Kota Bharu. Four samples: 3 from Tumpat and 1 from Pasir Mas were detected as pathogenic *Leptospira*. Meanwhile, an amount of 32 samples (62 %) after the flood was found as positive for *Leptospira* under DFM: 15 from Tumpat, 6 from Pasir Mas and 11 from Kota Bharu. Eighth samples: 4 from Tumpat, 3 from Pasir Mas and 1 from Kota Bharu were detected as pathogenic *Leptospira*. Therefore, this study successfully isolated and identified pathogenic *Leptospira* spp in environmental samples affected by flood.

Abstrak

Leptospirosis ialah zoonosis di seluruh dunia, sumber utama adalah tikus. Manusia terjangkit berpunca daripada penglibatan dengan sungai yang tercemar atau tasik atau haiwan. Wabak leptospirosis di seluruh dunia telah dikaitkan dengan hujan lebat dan banjir. Peristiwa-peristiwa cuaca yang melampau meningkatkan risiko leptospirosis kepada mereka yang hidup dalam persekitaran yang tercemar dan juga tahap wabak. Kajian ini bertujuan untuk mengasingkan dan mengenal pasti patogen *Leptospira* spp dalam sampel alam sekitar semasa dan selepas banjir di tiga daerah iaitu Tumpat, Pasir Mas dan Kota Bharu. Sampel dikultur dalam EMJH cecair yang merupakan media terpilih untuk *Leptospira* dan dilihat melalui pemerhatian menggunakan mikroskop bidang gelap (DFM) untuk menentukan morfologi positif *Leptospira*. Hanya sampel positif di bawah DFM telah diteruskan untuk mengesan *Leptospira*. PCR mensasarkan *LipL32* telah dijalankan, Ia hanya terdapat pada patogenik *Leptospira* spp. Hasil kajian menunjukkan 35 sampel semasa banjir adalah positif untuk *Leptospira* di bawah DFM: 19 dari Tumpat, 10 dari Pasir Mas dan 6 dari Kota Bharu. Empat sampel: 3 dari Tumpat dan 1 dari Pasir Mas telah dikesan sebagai patogen *Leptospira*. Sejumlah 32 sampel selepas banjir ditemui sebagai positif bagi *Leptospira* di bawah DFM: 14 dari Tumpat, 6 dari Pasir Mas dan 12 dari Kota Bharu. Lapan sampel: 4 dari Tumpat, 3 dari Pasir Mas dan 1 dari Kota Bharu telah dikesan sebagai patogen *Leptospira* spp. Oleh itu kajian ini telah berjaya mengasingkan dan mengenal pasti patogen *Leptospira* spp pada sampel alam sekitar yang terjejas oleh banjir.

Chapter 1: Introduction and Literature review

1.1 Introduction of Leptospirosis

Leptospirosis is a zoonotic infection caused by the spirochete *Leptospira* spp, and human is incidental hosts. Infection occurs when humans come in contact with urine (directly or indirectly) of infected carrier hosts. Pathogenic *Leptospira* spp are responsible for a worldwide zoonosis as shown in Figure 1.1, leptospirosis, in which humans are occasional hosts in a cycle involving wild and domestic animals. The animal reservoir includes mostly rodents; they excrete *Leptospira* in their urine and thus contaminate the hydric environment, transmitting the disease to other animals or to humans (Levett PN, 2001). *Leptospira* belong to the phylum of spirochetes that presents unique morphological characteristics in the bacterial world. *Leptospira* are helix shaped and have an internal locomotor organ, the endo flagellum, which gives them a great mobility, even in the most viscous media includes 20 *Leptospira* spp and more than 300 serovars, grouped in 20 serogroups, have been portrayed as shown in Figure 1.2. The lipopolysaccharide (LPS) structure is the primary serovar determinant. *Leptospira* are classified into three groups according to their phylogenic and pathogenicity (Cerqueira & Picardeau, 2009). There are six saprophytic species (environmental non-pathogenic strains), nine pathogenic species (strains isolated from humans or animals) and five intermediate species, which are distinct from pathogens and saprophytes, according to their 16S rRNA sequence (Picardeau, 2013).

