DETECTION AND MOLECULAR CHARACTERIZATION OF *Leptospira* spp. FROM ENVIRONMENTAL SAMPLES IN KELANTAN

by

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DEDICATION

To my late father.
To my mother, my siblings and the whole family.
To my teachers, lecturers and professors.

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Alhamdulillah, all praise is due to Allah, the Sustainer of the whole world. This thesis wouldn't be possible without his mercy and guidance. Shalawat and Salam are addressed to the beloved and the last messenger, the prophet Muhammad SAW, his Family, his Companions and all those who follow them sincerely until the Day of Judgement.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
µg	microgram
µm	micromole
µM	microMolar
µl	microliter
>	more than
<	less than
≥	more than equal to
≤	less than equal to
°C	degree Celcius
bp	base pair
AKI	acute kidney injury
AP-PCR	arbitrarily primed polymerase chain reaction
AUFI	acute undifferentiated febrile illness
BLAST	basic local alignment search tool
Ca^{2+}	calcium
CSTE	Council of State and Territorial Epidemiologists
DNA	deoxyribonucleic acid
D-HPLC	denaturing high pressure liquid chromatography
dNTP	deoxynucleotide triphosphate
ECDC	European Centre for Disease Prevention and Control
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
et al.	*ET alii*
GA	Georgia
GI	genomic island
g	gram
gyrB	gyrase subunit B

HRPZ	Hospital Raja Perempuan Zainab
HGT	horizontal gene transfer
HUSM	Hospital Universiti Sains Malaysia
HVHF	high-volume hemofiltration
IgG	immunoglobulin G
IgM	immunoglobulin M
IS	insertion sequences
kb	kilo base pair
LAMP	loop mediated isothermal amplification
ligA	leptospiral immunoglobulin-like A
ligB	leptospiral immunoglobulin-like B
lipl32	lipoprotein 32
lipl41	lipoprotein 41
loa22	outer membrane protein A-like 22
Lsa23	leptospiral surface adhesions 23
Lsa26	leptospiral surface adhesions 26
Lsa36	leptospiral surface adhesions 36
MAT	microscopic agglutination test
mg	miligram
$MgCl_2$	magnesium chloride
min(s)	minute(s)
ml	millilitre
MLVA	multi locus variable number of tandem repeats analysis
MOH	Ministry of Health
MPKB-BRI	Majlis Perbandaran Kota Bharu-Bandaraya Islam
MLST	multiple locus sequence typing
MST	multispacer sequence typing
MUSCLE	multiple sequence comparison by log-expectation
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
nm	nanometer
OmpL1	outermembrane protein L1

PAI	pathogenicity island
PBS	phosphate buffered saline
PDR	People’s Democratic Public
PFGE	pulsed-field gel elctrophoresis
PRDH	Puerto Rico Department of Health
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
REA	restriction endonuclease analysis
rpm	revolutions per minute
secY	main transmembrane subunit of the eubacterial Sec
rRNA	ribosomal ribonucleic acid
Taq	*Thermos aquaticus*
TAS	toxin-antitoxin systems
TBE	tris borate EDTA
UPGMA	unweighted pair group method with arithmetic mean
US	United States
VNTR	variable number of tandem repeats
Vol	volume
WGS	whole genome shotgun
WHO	World Health Organization
x	times

PENGESANAN DAN PENCIRIAN MOLEKUL KE ATAS *Leptospira* spp. DARIPADA SAMPEL ALAM SEKITAR DI KELANTAN

ABSTRAK

Leptospirosis adalah penyakit global yang berlaku di seluruh benua kecuali antartika. Di Malaysia, *Leptospira* spp. dikesan daripada manusia, ternakan, sampel alam sekitar dan haiwan pengerat. Spesies saprofitik biasanya dikaitkan dengan alam sekitar. Walau bagaimanapun, satu spesies patogenik yang baru, *Leptospira kmetyi*, telah diisolasikan dari tanah di Malaysia. Oleh itu, kajian ini bertujuan untuk mengisolasi *Leptospira* spp. dari tanah dan air di kawasan persekitaran terpilih, mengesan isolat patogenik dan menentukan perkaitan genetik dikalangan isolat tersebut.

Kajian ini adalah satu kajian keratan rentas deskriptif. Sampel air dan tanah telah diambil dari kawasan pasar dan pusat rekreasi terkenal di Kelantan. Kesemua sampel ditapis dan diinokulasi ke dalam medium Ellinghausen dan McCullough yang telah dimodifikasi dan ditambah dengan 5-fluorouracil. Kultur tersebut diinkubasi pada suhu 30°C selama 30 hari dan diperiksa di bawah mikrsoskop latar belakang gelap. Ujian aglutinasi mikroskopik (MAT) telah dijalankan untuk menentukan serovar kultur positif. PCR kemudian dijalankan ke atas kultur positif menggunakan primer G1/G2, B64-I/B64-II and Sapro1/Sapro2. Kehadiran gen virulen juga telah ditentukan. Sebahagian jujukan gen 16S RNA bagi isolat telah didapatkan untuk pengecaman isolat secara molekul. Analisis filogenetik juga dijalankan untuk menentukan perkaitan genetik antara isolat.

