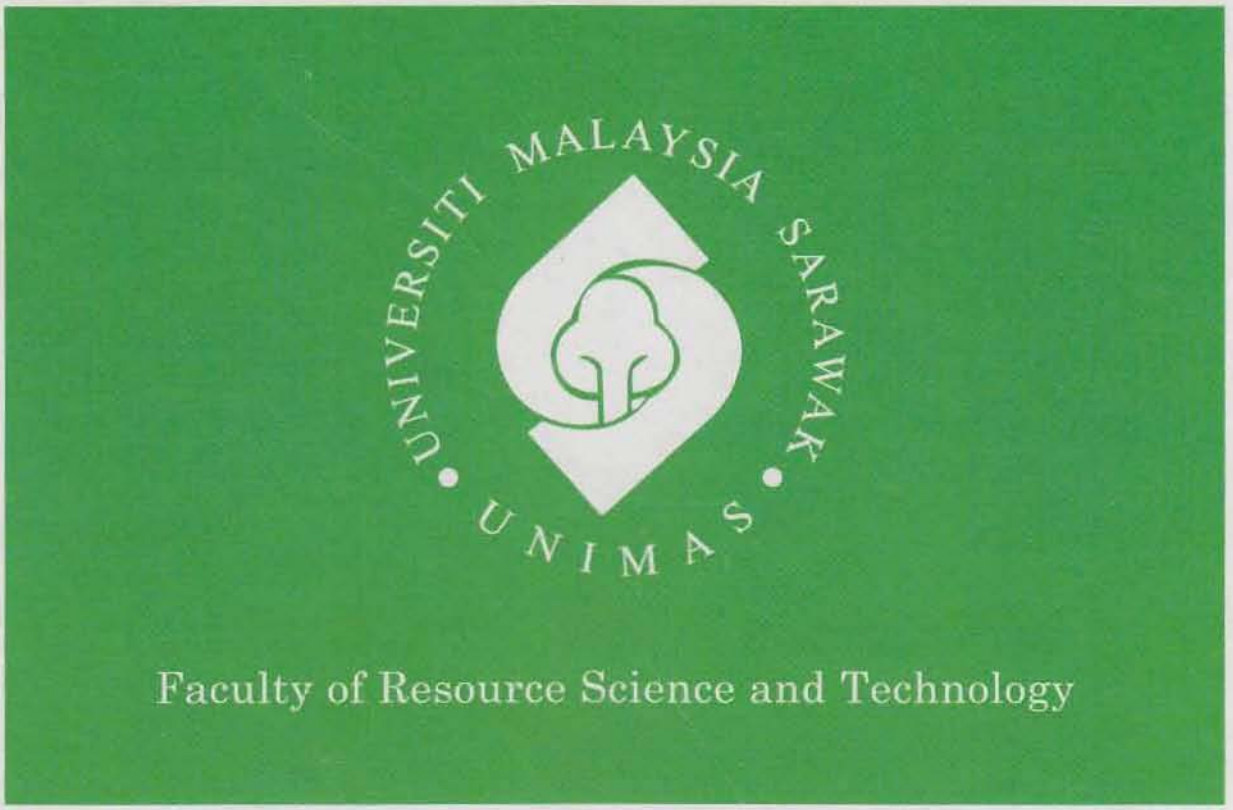

Survival of Pathogenic *Leptospira* in Different Types of Soils

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(45975)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

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This project is submitted in partial requirement for degree of Bachelor Science with Honours

Supervisor: Dr. Lesley Maurice Bilung

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Resource Biotechnology Programme
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Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2016/2017

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LIST OF ABBREVATIONS

EMJH	-	Ellinghausen McCullough Johnson Harris
ml	-	millilitre
µm	-	micrometer
°C	-	degree Celcius
kg	-	kilogram
cm	-	centimeter
sp.	-	species
%	-	percent

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Survival of Pathogenic *Leptospira* in Different Types of Soils

