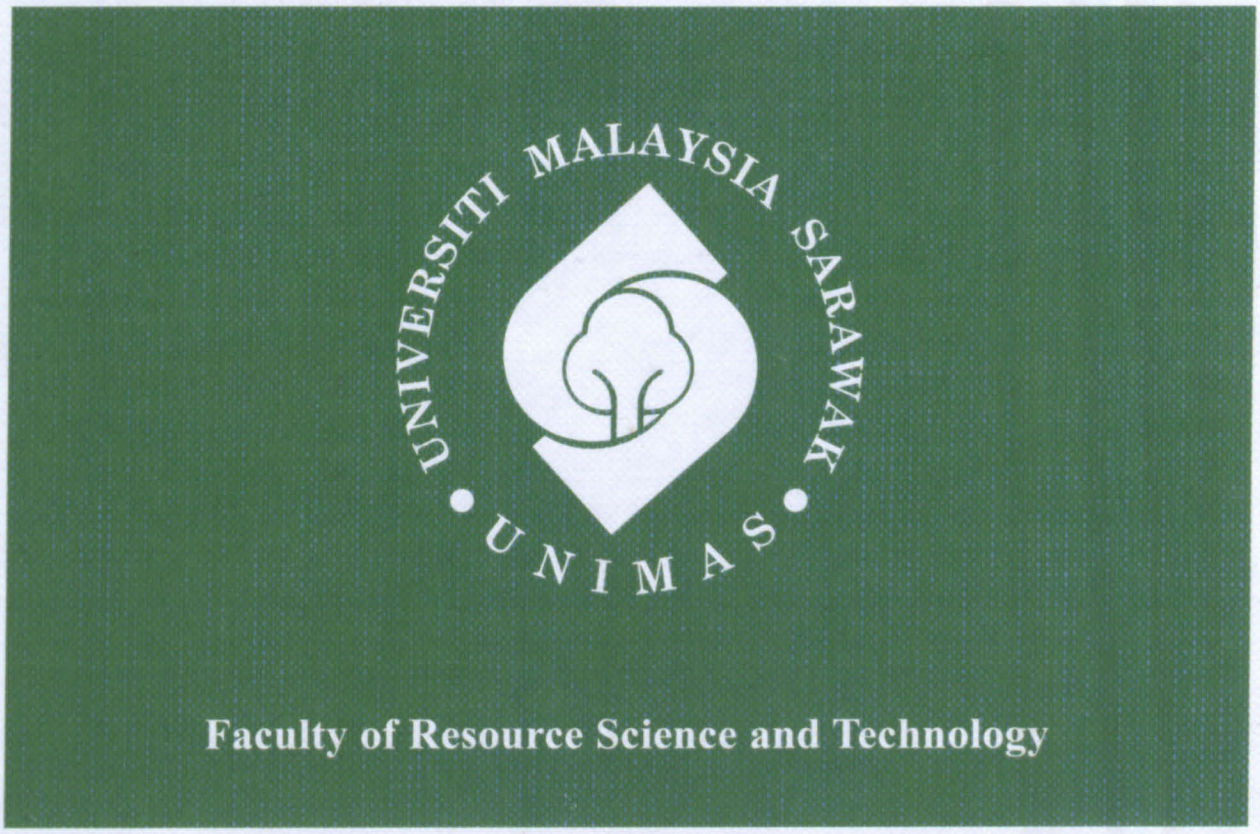

Optimization of Pulsed-Field Gel Electrophoresis Method for the Identification of Genetic Relatedness among Intermediate and Saprophytic *Leptospira* Species

Lai Siaw Ling (52204)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

Optimization of Pulsed-Field Gel Electrophoresis Method for the Identification of Genetic Relatedness among Intermediate and Saprophytic *Leptospira* Species

Lai Siaw Ling
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A thesis submitted in fulfillment of the requirement for the Degree of
Bachelor of Science with Honours

Supervisor: Dr. Lesley Maurice Bilung
Co-supervisor: Dr. Elexson Nilian

Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
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05/06/2018
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LIST OF ABBREVIATIONS