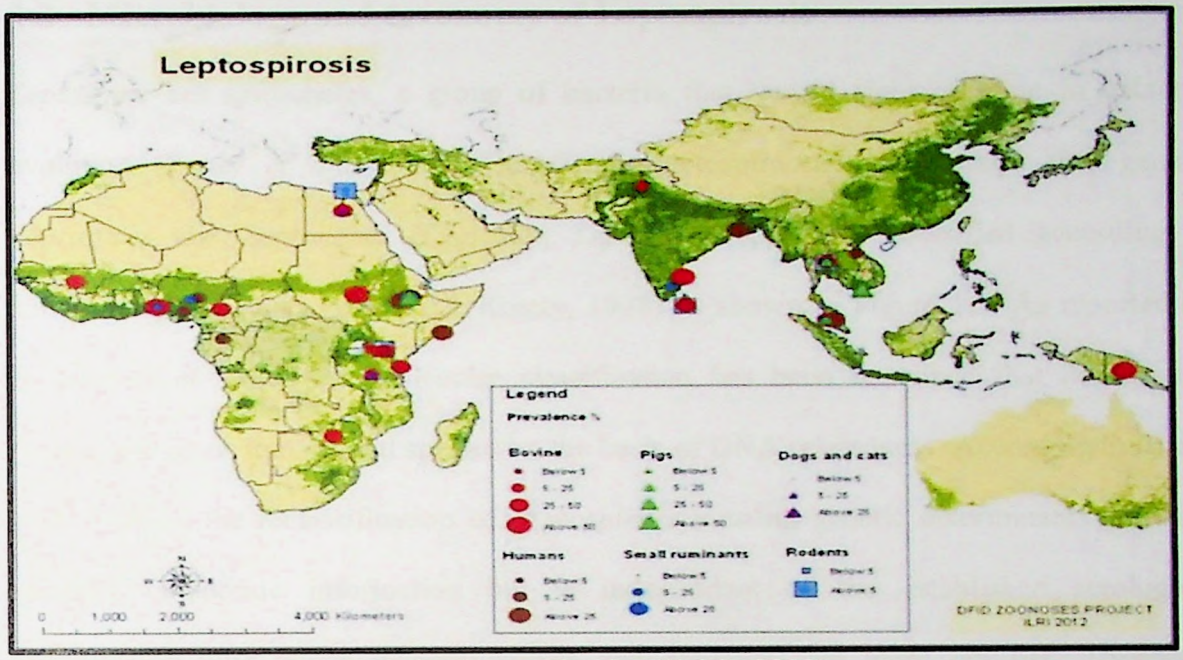

Figure 1.1: Prevalence leptospirosis worldwide (Walsh, 2013)

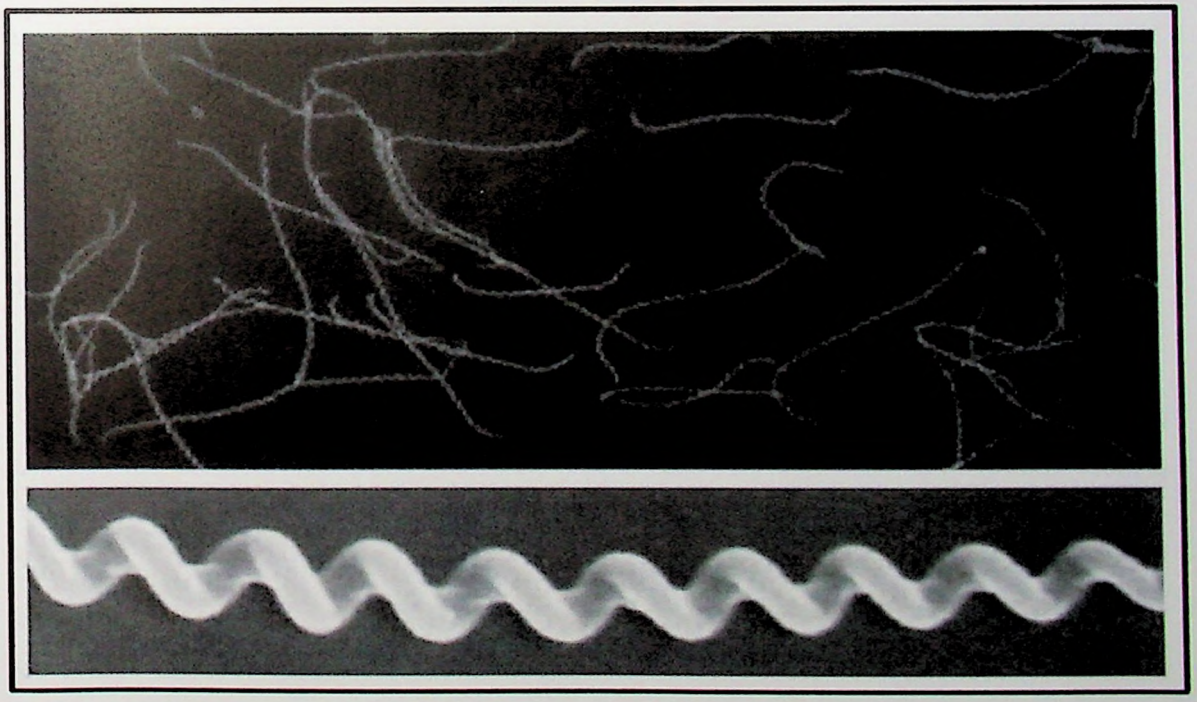

Figure 1.2: Electron micrographs of *Leptospira* (Picardeau, 2013)