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ABSTRACT

Leptospira interrogans are shed into the environment by rats and rodents can cause leptospirosis disease among humans. This study investigated the survival of pathogenic *Leptospira* in different types of soils. In this study, samples of alluvial, arenaceous, peat and red-yellow podzolic soils at different water concentration (20%, 30% and 40%) were inoculated with 10^8 CFU/ml of *Leptospira interrogans.* The samples were withdrawn at day 3,6, 9 and weekly at day 16 until day 30. This study shows significant difference ($p<0.05$) in the survival of *Leptospira interrogans* in alluvial, arenaceous, peat and red-yellow podzolic soils at different water concentration (20%, 30% and 40%). *Leptospira interrogans* have the highest survival in alluvial soil at 40% water concentration. Result of this study proved that the types of soil and water concentration can affect the survival of *Leptospira interrogans* in environmental soil. Therefore, the result from this study can be used as the background data for future studies related to the survival on pathogenic *Leptospira.*

Key words: *Leptospira interrogans,* survival, types of soils.

ABSTRAK

Leptospira interrogans yang dibebaskan ke persekitaran melalui tikus boleh menyebabkan penyebaran penyakit leptospirosis dalam kalangan manusia. Kajian ini menyelidik tentang kemandirian *Leptospira* patogenik dalam jenis-jenis tanah berbeza. Dalam kajian ini, sampel tanah aluvial, arenaceous, paya dan podzolik merah-kuning dengan kandungan air (20%, 30% dan 40%) telah diinokulasi dengan *Leptospira interrogans* sebanyak 10^8 CFU/ml. Sampel-sampel tersebut diambil pada hari ke-3,6,9 dan setiap minggu pada hari ke-16 sehingga hari ke-30. Kajian ini menunjuk perbezaan bererti ($p<0.05$) pada kemandirian *Leptospira interrogans* dalam tanah aluvial, arenaceous, paya dan podzolik merah-kuning dengan kandungan air (20%, 30% dan 40%). *Leptospira interrogans* mempunyai kemandirian yang tinggi dalam tanah aluvial dengan kandungan air 40%. Keputusan dalam kajian ini menunjukkan jenis-jenis tanah dan kandungan air mempengaruhi kemandirian *Leptospira interrogans* dalam tanah persekitaran. Oleh itu, hasil kajian ini dapat digunakan sebagai data latar belakang untuk kajian berkaitan kemandirian *Leptospira* patogenik pada masa hadapan.

Kata Kunci: *Leptospira interrogans,* kemandirian, jenis-jenis tanah.

1.0 Introduction

One of the common and major habitat of microorganism are soils. Madigan and Martinko (2006) explained that microorganism inhabit every conceivable habitat that will sustain life and their habitat is very diverse and rapidly changing. Soils are divided into types including organic, red-yellow podzolic, alluvial and arenaceous. Each soil types vary in properties such as water-retaining capabilities and pH. All of these combined factors affects the diversity as well as the number of microorganism present. Following that, soil types do not only influence the natural soil inhabitant but also the non-soil inhabitant microorganism that are capable to survive in soil such as the *Leptospira* species.

Previous studies have documented the role of environmental soils as the long-term reservoir for spirochetes *Leptospira.* Mohammed *et al.* (2011) explained that genus *Leptospira* is divided into two species of which *Leptospira interrogans* includes pathogenic strains and *Leptospira biflexa* includes saprophytic strains. According to Benacer *et al.* (2013), saprophytic *Leptospira* are naturally present in the environmental soil and water whereas the main reservoir of pathogenic *Leptospira* are rats and rodents. Pathogenic *Leptospira* exist in soil when leptospires harboured in the kidneys of hosts are released into the environment. Paixao *et al.* (2014) stated that rodents are considered as the important reservoirs because these organisms can remain infected for a long period of time without manifesting the disease and can transmit the pathogenic agents to human and other animals.

Pathogenic leptospires can survive in the environment outside the host under favorable conditions; the moisture and pH value of the soils as well as the surrounding temperature influence pathogenic leptospires survivability (Ridzlan *et al.*, 2010). Trueba *et al.* (2004) also

mentioned that pathogenic leptospires are able to survive for long periods of time in moist soil and fresh water, especially when the pH is slightly alkaline. The survival potential of pathogenic leptospires in the environment especially in the soil increases the possibility of leptospirosis outbreaks among humans.