PFGE	Pulse-Field Gel Electrophoresis
FIGE	Field-Inversion Gel Electrophoresis
TAFE	Transverse Alternating Gel Electrophoresis
CHEF	Contour Clamped Homogeneous Electric Field
OFAG	Orthogonal-Field Alternation Gel Electrophoresis
PHOGE	Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis
PACE	Programmable Autonomously-Controlled Electrodes
TE	Tris-EDTA
TBE	Tris-borate EDTA
CLB	Cell Lysis Buffer
CDC	Centers for Disease Control and Prevention
LERG	Leptospirosis Burden Epidemiology Reference Group
CAAT	Cross Absorption Agglutination Testing
MAT	Microscopic Agglutination Testing
UV	Ultraviolet

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Optimization of Pulsed-Field Gel Electrophoresis Method for the Identification of Genetic Relatedness among Intermediate and Saprophytic *Leptospira* Species

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ABSTRACT

Leptospira species is the main causative agent of leptospirosis, one of the most widely spread zoonotic disease that can lead to mortality in both infected human and animals. Unfortunately, information about the epidemiology of this disease is still very inadequate. Molecular characterization of *Leptospira* species is vital for better understanding of leptospirosis epidemiology as well as for the diagnosis of *Leptospira* strains in infected patient for early treatment of leptospirosis.The main aim of this study was to determine the genetic relatedness among intermediate and saprophytic *Leptospira* species respectively. A total of 10 intermediate and 10 saprophytic *Leptospira* strains isolated from soil, water and rat in selected places of Sarawak, Malaysia were analysed molecularly through pulsed-field gel electrophoresis (PFGE) method. DNA fragments digestion during electrophoresis was done using *NotI* restriction enzyme. This study suggested some modifications that can be done to current PFGE protocol in order to obtain a typeable DNA profile for *Leptospira* species isolated from Sarawak. The result of the study indicated the effectiveness of PFGE method in molecular typing of *Leptospira* strains isolated from different sources in Sarawak.

Keyword: *Leptospira*, genetic relatedness, PFGE, molecular typing

ABSTRAK

Leptospira spesies merupakan agen penyebab utama leptospirosis, satu penyakit zoonotic yang merebak secara meluas yang boleh membawa kepada kematian dalam manusia dan juga haiwan. Walau bagaimanapun, maklumat mengenai epidemiologi tentang penyakit ini masih tidak mencukupi. Pencirian molekul spesies Leptospira adalah sangat penting bagi pemahaman mendalam mengenai epidemiologi dan juga untuk diagnosis spesies Leptospira dalam pesakit yang dijangkiti untuk rawatan awal leptospirosis. Tujuan utama kajian ini adalah untuk mengenai hubungan genetik antara spesies Leptospira perantaraan dan saprofitik masing-masing. Sebanyak 10 perantaraan dan 10 saprofitik Leptospira yang telah diambil dari tanah, air dan tikus di kawasan terpilih di Sarawak, Malaysia telah dianalisis molekul melalui pulsed-field gel electrophoresis (PFGE). DNA serpihan penghadaman semasa elektroforesis telah dilakukan dengan menggunakan NotI restriction enzyme. Kajian ini mencadangkan beberapa pengubahsuaian yang boleh dilakukan terhadap protokol PFGE semasa untuk mendapatkan profil DNA bagi spesies Leptospira yang didapati dari Sarawak. Hasil kajian ini menunjukkan bahawa keberkesanan kaedah PFGE dalam pencirian molekul Leptospira spesies diambil dari pelbagai sumber di Sawarak.

***Kata kunci**: Leptospira, hubungan genetik, PFGE, pencirian molekul*

1.0 INTRODUCTION

Leptospira species are flexible, coiled, obligate aerobic Gram-negative bacteria which can be distinguished by its distinct mode of motility (Johnson, 1996). These *Leptospira* species can be found in many wild animals as well as in environment such as soil and water (Benacer, Woh, Mohd Zain, Amran, & Thong, 2013). *Leptospira* species are spirochetal bacteria categorized under the family of Leptospiraceae (Mende *et al.*, 2013). Among the 21 recognised species, nine are considered as pathogenic *Leptospira* species, five are intermediate *Leptospira* species and the remaining are non-pathogenic or also known as saprophytic *Leptospira* species (Moreno *et al.*, 2016). Various studies had been done on pathogenic *Leptospira* species. Yet, there are inadequate amount of researches on intermediate *Leptospira* species despite their vital role in causing disease outbreak (Thayaparan *et al.*, 2015).