1.2 Microbiology and taxonomy of Leptospirosis

Leptospira are spirochetes, a group of bacteria that veered right on time in bacterial evolution (Paster *et al.*, 1991). The family leptospiraceae incorporates two genera, *Leptospira* and *Leptonema*. Typically, *Leptospira* spp were classified according to antigenic determinants (Dikken & Kmety, 1978) as shown in Figure 1.3. As reported by Brenner *et al.* (1999), a molecular classification has been described that divides the *Leptospira* genus into several species on the basis of DNA relatedness. According to Bharti *et al.* (2003), the reclassification of *Leptospira* spp using genetic determinants provides valuable taxonomic information but is independent of the established serological classification with which epidemiologists and clinicians are more familiar. Therefore, serovar and serogroup designations will continue to be used for the foreseeable future.

Species	Serogroup	Serovar	Strain
Pathogenic			
L. interrogans	Icterohaemorrhagiae	Copenhageni	Fiocruz LI-130
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V
L. noguchii	Panama	Panama	CZ 214 K
L. borgpetersenii	Sejroe	Sejroe	M84
L. weilii	Celledoni	Celledoni	Celledoni
L. santarosai	Tarassovi	Atlantae	LT81
L. alexanderi	Manhao	Manhao 3	L 60
L. alstonii	ND	Sichuan	79.601
L. kmetyi	ND	ND	Bejo-Iso 9
Intermediate			
L. wolffii	ND	ND	Korat-H2
L. licerasiae	ND	Varillal	VAR010
L. inadai	Tarassovi	Kaup	LT64-68
L. fainei	Hurstbridge	Hurstbridge	BUT6
L. broomii	Undesignated	ND	5399
Saprophytes			
L. wolbachii	Codice	Codice	CDC
L. meyeri	Semaranga	Semaranga	Veldrat
L. biflexa	Semaranga	Patoc	Patoc 1
L. vanthielii	Holland	Holland	WaZ Holland
L. terpstrae	ND	ND	LT 11-33
L. yanagawae	Semaranga	Saopaulo	Sao Paulo

ND: not determined.

Figure 1.3: List of referent strains for the 20 species described in the genus *Leptospira* spp (Picardeau, 2013)

1.3 Epidemiology of Leptospirosis

Various animals, primarily mammals, are sources of human infection. Rodents are the most important and widely distributed reservoirs of *Leptospira*. Some serovars are associated with a particular species of natural maintenance host. Leptospirosis in humans is always obtained from an animal source (Adler & Moctezuma, 2010). The usual mode of entry is scraped areas or cuts in the skin or via the conjunctiva through direct or indirect contact with urine or tissues of infected animals (Levett, 2001). Other modes of infection, such as inhalation of water or aerosols, animal bites, or interhuman transmission, have been rarely demonstrated. Leptospirosis is an occupational disease for veterinarians, farmers, abattoir workers, butchers, hunters, rodent control workers, and other occupations requiring contact with animals. Indirect contact with contaminated wet soil or water is responsible for the great majority of cases in the tropics, either through occupational exposure as in rice or taro farming, flooding after heavy rains, or exposure to damp soil and water during avocational activities. Infection due to recreational exposures is increasing, often in association with adventure tourism in tropical endemic areas. Three epidemiological patterns have been defined: i) in temperate climates where few serovars are involved and human infection occurs by direct contact with infected animals; ii) in tropical wet areas where there are many more serovars infecting humans and animals and larger numbers of reservoir species; and iii) in the urban environment as a rodent-borne infection (Faine, 1994).

1.4 Pathogenesis of Leptospirosis

Leptospira enter the body through small cuts or abrasions, via mucous membrane such as the conjunctiva or through the wet skin (Adler & Moctezuma, 2010). *Leptospira* entering the body relocate to the lymphatic and circulatory system within minutes. However, the growth rate of *Leptospira* is slow, and therefore after an incubation period of 2–20 days, symptoms emerge. The organisms have a particular predilection for kidneys and liver. In the kidneys, it relocates to the interstitium, renal tubules, and lumen causing interstitial nephritis and acute tubular necrosis (ATN). Direct leptospire injury may cause ATN, whereas interstitial nephritis occurs due to antigen–antibody complexes of the immune phase. In the liver, centrilobular necrosis with the proliferation of Kupffer cells in seen resulting in hepatic dysfunction. *Leptospira* also disrupt the endothelial lining of blood vessels leading to capillary leaks and haemorrhages. Capillary vasculitis is found in every affected organ system. There may be associated thrombocytopenia. Despite the possibility of severe complications, the disease is usually non-fatal and self-limiting. Leptospire-mediated injury characterises the initial phase of the disease which is also called septicemic phase, and host immune response marks the onset of the second phase of symptoms called immune phase of the disease(Shah & Katira, 2007). The mechanism of *Leptospira* causes host tissue damage and disease is not well defined. There have been reports of pathogenic mechanisms, but in almost all cases the specific leptospiral component responsible for the activity investigated was not identified (Adler & Moctezuma, 2010).