Leptospirosis is a disease of acute febrile septicemic and is a re-emerging zoonotic disease of global distribution with a broad spectrum of host range which is caused by spirochetes *Leptospira interrogans* (Naubade *et al.*, 2002). The infection occurs in humans via direct contact with animal reservoirs contaminated urine or indirect contact with contaminated water and soil (Benacer *et al.,* 2013). Following that, leptospirosis is a notifiable disease in Sarawak and has become a major public health concern. Thayaparan *et al.* (2013) mentioned that in the year 2004 to 2009, there is a gradual increase in the number of leptospirosis cases. However, in the year 2011 and 2012, the number of cases have increased drastically. Sman (2016) reported that 657 leptospirosis cases has occured between 1st January to 28th September 2016; recording Sarawak with the second highest number of leptospirosis cases in Malaysia.

Previous studies have shown that pathogenic *Leptospira* were isolated from the environmental soil. Ridzlan *et al.* (2010) study has indicated the presence of pathogenic leptospires from environmental soil and water in Terengganu and Kelantan meanwhile Azali *et al.* (2016) isolated a pathogenic strain (*Leptospira alstonii)* from a soil sample collected at a market in Kelantan. Research by Pui *et al.* (2015) conducted in national parks in Sarawak has indicated presence of pathogenic leptospires. Although precise identification and characterisation are important for epidemiological and public health surveillance, yet many aspects of leptospirosis epidemiology remains unexplored due to limited knowledge of the

environmental factors (Mohammed *et al.*, 2011; Chiriboga *et al.*, 2015). Besides, few information exists on the complexity of the disease as the pathogenic mechanism in leptospirosis is still poorly understood (Ricaldi *et al.*, 2012).

Therefore, the main aim of this study is to compare the survival of pathogenic *Leptospira* in different types of soils and different water concentration as a precaution measure to avoid potential leptospirosis. This study also aims to provide a background data for the future studies of transmission and prevalence of pathogenic *Leptospira.* The hypothesis for this research is there are significant difference between the types of soils and water concentration toward the survival of pathogenic *Leptospira.* The specific objectives of this study are as follows:

1. To investigate the survival of pathogenic *Leptospira* in organic, red-yellow podzolic, alluvial and arenaceous soils.

2. To evaluate the water content factors that influence the survivability of pathogenic *Leptospira* in organic, red-yellow podzolic, alluvial and arenaceous soils.

2.0 Literature Review

2.1 Morphology of *Leptospira*

Genus *Leptospira* classified under spirochetes are gram-negative, tightly coiled bacteria, typically are slender and flexuous with hooks present at both ends (Madigan and Martinko, 2006). The size of *Leptospira* sp. is about 0.1 μm in diameter and 6 to 20 μm in length. *Leptospira* are mobile with endoflagella that extend about two-third of the cell length; the endoflagella and the protoplasmic cylinder are surrounded by a multilayered-flexible membrane namely the outer sheath (Madigan and Martinko, 2006). Besides, *Leptospira* have a double typical membrane in common with other spirochetes; the wall of cytoplasmic membrane and peptidoglycan are closely associated and are overlaid by an outer membrane (Levett, 2001). The leptospiral lipopolysacharide (LPS) composed as the main antigen within the outer membrane of the *Leptospira.* Mohammed *et al.* (2011), mentioned that *Leptospira* supports alkalinization at pH 7.8 and the optimum growth temperature is 28 - 30 °C.

2.2 Classification of *Leptospira*

There are eight genera classification of spirochetes and one of them is the genus *Leptospira.* Below are the serotypic and genotypic classification of *Leptospira.*

2.2.1 Serotypic Classification

Leptospira are classified based on the expression of the surface exposed epitomes in a mosaic of the LPS antigens (Muhammed *et al.*, 2011). According to Madigan and Martinko (2006), two major species are *Leptospira interrogans* (parasitic) and *Leptospira biflexa* (saprophytic); the strain of *Leptospira interrogans* are parasitic to human and animals. *L. biflexa* has over 60 serovars meanwhile *L. interrogans* has over 200 serovars

recognized (Levett, 2001). Serovars of related antigenicity are grouped into serogroups and is determined by a standard assay namely Cross-Agglutination Absorption Test (CAAT). Serovars are useful for epidemiological understanding (Levett, 2001).

