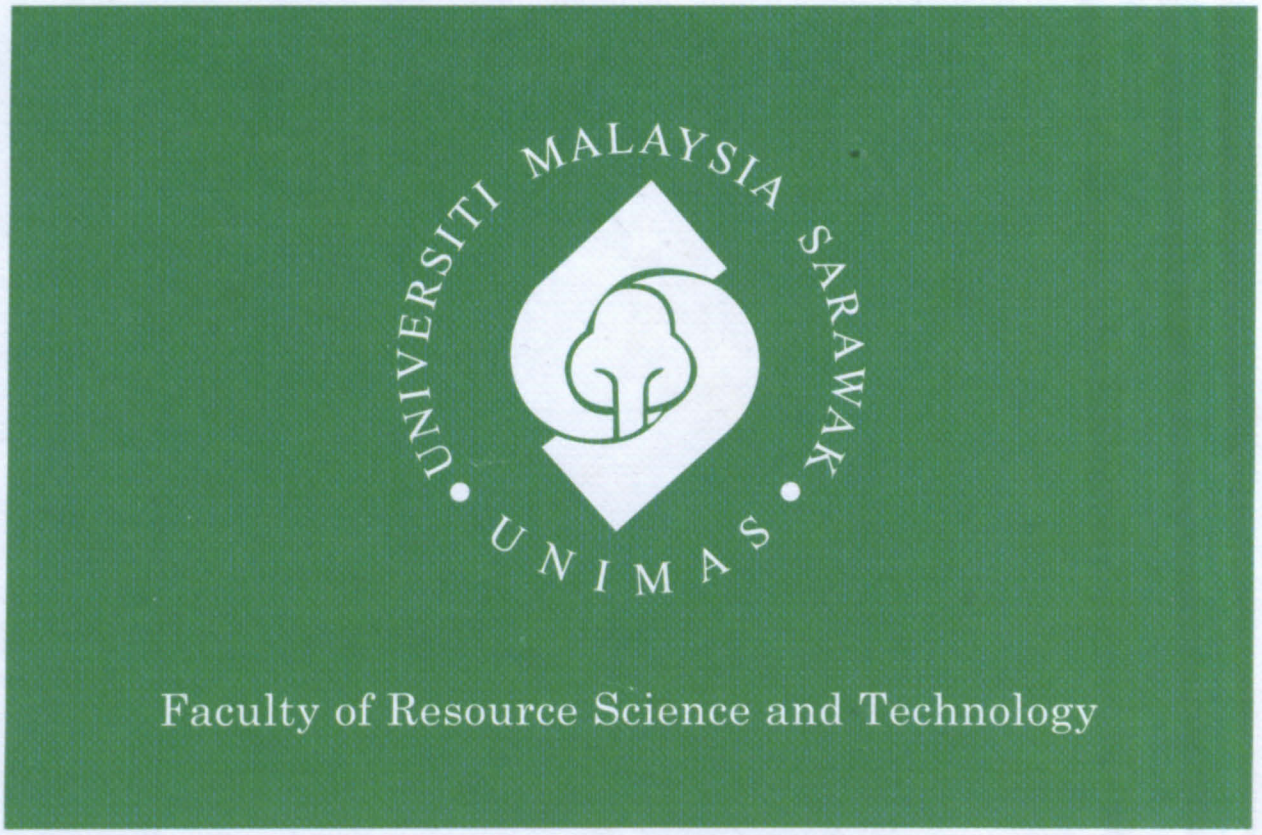

DETECTION OF *LEPTOSPIRA* SPECIES FROM ENVIRONMENT IN SANTUBONG NATIONAL PARK AND MULU NATIONAL PARK

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Bachelor of Science with Honours
(Resource Biotechnology)
2017

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

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LIST OF ABBREVIATIONS

EMJH	- Ellinghausen McCullough Johnson Harris
AGE	- Agarose Gel Electrophoresis
dNTPs	- Deoxynucleotide triphosphates
EtBr	- Ethidium Bromide
$MgCl_2$	- Magnesium Chloride
PCR	- Polymerase Chain Reaction
UV	- Ultraviolet
TBE	- Tris-borate-EDTA
DFM	- Dark Field Microscope
MAT	- Microscopic Agglutinantion Test
CAAT	- Cross Agglutinin Agglutinantion Test
PFGE	- Pulse Field Gel Electrophoresis
SNP	- Santubong National Park
MNP	- Mulu National Park

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Detection of *Leptospira* species from environment in

Santubong National Park and Mulu National Park.