1.5 Aim of Study

Leptospira spp are the parasitic bacterial organism related with the broad range of mammalian hosts and are responsible for severe cases of human leptospirosis. The epidemiology of leptospirosis is complex and dynamic (Ahmed *et al.*, 2006). Leptospirosis outbreak has recently been reported in some developing countries including Malaysia. *Leptospira* can survive for long period of time in the environment under favourable condition (Ridzlan *et al.*, 2010). According to Kuriakose *et al.* (1997), there is an association of leptospirosis and rainfall during monsoon. Thus, a molecular method was conducted for giving a more accurate result for isolation and identification of pathogenic *Leptospira* spp.

General objective:

To isolate and identify the pathogenic *Leptospira* spp from environmental sample during and after the flood.

Specific objectives of this study are:

1. To isolate *Leptospira* spp in an environmental samples during and after the flood
2. To identify pathogenic *Leptospira* spp using PCR

Chapter 2: Materials and methods

2.1 Media and solutions

The ingredients of media as shown in Table 2.1

Table 2.1: Media used in this study

Medium	**Ingredients/Reagents**
Liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) Media	1.15 g of EMJH powder in a final volume of 500 ml distilled water 5 ml of 10mg/ml 5-Flourouracil 50 ml of enrichment EMJH

2.2 Chemical and reagents

The preparation and utilisation of the various chemicals, reagents and kits were performed according to the supplier's recommendation. Various chemicals, reagents and kits used in this study are listed in Table 2.2.

Table 2.2: List of chemicals, reagents and kits used in this study

Chemicals, reagents, kits	Brand
General	
UltraPure™ DISTILLED WATER	Invitrogen, UK
Ethanol	Hamburg Indus inc.
Microbiological	
Ellinghausen-McCullough-Johnson-Harris (EMJH) medium	Difco
DNA manipulation	
100 bp DNA ladder	Promega, USA
Loading Dye	Promega, USA
peqGREEN dye	Peqlab
DNA extraction kit (NucleoSpin® Microbial DNA)	Macherey-Nagel kit

2.3 Instrument and apparatus

Various instruments and apparatus used in this study were listed in Table 2.3.

Table 2.3: List of instruments and apparatus used in this study.

Intruments/apparatus	Brand
NanoDrop (ND-1000) Spectrophotometer	Thermo Fisher Scientific inc, US
PCR Gradient Cycle	MJ Research
Image analyzer gene flash	SYNGENE
DNA Electrophoresis	Bio-Rad
Microwave Oven	Panasonic
Balance-Analytical	AND GR-300
Pipette	Eppendorf, USA/Gilson
Incubator (37°C)	Memmert
Incubator shaker (200 rpm, 18 hours, 37°C)	Thermo electron corporation/Innova 4000
Refrigerator (20°C, -80°C)	Thermo Scientific, USA
Water bath (56°C, 70°C)	Memmert
Centrifuge	MicroONE Tomy, SIGMA
Vortex/shaker	Fisher Scientific
1.5 ml Eppendorf tube	Axygen, USA
Vacuum filter unit (PES membrane filter 0.22 μm)	Millipore
Stainless steel soil scoop	
Stainless steel bucket	