Sejumlah 144 sampel yang terdiri daripada tanah (pasar, n=36; pusat rekreasi, n=36) dan air (pasar, n=36; pusat rekreasi, n=36) telah diambil. Berdasarkan pemerhatian mikroskopik latar belakang gelap, kultur air dan tanah adalah positif terhadap *Leptospira* spp. masing-masing sebanyak 10% and 36%. Kesemua isolat adalah negatif bagi serum hiperimmun yang duji dalam MAT. Sebanyak 18 daripada 33 kultur positif memberikan keptusan positif terhadap esei menggunakan primer G1/G2 and B64-I/B64-II. Gen *LipL32* tidak dikesan pada kesemua isolat. Keptusan jujukan 16S rRNA menunjukkan 31 daripada 33 isolat dikenal pasti sebagai *Leptospira* spp. Spesies ini terdiri daripada satu spesies patogenik, *Leptospira alstonii* dan lapan spesies pertengahan, *L. wolffii* (n=7), dan *L. licerasiae* (n=1). Dua puluh dua isolat telah dikenal pasti sebagai spesies bukan patogenik, *L. meyeri*. Dua lagi spesies telah dikenal pasti sebagai spesies daripada genus yang berkait rapat, *Leptonema illini.* Berdasarkan analisis filogenetik, isolat *Leptospira* jelas terpisah untuk membentuk tiga cabang iaitu patogenik, pertengahan dan bukan patogenik.

Sebagai kesimpulannya, hanya satu spesies patogenik, *L. alstonii* telah diisolasikan daripada kawasan terpilih di Malaysia. Isolat yang lain adalah kumpulan pertengahan dan saprofitik. Kesemua isolat didapati tidak mempunyai salah satu gen *Leptospira* yang terpelihara dan dianggap virulen, gen *lipL32*.

DETECTION AND MOLECULAR CHARACTERIZATION OF *Leptospira* spp. FROM ENVIRONMENTAL SAMPLES

ABSTRACT

Leptospirosis is an important worldwide zoonotic disease caused by pathogenic leptospires. In Malaysia, *Leptospira* spp. have been detected in humans, livestock, environmental samples and rodents. Saprophytic species was usually associated with the environment. However, a novel pathogenic species, *Leptospira kmetyi* has been isolated from the soil in Malaysia. Therefore, the aim of this study is to isolate *Leptospira* spp. from the soil and water in selected environment, to detect the pathogenic isolates and to determine their genetic relationship.

This is a cross-sectional descriptive study. Soil and water samples were collected from well known markets and recreational areas in Kelantan. All samples were filtered and inoculated into modified Ellinghausen and McCullough medium supplemented with additional 5-fluorouracil. The cultures were incubated at 30°C for 30 days and examined under dark field microscope. Microscopic Agglutination Test (MAT) was performed to determine the serovar of the positive cultures. Positive cultures were then subjected to PCR using G1/G2, B64-I/B64-II and Sapro1/Sapro2 primers. The presence of virulence gene *lipL32* was also determined. Partial sequences of 16S rRNA gene of the isolates were obtained for molecular identification of the isolates. Phylogenetic analysis was carried out to determine the genetic relatedness among isolates.

A total of 144 samples comprised of water (market, n=36; recreational area, n=36) and soil (market, n=36; recreational area, n=36) were collected. Based on dark

field microscopic observations, 10% water and 36% soil cultures were positive for *Leptospira* spp. All isolates were negative for the hyperimmune sera tested in MAT. A total of 18 out of 33 cultures gave positive PCR assay results using G1/G2 and B64-I/B64-II primers. *LipL32* gene was not detected in all of the isolates. 16S rRNA sequencing results showed that 31 out of 33 isolates were identified as *Leptospira* spp. There were one pathogenic species, *Leptospira alstonii* and eight intermediate species, *L. wolffii* (n=7), and *L. licerasiae* (n=1). Twenty two isolates were identified as nonpathogenic species, *L. meyeri*. The remaining two isolates were identified as species from other closely related genus, *Leptonema illini*. Based on phylogenetic analysis, the leptopsiral isolates were clearly separated to form three major clades namely pathogenic, intermediate and nonpathogenic clades.