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ABSTRACT

Leptospira is tightly coiled spirochete and obligate aerobic bacteria that can cause leptospirosis. It can cause global health impact on human and animals. *Leptospira* is divided into three major groups, pathogenic, saprophytic and intermediate *Leptospira*. In previous studies, the presence of pathogenic *Leptospira* from environment in Peninsular Malaysia has been detected. The main purpose of this study was to isolate and detect pathogenic, saprophytic and intermediate *Leptospira* in water and soil samples from Santubong National Park and Mulu National Park. A total of 35 environment samples, soil and water were collected from two selected national parks of Sarawak. The samples were cultured in EMJH medium supplemented with of 5-fluorouracil for 30 days. Extraction of DNA was done using Wizard ™ Genomic DNA purification Kit. The cultures were analysed using polymerase chain reaction (PCR) for detection of *Leptospira* species. Approximately 25.7% (9 out of 35) samples were detected positive towards *16S rRNA* gene which is specific to intermediate *Leptospira*. The findings of this study indicated the presence of *Leptospira* in soil sample is higher than water sample and the distribution of *Leptospira* is higher at Santubong National Park compared to Mulu National Park due to environment condition. Thus preventive measure and control should be taken and increase awareness among public regarding leptospirosis.

Key words: *Leptospira*, leptospirosis, polymerase chain reaction, *16S rRNA* gene.

ABSTRAK

Leptospira adalah Spirochete yang bergelung ketat dan bakteria aerobik yang wajib boleh menyebabkan leptospirosis. Ia boleh menyebabkan impak kesihatan global pada manusia dan haiwan. Leptospira dibahagikan kepada tiga kumpulan utama, patogenik, saprofitik dan perantaraan Leptospira. Dalam kajian sebelum ini, kewujudan patogenik Leptospira dari persikataran di semenanjung Malaysia telah dikesan. Tujuan utama kajian ini adalah untuk mengasingkan dan mengesan patogenik, saprofitik dan perantaraan Leptospira dalam sampel tanah dan air dari Taman Negara Santubong dan Taman Negara Mulu. Sebanyak 35 sampel, tanah dan air telah diambil dari dua Taman Negara yang telah dipilih di Sarawak. Sampel dikultur dalam media EMJH yang ditambah 5-fliorouracil untuk jangka 30 hari. DNA telah diekstrak menggunakan Wizard ™ Genomic DNA purification Kit. Kultur itu dianalisis menggunakan polymerase chain reaction (PCR) untuk mengesan spesis Leptospira. Kira-kira 25.7% (9 daripada 35) sampel dikesan positif terhadap gen 16S rRNA dimana spesifik kepada Leptospira perantaraan. Melalui kajian ini, ia menunjukkan bahawa kehadiran Leptospira dalam sampel tanah lebih tinggi dari sampel air dan pengedaran Leptospira lebih tinggi di Taman Negara Santubong berbanding di Taman Negara Mulu disebabkan oleh keadaan persekitran. Oleh itu, langkah pencegahan dan kawalan perlu diambil dan meningkatkan kesedaran mengenai leptospirosis dalam kalangan orang awam.

Kata kunci: Leptospira, leptospirosis, polymerase chain reaction, 16S rRNA

1.0 INTRODUCTION

Leptospira is often associated with worldwide zoonotic disease known as leptospirosis. It causes a worldwide health impact on human and animals (Lehmann *et al.,* 2014). *Leptospira* has the ability to survive in a wide range of hosts including mammalians hosts where rats serve as the real bearer in most human leptospirosis (Coburn & Evangelista, 2010). However, *Leptospira* and its maintenance hosts has seem to experience adjustment to their surroundings, thus the preference and pathogenicity of these hosts additionally can change with time and geographical regions (Balamurugan, *et al.,* 2013). Leptospirosis generally can occur through direct contact with infected urine or indirect exposure to the organisms in damp soil or water (Levett, 2001).