The ability of *Leptospira* species to survive for long periods in warm and high humidity environment had led to the extremely high occurrence of leptospirosis in tropical regions as compared to temperate regions (Levette, 2001). In Malaysia, the first fatal case of leptospirosis was reported in 1925 (Fletcher as cited in Benacer *et al.*, 2013). The circumstances of having warm weather and heavy rainfall throughout the year in Malaysia provide conducive environment for the survival of *Leptospira* species (Benacer *et al.*, 2013). Recently, it was reported that from statistical analysis, the number of leptospirosis cases in Sarawak alone had increased dramatically from 49 cases in 2010 to 186 cases in 2011 (Thayaparan *et al.*, 2015). This had raised the awareness of scientists to do investigation on *Leptospira* species.

Pulsed-field gel electrophoresis (PFGE) is a molecular technique established by Centers for Disease Control and Prevention (CDC) as an alternative methodology of Cross Absorption Agglutination Testing (CAAT) due to its ability to overcome limitations faced by other serological methods (Mende *et al.*, 2013). Unlike other methodology which are applicable for the identification of *Leptospira* species up to their species level, standard PFGE method allows the determination of *Leptospira* species up to their serovars level (Galloway & Levett, 2010). Due to the close relationship between serovars with their specific hosts as well as leptospirosis severity, identification of *Leptospira* serovars is advantageous in assisting the treatment of leptospirosis (Moreno *et al.*, 2016).

In order to achieve genetic profile of *Leptospira* species which will be very useful in leptospirosis treatment, intermediate and saprophytic strains of *Leptospira* species isolated from different sources was obtained and the genetic relatedness among themselves were investigated by using PFGE method.

Recently, there is limited statistics on the current circulation of *Leptospira* species in Sarawak. Hence, information on the genetic relatedness among 10 intermediate and 10 saprophytic *Leptospira* species isolated from different geographical area in Sarawak was provided in this study. Besides, this study also suggested various modifications that can be done on the current PFGE protocol to produce an optimized PFGE running parameter that can generate distinct PFGE DNA profile for *Leptospira* species isolated from Sarawak.

The main objective of this study was:

a) To evaluate an optimized PFGE protocol for intermediate and saprophytic *Leptospira* species

b) To investigate the genetic relatedness among intermediate and saprophytic *Leptospira* strains recpectively.

2.0 LITERATURE REVIEW

2.1 Leptospirosis

Leptospirosis is a critical worldwide zoonotic disease that caused by infection of spirochetes from the genus of *Leptospira* (Benacer *et al.*, 2013). Ahmed *et al.* (2012) described that according to Leptospirosis Burden Epidemiology Reference (LERG) group that currently created by WHO, the average worldwide burden for endemic human leptospirosis of approximately an incidence of 5 per 100,000 populations was actually an underestimation as it was only reported those severe and frequently notified incidences but not included epidemic leptospirosis. As reported by the World Health Organization (WHO), there was more than one million severe human cases per year caused by this disease and with an increasing number of countries reporting its outbreak (Cosson *et al.*, 2014). Hence, various studies and researches were conducted to investigate the epidemiology of leptospirosis for better prevention of treatment to this disease.