2.2.2 Genotypic Classification

Genotypic classification has replaced phenotypic classification of which DNA hybridization study has led to the definition of ten *Leptospira* genomospecies (Levett, 2001). Currently, molecular characterisation separates *Leptospira* into 20 species consisting of nine pathogenic, five intermediate and five saprophytic including one species of *L. meyeri* (Ahmed *et al.*, 2012). However, the genomic characterisation do not corespond to the existing serological classification of serovars and serogroups; pathogenic and nonpathogenic occur within the same species suggesting that genes determining the serotypes may be transferred horizontally among species (Levett, 2001; Cerquiera and Picardue, 2009; Ahmed *et al.*, 2012).

2.3 Transmission of *Leptospira*

According to the World Health Organization's (WHO) - International Leptospirosis Society (ILS) survey, the estimated annual worldwide number of leptospirosis cases is 350,000-500,000 cases (Scheirer *et al.,* 2009). The reservoir of the pathogenic *Leptospira* are rats, rodents and wild animals; *Leptospira* infect the renal tubules of the host and are shed to the environment when the host urinates (Ghane and Yasouri, 2013; Saito *et al.*, 2013). Infections occur mainly via skin and mucous membrane when human or other animals encounter leptospires-contaminated environment such as flood water, moist soil or vegetation contaminated by urine of infected animals (Barbosa *et al.*, 2009; Saito *et al.*,

2013). Pathogenic *Leptospira* efficiently colonize target organs and capable to multiply in blood, adhere to host cells and penetrate into tissue meanwhile the symptoms are broad-range and mimics the clinical presentations of other diseases including malaria and dengue fever (Barbosa *et al.*, 2009; Cerquiera and Picardeu, 2009).

2.3.1 *Leptospira* in Environment

Studies have been conducted to detect and isolate *Leptospira* in the environment of Malaysia and Sarawak. Slack *et al.* (2009) isolated *Leptospira kmetyi* sp. nov. which is a novel species of genus *Leptospira* from soil in Johor. Meanwhile, Benacer *et al.* (2013) isolated two pathogenic, one intermediate and five saprophytic *Leptospira* species at selected urban sites in Malaysia. In Sarawak, Pui *et al.* (2015) reported on the presence of *Leptospira* in the environmental soil and water sampled from two national parks; 0.9 % are the pathogenic strain and 5.5 % are the intermediate strain. Malaysia is a tropical country with high seasonal rainfall, warm temperatures with wet and humid climate, therefore the conditions support the survival of leptospires in the environment (Azali *et al.*, 2016).

2.3.2 Leptospirosis Outbreaks in Sarawak

Several cases of leptospirosis outbreak has been reported in Sarawak. According to Thayaparan *et al.* (2013), leptospirosis outbreak occurred at RSAT Army camp in Kuching on 12th December 2011 where five army recruits was showing leptospirosis symptoms and the disease was serologically confirmed subsequently of which the infection source was identified as drinking and bathing activities in a small river near the camp. On 30th December 2011, another leptospiral outbreak occured at Kem Semenggok,

Kuching where two army recruits were serologically positive for leptospirosis (Thayaparan *et al.*, 2013). In January 2010, a leptospirosis-contaminated pond was closed at Junaco Park National Service Training Camp, Sibu (Bernama, 2010). In the year 2016, 657 leptospirosis cases has occured between 1st January to 28th September 2016 thus recording Sarawak with the second highest number of leptospirosis cases in Malaysia (Sman, 2016).