Leptospira are firmly coiled spirochaete which have two perisplasmic flagella with polar insertion which are located in the periplasmic space that help them to move. *Leptospirae* are also obligates aerobes that grow optimally at temperature between 28 to 30 ℃ only (Levett, 2001). Currently, there are 20 types of *Leptospirae* with more than 300 serovars and the serovars are categorized into 20 serogroups (Mayer *et al.,* 2014). There are three species of *Leptospira* which are pathogenic, intermediate and saprophytic. The pathogenic *Leptospira* has the potential to cause disease in animals and humans. Meanwhile saprophytic *Leptospira* is free living and generally considered no role in human infection because these organisms are not necessarily resided in host and they feed organic matter in water (Lim *et al.,* 2011). In fact, saprophytic *Leptospira* lack of genes for virulence (Vinetz, 2012). *Leptospira* require hosts for survival and reproduction and they are particularly discovered in renal tubules of maintenance hosts (Lim *et al.,* 2011).

Additionally, leptospirosis is considered as an endemic disease particularly in tropical countries that experience seasonal rain and floods (Dutta & Christopher, 2005). Inadequate sanitation and climate factors in Malaysia have increased leptospirosis outbreaks which can lead to high risk leptospirosis infection (Thayaparan *et al.,* 2013). Ministry of Health Malaysia (2015) had reported the increase in total cases of leptospirosis by 40% from 3,289 in 2014 to 5,370 in 2015. As mentioned by Ridzlan and collegues (2010), Kelantan, Selangor, Sarawak, Kedah and Terengganu have recorded the highest case of leptospirosis in Malaysia.

Leptospirosis is a notifiable disease in Sarawak (Pui *et al.,* 2015). As reported by Sarawak Heath Department (2015), leptospirosis had cause 616 cases and 24 deaths in 2014. The number of cases and deaths dropped to 163 and six respectively as of 7 March 2015. In previous studies have reported the presence of pathogenic *Leptospira* from selected environment in Kuala Lumpur, Selangor and Johor (Ridzalan *et al.,* 2010; Benacer *et al.*, 2013). Moreover, Benacer and co-workers (2013) have detected the presence of *Leptospira* from recreational parks and drain effluents in housing area. The occurrence of *Leptospira* in wildlife at Sarawak had been reported (Thayarapan *et al.,* 2013).

As reported by Pui and collegues (2015), there was a research being conducted in detecting *Leptospira* species from environment, soil and water at Bako National Park and Tanjung Datu National Park. However, there is no study has been done in detecting the prsence of *Leptospira* species at Santubong National Park and Mulu National Park. The factor affecting the presence of *Leptospira* in the soil and water are not fully been understood due to the limitation of the understanding about their ecology in their habitat. Hence, through this study, leptospirosis awareness can be promoted and preventive measures can be taken to control leptospirosis outbreak.

The objectives of this study are:

1. To isolate the pathogenic, saprophytic and intermediate *Leptospira* from water and soil in Santubong National Park and Mulu National Park
2. To detect the pathogenic, saprophytic and intermediate *Leptospira* in water and soil samples using Polymerase Chain Reaction (PCR) method, targeting on *lip*L32, *rrs, 16 SrRNA* genes.

2.0 LITERATURE REVIEW

2.1 Morpholgy of *Leptospira*

Leptospira have dimensions of approximately 0.1 μm in width by 6-20 μm in length (Levett, 2001). The bacteria have very small diameter and need very dim light in order to contrast the bacterium under the microscope. They also need oxygen which is also known as aerobic bacterium to stay alive. Hence, they cannot live in a dehydrated place and the maximum pH level of this bacterium can survive is only up to 7 (Haraji *et al.,* 2011). The shape of this bacterium is likely a shape of question mark which either one or both of its end are arched into discrete shape (Coburn & Evangelista, 2010). The bacteria have two perisplasmic flagella with polar insertion which are located in the periplasmic space that help them to move. In addition, the FlaA and FlaB complement flagella sheath and core separately. However, under an electron microscopy, a mutate FlaB is lack of endoflagella and inhibit the movement of the bacterium 8 (Haraji *et al.,* 2011). On the outer surface of this bacterium, it has a double membrane structure and LPS and adjacent association between peptidoglycan cell wall and cytoplasmic membrane which characterize them into gram negative and gram positive (Haake 2000). However, gram positive bacteria has a lower endotoxic activity compared to gram negative bacteria (Levett, 2001).