2.2 *Leptospira* species

2.2.1 Morphology of *Leptospira* species

Leptospira species are Gram-negative bacteria which grow in corkscrew-shaped with a size of about 0.1 μm in diameter and 6 – 20 μm by length (Mohammed, Nozha, Hakim, Abdelaziz & Rekia, 2011). They are enveloped by typical double membrane structure consisting of the closely linked cytoplasmic membrane and peptidoglycan cell wall, covered by an outer membrane (Adler, 2015). Freeze fracture electron microscopy studies conducted previously had displayed that the outer membrane of *Leptospira* species constitutes of transmembrane outer membrane protein and lipoproteins. Current studies show that the outer membrane

proteins of *Leptospira* species are able to form heat-modifiable porin that present in small amount within the outer membrane, assisting in their survival in harsh condition (Cullen, Cordwell, Bulach, Haake & Adler, 2002). Besides, these outer membrane proteins also crucial in the adhesion of *Leptospira* to host tissues, contributing a crucial role in their colonization of host tissues (Barbosa *et al.*, 2006).

Meanwhile, the lipoproteins within the outer membrane of *Leptospira* species are comparatively abundant and they are associated with the outer membrane through their N-terminal fatty acids (Collen *et al.*, 2002). As stated by Palaniappan *et al.* (2001), the protein compositions within the outer membrane such as the lipoproteins of *Leptospira* species that cultured in vivo are significantly differ with those present within the *Leptospira* species found in an infected animal.

In addition, there are also numerous lipopolysaccharides found within the outer membrane. These lipopolysaccharides act as the main antigen of *Leptospira* species, contributing to their pathogenity as well as their specification (Mohammed *et al.*, 2011). These lipopolysaccharides are similar structurally and immunologically to those found in Gram negative organism (Mohammed *et al.*, 2011). Yet, these lipopolysaccharides confer relatively lower endotoxic activity as compared to other Gram negative organism (Levett, 2001). The lipopolysaccharides structure that are highly immunogenic and unique in *Leptospira* species led to the diverse serovars of *Leptospira* species (Levett, 2001).

There are also periplasmic flagella located between the outer membrane and the peptidoglycan layer (Johnson, 1996). These periplasmic flagella situated within the periplasmic space are responsible for the motility of *Leptospira* species (Picardeau, Brenot & Girons, 2001). According to Johnson (1996), due to its small size, *Leptospira* species are very

difficult to be detected under bright-field microscope but can be clearly visualized under dark-field or phase microscopy. All *Leptospira* species are morphologically indistinguishable. However, the morphology of *Leptospira* species subcultured in vivo are differ with those in the infected animal (Levett, 2001).

2.2.2 Habitat of *Leptospira* species

Leptospira species are obligate aerobic bacteria, which mean that aeration is necessary for its maximal growth (Johnson, 1996). According to Levett (2001), the optimum temperature for the growth of *Leptospira* species is 28 – 30 °C. Furthermore, *Leptospira* species can grow in alkaline condition, up to pH 8.0 (Mohammed *et al.*, 2011). They can be found either in water and soil as free-living organisms or occasionally found in mammalian hosts such as rodents (Johnson, 1996). Benacer *et al.* (2013) claimed that tropical and subtropical countries with high humidity and warm temperature throughout the years contributes an ideal condition for the survival of *Leptospira* in the environment.

However, the growth of *Leptospira* species on primary isolation is usually very time-consuming. For instance, *Leptospira* pure culture in liquid media often grow within 10 to 14 days (Levett, 2001). Agar may be added to the media at low concentration to form a semisolid media that will allow the growth of *Leptospira* species to reach its maximum density (Mohammed *et al.*, 2011). *Leptospira* cultures can be maintained by repeated subculture in similar media.

2.2.3 Classification of *Leptospira* species

In bacteriology, *Leptospira* species are grouped either by serological or genotypic classification.

2.2.3.1 Serological classification

Serovars can be defined as the agglutination reaction of the bacteria in the presence of homologous antigen (Mohammed *et al.*, 2011). Initially, *Leptospira* species was grouped through serological classification which classified according to their serological features. Till 1989, the genus *Leptospira* was divided into two species, *Leptospira interrogans* and *Leptospira biflexa* based on their metabolic characteristics (Galloway & Levett, 2008).