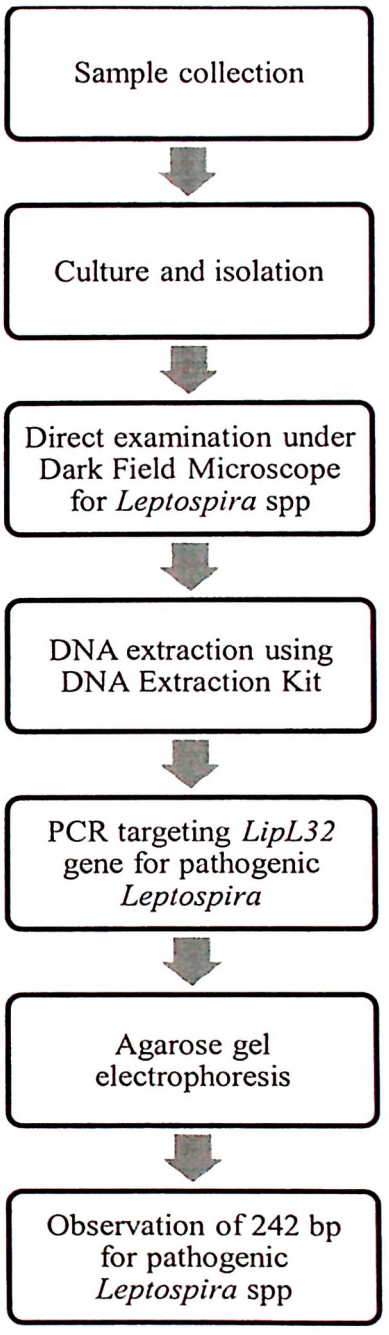

Figure 2.1: Flowchart of the overall study

2.4 Study location

Zainudin Abdul Wahab, (2015) reported that Kelantan region as the highest prevalence of a number of cases and death due to leptospirosis infection until July 2015 in Malaysia as shown in Figure 2.2, thus it was chosen for study location. Kelantan is positioned in the north-east of Peninsular Malaysia. It is bordered by Narathiwat Province of Thailand to the north, Terengganu to the south-east, Perak to the west, and Pahang to the south. To the north-east of Kelantan is the South China Sea. There are 10 districts in Kelantan, consist of Kota Bharu, Pasir Mas, Tumpat, Pasir Puteh, Bachok, Kuala Krai, Machang, Tanah Merah, Jeli and Gua Musang. This study was conducted in 3 different districts that affected by the flood occurred in January 2017 which are Kota Bharu, Tumpat and Pasir Mas for samples collection.

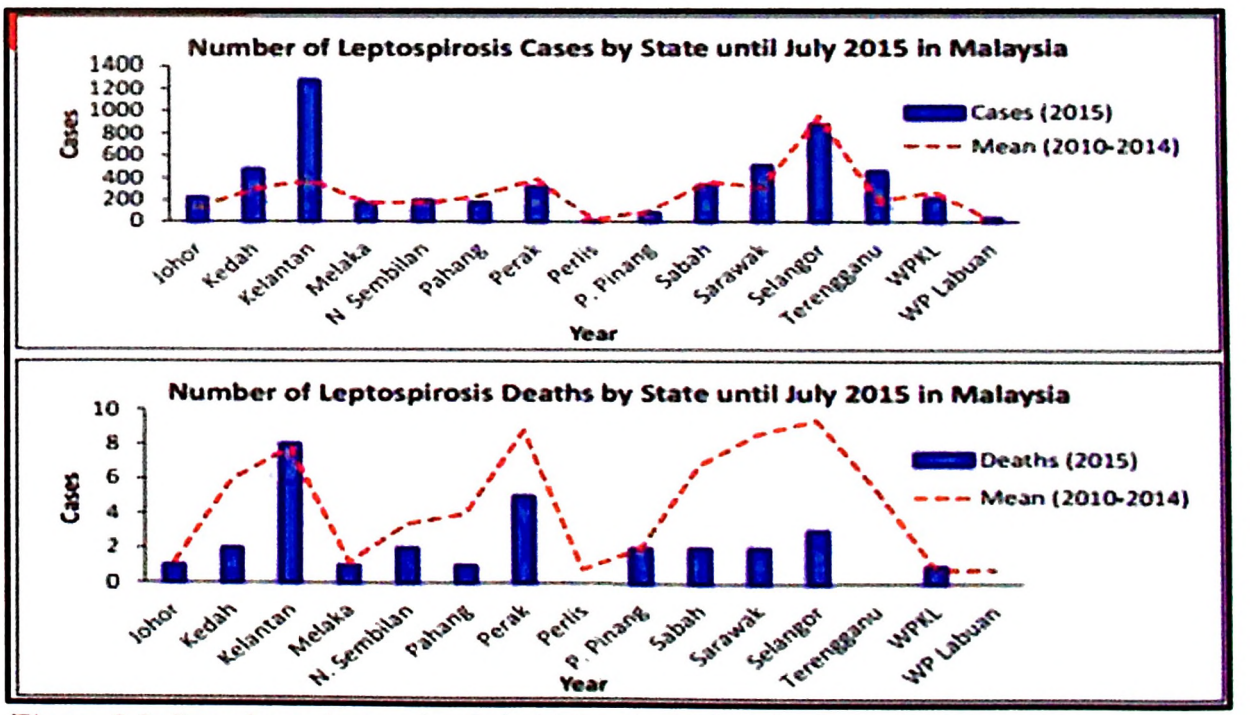

Figure 2.2: Prevalence leptospirosis in Malaysia (Zainudin Abdul Wahab, 2015)