2.2 Classification of *Leptospira*

Currentlly, *Leptospira* consists of 22 different species and the species are clustered into saprophytic, intermediate and pathogenic *Leptospira* (Dietrich *et al.,* 2015). According to Levett (2001), the genotypic classification has replaced phenotypic classification. The pathogenic *Leptospira* has the potential to cause disease in animals and humans. Meanwhile, the saprophytic *Leptospira* is freely living and generally considered do not cause diseases because of the lack of genes for virulence (Vinetz, 2012).

2.2.1 Serological Classification

Serological classification is a system that organizes individual bacterial strains of a species into smaller groups based on their cell surface antigens. The *Leptospira* genus is serologically classified into two species which are *Leptospira interrogans* and *Leptospira biflexa*. *L. interrogans* contains all pathogenic strains which can give disease to human neither animals while *L. biflexa* comprisies all saprophytic strains (Coburn & Evangelista, 2010). *According to Levett (2001), presence of* 8-azuaguanine and temperature of 13 °C are the factors that affect the growth ability of *L. biflexa* compared to *L interrogans*. On the other hand, *L. biflexa* cannot form cells in spherical shape in 1M sodium chloride (NaCl). Serovar is the basic taxon and *Leptospira* genus have more than two hundred serovars with a triple-layered cell envelope, consists of an outer membrane containing long sugar polymer extensions called lipopolysaccharides (Sclonczewski *et al.,* 2012), a peptidoglycan cell wall and an inner membrane. To determine the *Leptospira* serovar by serological classification, cross agglutinin absorption test (CAAT) or Microscopic agglutination test (MAT) is used (Romero *et al.,* 2009).

2.2.2 Genotypic Classification

The genotypic classification of *Leptospira* is based on DNA hybridization. According to the new genomic classification system, pathogenic species contains both pathogenic and nonpathogenic serovars as well as intermediate species such as *L.meyeri* and *L. inadai* (Morey, *et al.,* 2006). Curently, there are 22 species of *Leptospira* that are classified based on genotypic classification as shown in Table 1 (Dietrich *et al.,* 2015). Numerous studies have been done in order to understand the pathogenicity of the pathogenic, saprophytic and intermediate groups of *Leptospira*. But yet until now, there is still limited information on the intermediate *Leptospira* group compared to pathogenic and saprophytic groups.

Table 1: Species of *Leptospira* (Dietrich *et al.*, 2015).

Pathogenicity	Leptospira species
Pathogenic *Leptospira*	*L. interrogans, L. kirschneri, L. borgpetersenii, L. mayottensis, L. santarosai, L. noguchii, L. weilii, L.alexanderi, L. kmetyi, L. astonii*
Saprophytic *Leptospira*	*L. biflexa, L. idonii, L. meyeri, L. terpstrae, L. vanthielii, L. wolbachii L. yanagaweae*
Intermediate *Leptospira*	*L. broomii, L. fainei, L. inadai, L. licerasiae, L. wolffii*

2.3 Epidemiology of *Leptospira*

Leptospirosis is known as endemic disease that is very common in Southeast Asia, Latin America, Oceania, Caribbean and Indian subcontinent which in tropical and subtropical high rainfall. Usually, it can be found where humans have direct contact with the urine of infected animals or a urine-polluted environment (Coburn & Evangelista, 2010). According to the statistics, the annual incidence rate is evaluated from 0.1 to 1 per 100,000 populations in a moderate temperature regions and from 10 to 100 per 100,000 in tropical regions (Verma *et al.*, 2013). According to El Jalii & Bahaman (2004), in Southeast Asia, leptospirosis is commonly known as the important arising worldwide disease because of its warm and humid

climatic condition (Khairani-Bejo *et al.,* 2004). On the other hand, in Malaysia, *Leptospira* have been isolated from water and soils (Ridzlan *et al.,* 2010). In addition, the estimated incidence rate of leptospirosis in Malaysia ranges from 2 to 5 per 100,000 populations yet recently, the rate of incidence has increase significantly (Lim *et al.,* 2011). According to Ministry Health Malaysia (2011), incidence of leptospirosis had increased from 263 that cause 20 death in the year of 2004, to 1418 with 62 deaths in the year of 2009.