L. interrogans consist of all pathogenic strains which are the causative agent of leptospirosis. On the other hand, *L. biflexa* comprise of non-pathogenic or commonly known as saprophytic *Leptospira* strains which do not cause disease and can be found from environmental soil and water (Benacer *et al.*, 2013). These two *Leptospira* species were then further divided into serovars through serological classification, where grouping can be done according to their distinct serovars. Example of serological methodological involved are cross-absorption agglutination testing (CAAT) and microscopic agglutination testing (MAT) (Mende *et al.*, 2013). Serologically-related serovars that are categorized into serogroup do not exhibit taxonomic status (Ahmed *et* al., 2012). However, studies on serogroups of *Leptospira* provide an important data souce in leptospirosis epidemiology understanding.

2.2.3.2 Genotypic classification

Rapid development of molecular technologies allows the classification of *Leptospira* species according to their genotype characteristics. Molecular characterization classified Leptospira into species. Studies on genotypes of *Leptospira* had revealed that those strains with distinct serotypes may be classified under the same genetic group (Johnson, 1996).

Based on this novel genotypic classification approach, *Leptospira* species are divided into pathogenic species and intermediate species. It is found that pathogenic *Leptospira* species can involve those either with pathogenic or non-pathogenic that typically known as saprophytic serovar (Mohammed *et al.*, 2011). Intermediate *Leptospira* species are those conferring intermediate pathogenicity which are potentially to cause infections but with a lower tendency and variable clinical symptoms (Moreno *et al.*, 2016). Example of intermediate *Leptospira* species are *L. meyeri*, *L. inadai* and *L. fainei* (Mohammed *et al.*, 2011). Though the emergence of molecular technologies, it is apparently noticed that the classification of *Leptospira* through serological characteristics has no longer fully satisfactory since it may be unable to define clearly some crucial strains or isolates epidemiologically (Balamuragan *et al.*, 2013).

2.2.4 Ecology of *Leptospira*

Leptospira species can be detected in many places based on their mode of living. Saprophytic *Leptospira* such as *L. biflexa* are free living in environmental such as water and soil (Johnson, 1996). On the other hand, pathogenic *Leptospira* species require host for their survival. Example of reservoir hosts that act as the natural carrier for pathogenic *Leptospira* are rodents and domestic animals such as dogs, pigs and cattle (Priya *et al.*, 2007). As stated by Levett

(2001), survival of pathogenic *Leptospira* species in environment is limited due to various factors such as pH, temperature and the presence of inhibiting compounds. The ecology of leptospirosis is very complicated due to the complex relationship between the *Leptospira* species, animal reservoirs, human and the environment condition they coexist with (Priya *et al.*, 2007).

2.3 Source of *Leptospira* infection

Leptospirosis occurs when there is an infection by spirochetes belonging to pathogenic members from the genus *Leptospira* (Kositanont, Prasajak, Trakulsomboon, Sangjun & Phulsuksombati, 2012). Animals infected by *Leptospira* species will suffer from chronic renal infection and act as carrier animals (Galloway & Levett, 2008). Leptospirosis is considered as a zoonotic disease because rodents and other natural carriers of pathogenic *Leptospira* form an infection reservoirs that can further transmit this infection to other animals including humans (Priya *et al.*, 2007). Johnson (1996) stated that the case study of human to human transmission for leptospirosis is very low. Normally, human acquire *Leptospira* infection when they are directly or indirectly in contact with the urine of infected animals (Haake & Levett, 2015). Other uncommon modes of transmission are through contaminated water, aerosol inhalation, animal bites or sexual intercourse during convalescence (Levett, 2001).

The main portal of entry for *Leptospira* species into human are through mucosa or broken skin (Levett, 2001). After their entry inside the human body, they will be detected and removed by the immune response from blood and most of the tissue. However, some *Leptospira* species will remain and multiply in the kidney tubules due to the inefficient antibody-complement system in kidney (Johnson, 1996).