2.5 Sample collection of *Leptospira* spp

The total amount of 52 samples of water and soil were collected in the flood affected area of Kelantan region. The sample was collected during and after the flood from January 2017 to February 2017. Date, place of collection, and volume of each sample were recorded prior to the sampling. Samples during the flood were labeled as (A) as shown in Table 2.4, while samples after the flood were labeled as (B) as shown in Table 2.5. An amount of 250 ml of water was collected from potentially contaminated water source using a sterile steel water bucket and placed into Whirl-Pak bags. An approximate amount of 400 g surface soil (15 cm by 15 cm) was collected from wet and shaded areas using a sterile steel soil scoop. The sample then was placed in Whirl-Pak bags. Soil sample in Whirl-Pak bags was reduced until 250 g and confirmed the weight by using Balance-Analytical. An amount of 200 ml sterile distilled water was added into Whirl-Pak bags contain soil sample and shake for 5 minutes. Then the soil sample was kept for 30 minutes to form sediment. The soil sample that difficult to form sediment after rest for 30 minutes was placed in a sterile 50 ml falcon tube and centrifuged for 5 minutes at 2500xg.

Table 2.4: Study location and samples during the flood

STUDY LOCATION	SAMPLES
Tumpat, Kelantan	LS 001/17 (A)
	LS 002/17 (A)
	LW 003/17 (A)
	LW 004/17 (A)
	LS 005/17 (A)
	LW 006/17 (A)
	LW 007/17 (A)
	LS 008/17 (A)
	LW 009/17 (A)
	LS 010/17 (A)
	LW 011/17 (A)
	LS 012/17 (A)
	LS 013/17 (A)
	LW 014/17 (A)
	LS 015/17 (A)
	LW 016/17 (A)
	LS 017/17 (A)
	LW 018/17 (A)
	LS 019/17 (A)
	LW 020/17 (A)
Pasir Mas, Kelantan	LS 021/17 (A)
	LW 022/17 (A)
	LS 023/17 (A)
	LW 024/17 (A)
	LS 025/17 (A)
	LW 026/17 (A)
	LS 027/17 (A)
	LS 028/17 (A)
	LW 029/17 (A)
	LW 030/17 (A)
	LW 031/17 (A)
	LS 032/17 (A)
Kota Bharu, Kelantan	LS 033/17 (A)
	LW 034/17 (A)
	LS 035/17 (A)
	LW 036/17 (A)

	LS 037/17 (A)
	LS 038/17 (A)
	LW 039/17 (A)
	LW 040/17 (A)
	LS 041/17 (A)
	LW 042/17 (A)
	LS 043/17 (A)
	LW 044/17 (A)
	LS 045/17 (A)
	LW 046/17 (A)
	LS 047/17 (A)
	LW 048/17 (A)
	LS 049/17 (A)
	LW 050/17 (A)
	LS 051/17 (A)
	LW 052/17 (A)

** LW = Water sample ** LS = Soil sample ** (A) = Sample during flood

** Total sample collected 52; 20 samples from Tumpat, 12 samples from Pasir Mas and 20 samples from Kota Bharu

Table 2.5: Study location and samples after the flood

STUDY LOCATION	SAMPLES
Tumpat, Kelantan	LS 001/17 (B)
	LS 002/17 (B)
	LW 003/17 (B)
	LW 004/17 (B)
	LS 005/17 (B)
	LW 006/17 (B)
	LW 007/17 (B)
	LS 008/17 (B)
	LW 009/17 (B)
	LS 010/17 (B)
	LS 011/17 (B)
	LS 012/17 (B)
	LS 013/17 (B)
	LW 014/17 (B)
	LS 015/17 (B)
	LW 016/17 (B)
	LS 017/17 (B)
	LW 018/17 (B)
	LS 019/17 (B)
	LW 020/17 (B)
Pasir Mas, Kelantan	LS 021/17 (B)
	LW 022/17 (B)
	LS 023/17 (B)
	LW 024/17 (B)
	LS 025/17 (B)
	LS 026/17 (B)
	LS 027/17 (B)
	LS 028/17 (B)
	LW 029/17 (B)
	LW 030/17 (B)
	LW 031/17 (B)
	LS 032/17 (B)
Kota Bharu, Kelantan	LS 033/17 (B)
	LW 034/17 (B)
	LS 035/17 (B)
	LW 036/17 (B)

	LS 037/17 (B)
	LS 038/17 (B)
	LW 039/17 (B)
	LW 040/17 (B)
	LS 041/17 (B)
	LW 042/17 (B)
	LS 043/17 (B)
	LW 044/17 (B)
	LS 045/17 (B)
	LW 046/17 (B)
	LS 047/17 (B)
	LW 048/17 (B)
	LS 049/17 (B)
	LW 050/17 (B)
	LS 051/17 (B)
	LW 052/17 (B)

** LW = Water sample ** LS = Soil sample ** (A) = Sample after flood

** Total sample collected 52; 20 samples from Tumpat, 12 samples from Pasir Mas and 20 samples from Kota Bharu

2.6 Culture and isolation of *Leptospira* spp

Each sample was filtered using sterile 0.22 µm polyethersulfone membrane filter with help of vacuum pump. Then, one ml of filtered sample was pipetted into eight ml liquid EMJH medium. This medium was incorporated with 5- fluorouracil (5-FU) at a concentration of 5 mg/ml to minimize bacterial contamination.