2.4 Clinical manifestation

According to Dutta and Christopher (2005), the clinical symptom of leptospirosis can be classified into two forms in which 90% of patients present anicteric side effects and 10% experience the ill effects of icteric disorder. Anicteric leptospirosis is a mild form of disease which only exhibits as influenza but when contrasted with icteric leptospirosis, its can lead to severe disease like jaundice, renal impairment and haemorrhage. The anicteric and icteric leptospirosis can be exhibited in two stages, which are septicemic phase and immune phase. Levett & Haake (2009) had stated, in septicemic phase, patients can have febrile sickness for around a week and *Leptospira* can be recuperated from cerebrospinal fluid (CSF) and blood, though in immune phase, antibody are produced and *Leptospira* can be recognized In the tissue, organs and urine of patients (Gamage *et al.,* 2011). For few patients, the two particular phase of leptospirosis can be shown with initial septicemic phage took after by brief decrease in fever then followed by immune phase. However, most of the patients show side effects which are begun at second period of sickness (Levett & Haake, 2009).

2.5 Mode of transmission

The leptospirosis transmission can occur directly whereby through contact with animal reservoirs, blood and urine or indirectly through water contaminated with *Leptospira* that are shed from reservoir mammals (Camahuali 2009). *Leptospira* usually enters the body through

the mucous membrane of the respiratory duct, abraded skin, ingested in contaminated food or water and inhalation of aerosols (Levett, 2001). Skin that has been immersed in contaminated water even for quite a while also is a way for *Leptospira* to penetrate onto the skin. For animals, *Leptospira* can be found in immature or stillborn babies as well as in normal babies or vaginal discharges after giving birth yet in humans, they additionally can be transmitted by sexual contact or breastfeeding (The Centre for Food Security & Public Health, 2013). Another uncommon route of exposure in humans includes rodent bites and laboratory accidents (Williams & Barker, 2008).

2.6 Laboratory diagnosis

2.6.1 Isolation using Ellinghausen-McCullough-Johnson-Harris (EMJH)

Leptospira can be isolate using culturing method which requires specific media. *Leptospira* can grow and survive in media supplemented with vitamin, ammonium salts and long chain fatty acids which acts as the sole carbon source for *Leptospira* through metabolism with beta oxidation. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is the most commonly medium for culturing *Leptospira* (Levett, 2011). However, the non-selective EMJH medium is not able to recover high percentage of *Leptospira* (Miraglia *et al.*, 2009). Hence, Ridzlan and colleagues (2010) had mentioned by adding a selective agent called 5-flourouracil in media can minimize the growth of contaminants in media.

Johnson & Rogers (1964) stated that 5-fluorourocil is a pyrimidine analogue and able to inhibit the growth of many bacteria due to the strong bacteriostatic action. Besides, pyrimidine analogue can be used specifically to isolate *Leptospira* from the contaminants as *Leptospira* do not take up fluorouracil because fluorouracil may not enter the cells. Isolation of *Leptospira* from the environmental using EMJH medium which supplemented with 5-fluorourocil was successful in the most previous studies (Issazadeh *et al.*, 2009). The

recommended concentrations of 5-fluorourocil for selective isolation of *Leptospira* is between 100 μg/mL and 400 μg/mL (Oie *et al.,* 1986). The growth of pathogenic *Leptospira* may be inhibited if the concentration of 5-fluorourocil too high (Tansuphasiri *et al.,* 2006). From the past studies, all serovar of Leptospirae can grow in the concentration of 5-fluorourocil at 100 μg/mL (Oie *et al.,* 1986). EMJH medium supplemented with 5-fluorourocil was used in most previous studies for isolation of *Leptospira* from water and soil (Issazadech *et al.,* 2009, Yuwvaranni & Thiruvengadam, 2010, Benacer *et al.,* 2013). Levett & Haake (2009) mentioned that usually *Leptospira* takes several weeks to grow up because they grow quite slow in the medium.