2.3.3 Clinical Symptoms of Leptospirosis

The symptoms of leptospirosis are often variable and this leads to the unawareness of the disease. Thayaparan *et al.* (2015) mentioned that the clinical symptoms of leptospirosis may not occur in every case but severe fever is the important sign of acute leptospirosis. The symptoms comprise of fever, severe headache, chills, diarrhea, nausea and vomiting, joint paint, jaundice, conjunctival suffusion, aseptic meningitis, haemorrhages, oliguria/annuria, cough, cardiac arrhythmia, psychosis and/or delirium (Hartskeerl *et al.*, 2011). Amilasan *et al.* (2012) stated in her research that the most common clinical symptoms are myalgia and conjunctival suffusion, abdominal pain and oliguria. Based on her research, death due to leptospirosis were primarily caused by pulmonary hemorrhage and acute respiratory distress syndrome, followed by acute renal failure and multiple organ failure (Amilasan *et al.*, 2012).

2.4 Types of Soils

Soil differs by type based on their components. According to McCauley *et al.* (2005), soil components are minerals, soil organic matter, water and air and the composition and proportion influences soil physical properties (texture, structure and porosity). The texture

of the soil is the proportion of mineral particles which are clay, silt and sand; sand particles are coarse, silt is medium and clay is fine and they determine the soil air-water relationship (Osman, 2013). Soil structure determines the pore-size distribution in soil; the pore-size distribution affects the water flow and microbial behaviour in soil (Osman, 2013). Whereas, the porosity of soil affect water and air movement in soil (McCauley *et al.*, 2005). Soils are further classified to groups. The soils in Sarawak are classified into eleven groups (Teng, 2004). Four of the soil groups are peat soil, red-yellow podzolic soil, arenaceous soil and alluvial soil. Referring to the Soil Map of Sarawak 1968, the largest distribution of soil in Sarawak are red-yellow podzolic and peat soils whereas the smallest distribution is podzol soil.

Peat soils are categorized under organic soils. Soils that contain proportion of organic matter are grouped as organic soil. This soils occur at areas of swamp and riverine. Peat soils can be recognized by the characteristic of blackish colour and the fiber presence. The important characteristic which determines the bulk density, water retention and the drainable porosity is the fiber content in organic soils (Verry *et al.*, 2011).

Tie (1982) mentioned that the red-yellow podzolic soil has cambic or an argillic horizon. The soils are suitable for agriculture use for various types of plants (Mohidin *et al.*, 2009). The soils occur at variety of terrains and hilly areas (Mohidin *et al.*, 2009). Red-yellow podzolic soils have several types of textures which includes coarse loamy to fine clayey. However the soil can be recognized by its yellowish-red colour.

According to Tie (1982), alluvium is the variety of materials which have been moved

and deposited by water. Alluvial soils are soils developed in accreting alluvium (riverine). This type of soils occur in riverbed and interior floodplain. The soil can be recognized by its brownish or dark-brown colour.

Arenaceous soils are mineral soils that have a sandy particle-size class in the upper 50 cm or more of the soil particle (Teng, 2004). These soils are derived from marine alluvium, riverine alluvium and non-accreting alluvium (Tie, 1982). Arenaceous soil can be recognized by its yellow colour, red or dark-red colour depending on the types of either alluvium derived or non-accreting alluvium.

2.4.1 *Leptospira* and Types of Soils

Few studies have been conducted on the relationship of *Leptospira* and types of soils. Kingscote (1970) had studied on the correlation of bedrock type and leptospirosis in Canada. The research showed clinical leptospirosis occurred mostly at areas underlain by bedrock of Paleozoic sedimentery compared to Precambrian bedrock. The major difference between the two rock is the calcium or magnesium carbonate content and form (Kingscote, 1970). Kingscote (1970) also mentioned the distribution of leptospirosis appears to follow the bedrock type. Bejo *et al.* (2004) conducted a study on the survival of *Leptospira interrogans* in Malaysian environment which includes different types of soils. The study has demonstrated that environmental temperature and water content in different types of soils (clay, loam and sandy) influenced the survival time of *Leptospira interrogans.* Meanwhile, Schneider *et al.* (2012) mentioned in her study that critical areas of leptospirosis cases in Nicaragua corresponds to the type of soil combination (cambisol over pyroclastic bedrock and lava strata; andosol over volcanic ash).