2.7 Direct examination of *Leptospira* spp under dark field microscope (DFM)

In the total of a month of complete culture incubation, observation under DFM is conducted during the second week, third week and fourth week. A volume of three µl of bacterial culture was pipetted from the culture medium onto a glass slide. Then it was examined under dark field microscope at 10x and 40x magnification to detect a presence of bacteria based on physical morphology. The presence of bacteria that has a similar characteristic (motile and helix shaped) of *Leptospira* was assumed as a positive result.

2.8 DNA extraction of *Leptospira* spp

The DNA was extracted from bacterial cultures by Nucleospin™ DNA Extraction Kit. A total volume of one ml of culture was transferred into 1.5 ml microcentrifuge tube. The tube was centrifuged for five minutes at 8,000 xg and the supernatant was discarded. An amount of 180 µL Buffer T1 added to the pellet. The pellet was then resuspended. 25 µL of proteinase K was added and mixed by using a digital vertical mixer. Then the sample was resuspended thoroughly by vortex and incubated at 56 °C water bath for two hours. An amount of 200 µL Buffer B3 was added to the sample then mix thoroughly by vortex and

incubated at 70 °C for 10 min. DNA binding condition was adjusted by adding 210 µL ethanol (96–100 %) to the sample and vortex vigorously. The sample was applied into the NucleoSpin® Tissue Column that placed into a collection tube and centrifuged for one minute at 11,000 xg. The solution in the collection tube was discarded. Two step of washing silica membrane was done, a total volume of 500 µL Buffer BW was added to the column and centrifuged for one minute at 11,000 xg. The flow through was discarded and 600 µL of Buffer B5 was added and centrifuged for one minute at 11,000 xg. The flow through was discarded. Then, the column was centrifuged again to dry the silica membrane for one minute at 11,000 xg. The collection tube was discarded and the column was transferred to a new 1.5 ml centrifuge tube. A volume of 100 µL of pre-warmed buffer BE was added directly onto the column membrane, incubated for one minute and centrifuged at 11,000 xg for one minute. The eluted solution was used as the DNA template and the spin column was discarded.

2.9 Quantification of DNA

The concentration and purity of DNA samples that have been extracted with NucleoSpin DNA Extraction kit were determined by measuring the absorbance at 260 and 280 nm using NanoDrop (ND-1000) Spectrophotometer.

2.10 Amplification of *Leptospira spp* DNA

The extracted DNA was amplified using polymerase chain reaction (PCR). The PCR reaction mixture consisted of 9.2 µl sterile dH_2O, 4 µl 1X PCR buffer, 0.6 µl forward primers, 0.6 µl reverse primers, 0.4 µl dNTPs, 2 µl $Mgcl_2$, 0.2 µl Taq polymerase enzyme and 3 µl sample DNA. The summary of the temperature profile used was listed in Table 2.6. The sequence of the primers used in this study is listed in Table 2.7. The PCR reaction involved denaturation at 95°C for 30 seconds, annealed at 52°C for 30 seconds, and elongated at 72°C for 30 seconds. This reaction was carried out for 29 cycles. An initial cycling step of denaturation at 95°C for 5 minutes, final annealing at 52°C for 30 seconds and final elongation at 72°C for 5 minutes was included as well. A volume of 3 µl of the PCR product was electrophoresed on 2% agarose gel to profile the amplified DNA.

Table 2.6: Temperature profile used for DNA amplification by PCR

Stage	Temperature (°C)	Time	Number of cycle
Initial denaturation	95	5 minute	1
Denaturation	95	30 second	29
Annealing	52	30 second	29
Elongation	72	30 second	29
Final annealing	52	30 second	1
Final elongation	72	5 minute	1
Hold	4	Forever	-

Table 2.7: Primer used for PCR amplification of sample DNA

No	Gene	Primers	5'-oligonucleotide-3'	Product length (bp)
1	LipL32	LipL32-45 (F)	AAG CAT TAC CGC TTG TGG TG	242
		LipL32-286 (R)	GAA CTC CCA TTT CAG CGA TT	