2.6.2 Molecular diagnosis and typing

2.6.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a molecular techniques that amplify DNA to generate millions copies of DNA of particular DNA sequence. Advancement of PCR adds to gene analysis, diagnosis of genetic disease as well detection of pathogens such as bacteria, virus and fungi (Valones *et al.,* 2009). Many molecular studies have been carried out to study the presence of *Leptospira* including Microscopic Agglutination Test (MAT), Dark Field Microscopy (DFM), Cross Agglutinin Agglutination test (CAAT) and Pulse Field Gel Electrophoresis (PFGE). However, PCR performs better than other tests because PCR can detect *Leptospira* even in early stage where immunoglobulin M (IgM) antibodies have not been appeared yet in serum (Levett & Haake 2009).

Obviously, PCR has widely used in diagnostic pathogenic strains of *Leptospira* from suspected sources like water and soil including rodents and bats (Tulsiani *et al.,* 2011). As mentioned by Tulsiani and collegues (2011), PCR is one of the best methods as its has high sensitivity and specificity in detecting and identifying *Leptospira* which is classified as

fastidious organism. PCR is also very useful in diagnosing leptospirosis especially in human acute syndrome cases while other techniques such as MAT and bacterial culture may give false-negative results and time consuming (Vitale *et al.,* 2005). Hence, PCR is the best molecular approach for *Leptospira* detection especially in geographical region where the prevelance of leptospirosis is still unkwon (Mgode *et al.,* 2005)

For amplifying the target gene, specific primers are used to detect the *Leptospira* species. Moreover, different species need different primers to detect it. Pathogenic *Leptospira* can be detected using *lip*L32-270F or *lip*L32-692R primer which target on *lip*L32 gene whereas *Sapro1* or *Sapro2* are the two primers that are used to target rrs gene for saprophytic *Leptospira* detection and confirmation (Vein *et al.,* 2012). The third primer which is *rrs*F and *rrs*R will be targetted 16 SrRNA to indicate the intermediate *Leptospira* (Benacer *et al.,* 2013). From the past studies, specific PCR that targets *lip*L32 has been fully detected pathogenic *Leptospira* from the ecological sample which is water and soil (Yasouri *et al.,* 2013).

3.0 MATERIALS AND METHODS

3.1 Materials

The list of materials and apparatus used in this study are stated in Appendix 1.

3.2 Methods

3.2.1 Study sites

In the present study, Santubong National Park and Mulu National Park were chosen as the sampling sites for water and soil collection. These two sites are recreational sites and it is tourist attraction spot which consists of outdoor activity such as mount climbing, jungle trekking and bird watching. A total of 35 samples, water (n= 18) and soil (n=17) were collected at different location within Santubong National Park and Mulu National Park. The water samples were mainly collected from the puddle, jungle ponds, unflowing river and bat's route which all the bats fly frequently. Meanwhile for soil samples, all samples were collected from wet, moist site and rodent's route which rats or squirrels walk or excrete urine on the soil. The details of water and soil samples collected from the sampling sites were shown in Table 7 (Appendix 2).

3.2.2 Sample collection

Water and soil samples were collected from Santubong National Park and Mulu National Park. Fifty millimetres of water samples were collected using sterile 50 mL Falcon tube. Approximately 20 g of topsoil were collected using spatula and placed in sterile 50 mL Falcon tube. The temperature and pH values of water and soil were measured at the site using portable pH meter (Extech PH100-ExStik, USA).

3.2.3 Sample processing and enrichment

All the collected samples were processed with some modification as described by Benacer and colleagues (2013). During sample processing in the laboratory, the pH values of water and soil samples were measured using Extech PH100-ExStik, USA. Fifty millimetres of water sample were filtered using Minisart NML syringe filter (Sartorious AG, Germany) with pore size in 0.20 µm. After that, 1 mL of filtered water were inoculated into modified liquid EMJH medium (Difco) which supplemented with 5-flolorouracil. Soil samples were soaked in distilled water which was about three times the volumes of samples. Then, the sample was shaken vigorously and settled for 15 to 20 minutes. Lastly, the filtered water from the soil was inoculated into EMJH culture medium.