2.4 Clinical symptom of *Leptospira* species infection

The clinical symptom of human infected leptospirosis was reported to be varied in a wide range. The sign of leptospirosis may range from mild, flu-like sickness to severe illness type (or commonly known as Weil disease) which can cause high mortality, showing the symptom of jaundice, acute failure in renal and hepatic system, pulmonary difficulty as well as severe hemorrhage (Evangelista & Coburn, 2010). In *Leptospira* infection, the organ systems that are most frequently involved are central nervous system, kidneys and liver (Johnson, 1996). Unfortunately, the clinical symptoms of leptospirosis with a general febrile sign are inadequate for its diagnosis. This will eventually lead to misdiagnosed of leptospirosis as meningitis or hepatitis at its initial stage of infection (Johnson, 1996).

2.5 Pulsed-Field Gel Electrophoresis (PFGE) Method

PFGE is a newly developed technique used for the separation of larger DNA fragments (Herschleb, Ananiev, & Schwatz, 2007). Conventionally, microscopic agglutination test (MAT) and cross-agglutination absorption test (CAAT) are utilized to detect *Leptospira* species. Nevertheless, both techniques are labour-intensive and time-consuming, requiring maintenance of a wide-range reference strains and correspondent rabbit antisera (Romero, Blanco, & Galloway, 2009). As compared to traditional agarose-based DNA electrophoresis which usually resolves DNA fragments up to approximately 50 kb, PFGE method which was designed by Schwatz and Cantor in 1983, allows the separation of larger DNA molecules up to 10 Mb (Herschleb *et al.*, 2007). This is very useful in resolving large DNA fragments of *Leptospira* species, for example, *Leptospira interrogans* which are approximately 4750 kb in size (Zuerner, 1991).

Various serological and molecular techniques had been proposed for the identification of *Leptospira* species since 1980 (Turk *et al.* as cited in Miraglia *et al.*, 2013). For instance, CAAT and MAT which are utilised to diagnose and categorise *Leptospira* serovars serologically (Mende *et al.*, 2013). However, these methods have several limitations such as laborious, time-consuming and may cause misidentification of the strains (Mende *et al.*, 2013).

PFGE has been commonly applied in DNA-based typing and is considered as the 'gold standard' molecular fingerprinting technique for epidemiological researches in many bacteria species (Ribeiro *et al.*, 2009). PFGE method allows the fractionation of large DNA fragments to be done in a higher resolution compared to that of traditional agarose gel electrophoresis technique. PFGE method applied a totally different mechanism in its separation of DNA fragments.

As stated by Basim & Basim (2000), PFGE fractionates large molecules of DNA by using two alternating electric field. Unlike in conventional DNA electrophoresis which applies a constant electrical charge across a slab of agarose gel, the direction of electrical charge applied in PFGE method is altered periodically (Herschleb, Avaniez, & Schwartz, 2007). This makes PFGE method become more advantageous in the separation of large DNA fragments by preventing them to become floppy and allowing the large DNA fragments to move size-dependently. The use of electric charge with speed and changing with period allows the introduction of various types of PFGE method such as FIGE, TAFE, CHEF, OFAG, PHOGE and PACE which are also used in isolation and typing of large DNA molecules (Parizad, Parizad, & Valizadeh, 2016).

3.0 MATERIALS AND METHODS

3.1 Materials

The media and materials used in this study are listed in *Appendix* section.

3.2. *Leptospira* strains

Ten intermediate and 10 saprophytic *Leptospira* strains were obtained from the *Leptospira* culture collections at Molecular Microbiology Laboratory in UNIMAS. Table 3.1 shows the intermediate and saprophytic *Leptospira* strains used as positive control in this study. The lists of the tested intermediate and saprophytic *Leptospira* strains are shown in Table 3.2 and Table 3.3, respectively. Meanwhile, *Salmonella* serotype Braenderup and *Saccharomycess cerevisae* were used as the universal size standard in this study.

Table 3.1: Positive control of *Leptospira* tested

Type of *Leptospira* strain	**Species**	**Serovars**	**Strains**	**Source**	**Location**
Intermediate *Leptospira* (positive control)	*L. wolffii*	Khorat	Khorat-H2	Soil	Tanjung Datu National Park
Saprophytic *Leptospira (positive control)*	*L. biflexa*	Patoc	Patoc 1 (Ames)	Water	Juara National Service Training Centre