** F: Forward ** R: Reverse (Stoddard *et al.*, 2009)

2.11 Agarose Gel Electrophoresis

Agarose gel (2%) was prepared using TAE buffer and pre-stained with peqGREEN dye. For a 2% gel, 1.6 g agarose was weight and resuspend in 80 ml 1x TAE buffer. The solution was heated to boil in the microwave for two minutes to dissolve the agarose. The gel was cool to around 50 °C before adding the dye. 3.2 µL of *peqGREEN* dye was added to the dissolved agarose and thoroughly mix by swirling gently. The slots on each side of the gel plate were placed in between the two dams of casting tray. The melted agarose was poured onto the gel plate in the casting tray. The present of bubbles was pop with a pipette tip. Next, the comb was placed. The small comb was used for more than 14 samples. The gel was rest to cool to room temperature for about 30 minutes. The comb was carefully removed from the casting tray. 1X TAE was poured into the gel tank to submerge the gel to 2-5 mm depth. Four µl of the marker was inserted into first well. Three µL of the negative control and DNA samples were then loaded into the other well of the gel and electrophoresed at 90 volts for 60 minutes. The samples were carefully placed into adjacent wells by using an Eppendorf pipette and a steady hand. The gel was then visualized under UV illuminator.

Chapter 3: Results

3.1 Direct Examination

Direct examination result of *Leptospira* spp under dark field microscope was shown in Table 3.1 and Table 3.2.

Table 3.1: Observation of *Leptospira* spp under dark field microscope for samples collected during flood.

STUDY LOCATION	SAMPLES	DARK FIELD MICROSCOPE
Tumpat, Kelantan	LS 001/17 (A)	√
	LS 002/17 (A)	X
	LW 003/17 (A)	√
	LW 004/17 (A)	√
	LS 005/17 (A)	√
	LW 006/17 (A)	√
	LW 007/17 (A)	√
	LS 008/17 (A)	√
	LW 009/17 (A)	√
	LS 010/17 (A)	√
	LW 011/17 (A)	√
	LS 012/17 (A)	√
	LS 013/17 (A)	√
	LW 014/17 (A)	√
	LS 015/17 (A)	√
	LW 016/17 (A)	√
	LS 017/17 (A)	√
	LW 018/17 (A)	√
	LS 019/17 (A)	√
	LW 020/17 (A)	√
Pasir Mas, Kelantan	LS 021/17 (A)	√
	LW 022/17 (A)	X
	LS 023/17 (A)	√
	LW 024/17 (A)	√
	LS 025/17 (A)	X
	LW 026/17 (A)	√

	LS 027/17 (A)	√
	LS 028/17 (A)	√
	LW 029/17 (A)	√
	LW 030/17 (A)	√
	LW 031/17 (A)	√
	LS 032/17 (A)	√
Kota Bharu, Kelantan	LS 033/17 (A)	√
	LW 034/17 (A)	X
	LS 035/17 (A)	X
	LW 036/17 (A)	X
	LS 037/17 (A)	√
	LS 038/17 (A)	√
	LW 039/17 (A)	X
	LW 040/17 (A)	X
	LS 041/17 (A)	X
	LW 042/17 (A)	X
	LS 043/17 (A)	√
	LW 044/17 (A)	X
	LS 045/17 (A)	√
	LW 046/17 (A)	X
	LS 047/17 (A)	X
	LW 048/17 (A)	X
	LS 049/17 (A)	√
	LW 050/17 (A)	X
	LS 051/17 (A)	X
	LW 052/17 (A)	X

** √ = Present of *Leptospira* under DFM

** X = No *Leptospira* spp detected

Table 3.2: Observation of *Leptospira* spp under dark field microscope for samples collected after flood.

STUDY LOCATION	SAMPLES	DARK FIELD MICROSCOPE
Tumpat, Kelantan	LS 001/17 (B)	√
	LS 002/17 (B)	√
	LW 003/17 (B)	X
	LW 004/17 (B)	X
	LS 005/17 (B)	√
	LW 006/17 (B)	√
	LW 007/17 (B)	√
	LS 008/17 (B)	√
	LW 009/17 (B)	X
	LS 010/17 (B)	√
	LS 011/17 (B)	√
	LS 012/17 (B)	√
	LS 013/17 (B)	X
	LW 014/17 (B)	√
	LS 015/17 (B)	√
	LW 016/17 (B)	X
	LS 017/17 (B)	√
	LW 018/17 (B)	√
	LS 019/17 (B)	√
	LW 020/17 (B)	√
Pasir Mas, Kelantan	LS 021/17 (B)	X
	LW 022/17 (B)	X
	LS 023/17 (B)	√
	LW 024/17 (B)	X
	LS 025/17 (B)	√
	LS 026/17 (B)	√
	LS 027/17 (B)	√
	LS 028/17 (B)	√
	LW 029/17 (B)	X
	LW 030/17 (B)	X
	LW 031/17 (B)	X
	LS 032/17 (B)	√
Kota Bharu, Kelantan	LS 033/17 (B)	√
	LW 034/17 (B)	X
	LS 035/17 (B)	√