For the preparation of EMJH, 2.3 g of EMJH medium powder was mixed with 900 mL of distilled water. Then proceed to the addition of 0.1 g 5-flolorouracil to minimize the growth of contaminants. After that, the medium was sterilized to ensure all the contaminants and bacteria are removed. The inoculated media were incubated aerobically at the room temperature for 30 days (Benacer *et al.*, 2013).

3.2.4 *Leptospira* DNA extraction

The genomic deoxyribonucleic acid (DNA) of *Leptospira* was extracted according to Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA) protocol with some modification. First, 1.5 mL overnight culture was transferred aseptically into 2 mL microcentrifuge tube and centrifuged for 5 minutes at 10700 rpm. Two layers were formed and the supernatant was removed. Six hundred microliters of Nuclei Lysis Solution was added and mixed vigorously by vortexing. Then it was incubated at 80 °C for 5 minutes in the water bath before cooled it at room temperature. Next, 3 µl of RNAse solution was added to the cell lysate and mixed it well before incubated in 37 °C for 30 minutes. Later, it was chilled it on ice for 5 minutes. Two hundred microliters of Protein Preparation Solution was added and vortex

vigorously before incubated it on ice for 5 minutes. After the incubation process, it was centrifuged again at 10700 rpm for 3 minutes. Six hundred microlitre of supernatant was transferred to 1.5 µl tube which contains 600 µl of isopropanol in a new tube. The mixture was centrifuged again at 10700 rpm for 2 minutes. The supernatant was poured out and the tube was drained on absorbent paper. Six hundred microliter of 70% ethanol was added in the tube and gently inverted several times. The tube was centrifuged again at 10700 rpm for 2 minutes and the supernatant was discarded. The tube was drained on clean absorbent paper and then air dried for 15 minutes. Lastly, 100 µl of DNA rehydrate solution was added to the tube and incubated overnight at 4 °C. The DNA was stored at -20 °C.

3.2.5 Detection of *Leptospira* species by Polymerase Chain Reaction (PCR)

Reaction mixture with total volume 25 µl, comprise of 5X green buffer, dNTPs, 25 mM $MgCl_2$, Go*Taq* DNA polymerase, forward and reverse primers, template DNA and distilled water. The *Leptospira* species was detected using PCR method using three sets of primers which are *lip*L32-270F/*lip*L32-692R, *Sapro*1/*Sapro*2 and *rrs*F or *rrs*R. Negative control which contains distilled water and positive control which contains *Leptopira* DNA were used. First primer set is *lip*L32-270F/*lip*L32-692R that targets on *lip*L32 gene to detect the presence of pathogenic *Leptospira* whereas second primer is *Sapro*1/*Sapro*2 that targets *rrs* gene for saprophytic *Leptospira*. The third primer are *rrs*F and *rrs*R were used to target *16S rRNA* for intermediate *Leptospira.* Table 2 indicates the three sets of primer that used for *Leptospira* species detection.

The thermal cycling condition starts with initial denaturation at 95 ^{0}C for 2 minutes, 35 cycles of 95 ^{0}C for 1 minute, 55 ^{0}C for 30 seconds, 72 ^{0}C for 1 minute and further extension at 72 ^{0}C for 5 minutes. PCR thermal cycle (Mastercycle gradient personal, Eppendorf, Germany) was set up to keep the temperature at 4 ^{0}C after all the steps were completed.

Table 2: Three sets of primer used to target specific gene of *Leptospira* in PCR

Primer Designation	Primer sequence 5' to 3'	Target		Amplicon size (bp)	Reference
		Species	Gene		
*Sapro*1	AGAAATTTGTGCTATACCGAATGT	Saprophytic *Leptospira*	*rrs*	240	Leon *et al.*, (2006)
*Sapro*2	GGCGTCGCTGTTCAGGCTTTCG				
*Rrs*F	GGCGGCGCGTCTTAAACATG	Intermediate *Leptospira*	*16S rRNA*	331	Benacer *et al.*, (2013)
*rrs*F	TTCCCCCCATTGAGCAAGATT				
*lip*L32-270F	CGCTGAAATGGGAGTTCGTATGATT	Pathogenic *Leptospira*	*lipL32*	423	Kucerova *et al.*, (2013)
*lip*L32-692R	CCAACAGATGCAACGAAAGATCCTTT				