2.5 Plate Count Method

Plate count method is a procedure for bacteria enumeration. Davey (2011) mentioned that it is the "gold standard" method which involves growing colonies on a nutrient agar surface during period of incubation. The method is based on the assumption that a single bacterium can grow and divide to give rise to a single colony; the amplification provides a high level of sensitivity detection of viable bacteria capability at densities of 10 per ml without the necessity for preanalysis concentration (Davey, 2011). Sutton (2011) mentioned that plate count method is the best data for interpretation of an approximation of the number of cells present. The number of cells analysed are typically based on plates with 30 to 300 cells (Davey, 2011).

2.6 Colony-Formation Unit

Colony-formation units is a unit used for bacterial cell count that allow quantification bacterial cells with the advantage of live-cells assessment (Hazan *et al.*, 2012). Viable count will be performed via colony-counting, with the assumption made in this type of counting that each viable cells can grow and divide to yield one colony (Madigan and Martinko, 2006). Brugger *et al.* (2012) also mentioned that the bacteria concentration of the original culture can be calculated based on the assumption that each colony has raised from one single bacterium, which is known as Colony-Forming Unit (CFU). Therefore, CFU is only the estimation of the number of cells present (Sutton, 2011).

3.0 Materials and Method

3.1 Soil Sample Preparation

The soils were obtained at different locations; alluvial soil were obtained from a river in Kampung Pueh, Lundu. Red-yellow soil were obtained from Pusat Islam Tun Abang Salahuddin (PITAS), UNIMAS. Organic (peat) and arenaceous soils were obtained from the Environmental Soil Science Laboratory, FRST, UNIMAS. The soils were collected by hand shovel at approximately 2 kg and were placed in plastic bags. The soils were later processed and prepared in the Environmental Soil Science Laboratory, FRST.

The soil samples were spread out on newspaper for air-drying process. The process of air-drying was carried out for a week. The air-dried soil samples were grinded by using mortal and pestle. Once done, the soils samples were then sieved by using a 2 mm pore sieve and stored in clean plastic bags. The air-dried soil samples were proceeded by drying in a hot-air oven. Three hundred gram from each air-dried soil types samples were weighed and placed in sterile beakers. The soil samples were then left in the hot-air oven at 40 °C for three days (Bejo *et al.*, 2004). Once oven-drying process were done, the soil samples were then placed in new and clean plastic bags. Twenty-eight sterile falcon tubes were prepared for each soil types and 10 g of each of the oven-dried soil types were placed in the falcon tubes.

For the negative control, 10 g of each types of soils that have been oven dried were placed in sterile conical flasks and 20 ml of sterile distilled water were added to each flasks. The flasks were shaken and were left for two hours. 0.1 ml of supernatant from each samples were spread on EMJH agar and incubated. There were no growth of colonies

recorded indicating the soils were free from *Leptospira* bacteria.

3.1.1 Soil pH Measurement

The pH of each soil types were determined after the soils were air-dried by using a pH meter (Eutech pH 700, USA). Five gram of the each types of air-dried soils were weighed and placed in sterile falcon tubes. A replicate were made for each soil types sample and the total soil samples tested were eight. Twenty-five ml of deionized water were placed in each of the falcon tubes containing the soil sample and were shaken using a shaker for one hour at 120/minute. Once the process shaking was finished, the suspension were immediately shaken again by hand before the pH electrode were inserted in the supernatant of each soil samples. The pH measurement were taken.

3.1.2 Soil-Water Concentration Level

Each falcon tubes containing different types of soil were added with 2g, 3g, 4g of sterile distilled water to obtain 20% , 30% and 40% of the total soil weight (10g) respectively. The tubes were left to stabilize in the environment for five days in shaded area (Bejo *et al.*, 2004).

3.1.3 Soil Texture Determination

The soils texture were determined using the feel method (Ritchey *et al.*, 2015). A small handful of each soil types was rolled into a small ball as the first step. The ball were shaped according to the procedure diagram (Ritchey *et al.*, 2015). The texture of all soil types were recorded.