In conclusion, only one pathogenic leptospires, *L. alstonii* was isolated from environment in selected areas in Malaysia. The remaining isolates were intermediate and saprophytic groups. All isolates were found not to contain one of the highly conserve putative leptospiral virulence gene *LipL32*.

CHAPTER 1

INTRODUCTION

1.1 Biology of *Leptospira* spp.

1.1.1 Morphology

Leptospires are very thin, helically coiled, about 0.1 μm in diameter by 6–20 μm in length, and highly mobile (Ko *et al.*, 2009; Adler and de la Peña Moctezuma, 2010; Evangelista and Coburn, 2010). The leptospires differ from other spirochaetes on the basis of their unique hook or question mark-shaped ends (Ko *et al.*, 2009).

1.1.2 Distribution in soil and water

Leptospira, particularly saprophytic species, are most abundant in soil with high moisture and organic matter content (Henry and Johnson, 1978). Pathogenic species are shed from the tubules of infected animals into the environment (Levett, 2001). The pathogenic leptospires can survive in low nutrient environment with salt concentration, pH and viscosity as critical factors (Evangelista and Coburn, 2010). Several mechanisms such as cell aggregation (Trueba *et al.*, 2004), interaction with other bacteria and biofilm formation (Barragan *et al.*, 2011) ensure the survival of this bacteria for weeks or even months outside their hosts (Ko *et al.*, 2009).

1.1.3 Animal carriers

In most cases, each serovar of *Leptopsira* is confined to certain mammalian hosts. For examples, rats are maintenance hosts for serovars of the serogroups lcterohaemorrhagiae and Ballum, mice are the maintenance hosts for serogroup Ballum, dairy cattle may harbor serovars hardjo, pomona, and grippotyphosa, pigs

may harbor pomona, tarassovi, or bratislava, sheep may harbor hardjo and pomona, and dogs may harbor canicola (Levett, 2001). However, the association is not absolute and have no basis even at molecular level (Adler and de la Peña Moctezuma, 2010).

1.2 Taxonomy and classification

Leptospira spp. belongs to the phylum Spirochaetes (Ko *et al.*, 2009). The genus *Leptospira* includes both saphrophytic and pathogenic species (Levett, 2001). To date, at least 13 species of pathogenic *Leptospira* have been identified namely *L. alexanderi, L. alstonii*, *L. borgpetersenii*, *L. inadai, L. interrogans, L. fainei, L. kirschneri*, *L. licerasiae, L. noguchi, L. santarosai, L. terpstrae*, *L. weilii*, *L. wolffii*, with more than 260 serovars and it was expected that additional new species exist (Adler and de la Peña Moctezuma, 2010). Saprophytic species of *Leptospira* include *L. biflexa, L. meyeri, L. yanagawae, L. kmetyi, L. vanthielii*, and *L. wolbachii*, and more than 60 serovars have been reported (Levett, 2001; Adler and de la Peña Moctezuma, 2010).

1.3 Leptospiral Genomics

1.3.1 Genome sequences

Complete genome sequences of standard quality for seven strains of *L. interrogans, L. borgpetersenii* and *L. biflexa* are available in GenBank (NCBI, 2013). Currently, many strains of *Leptospira* spp. have been sequenced using whole genome shotgun (WGS) techniques producing standard or high quality drafts (NCBI, 2013). More high quality sequences are expected to be published with the lower cost of genome sequencing.

1.3.2 Mobile Genetic Elements

Genetic changes in *Leptospira* occur by transmission of mobile genetic elements such as prophages, transposons, insertion sequences (IS) elements, plasmids and genomic islands (Lehmann *et al.*, 2014). This transmission may promote differences among *Leptospira* strain and introduce virulence factors.

The term prophage introduced by André Lwoff, is phage DNA integrated stably into the host chromosome through lysogenic cycle (Wilson, 2006;Wang *et al.*, 2010). Prophages have been detected in *L.interrogans*, *L. licerasiae* and *L. biflexa* (Bourhy *et al.*, 2005; Qin *et al.*, 2008; Ricaldi *et al.*, 2012). In *L. biflexa*, prophage is identified as extrachromosomal circular replicon (Bourhy *et al.*, 2005) and it shares homology with one prophage found in *L. licerasiae* strains VAR010^{T} and MMD0835 (Ricaldi *et al.*, 2012).

Toxin-antitoxin systems (TASs) are one of the mobile genetic elements of prokaryotic organisms that spread via plasmid-mediated horizontal gene transfer (HGT) (Van Melderen, 2010). The systems were associated with several functional roles such as gene regulation (Gerdes, 2000), programmed cell death (Engelberg-Kulka *et al.*, 2006) and anti-phage activity (Pecota and Wood, 1996; Hazan and Engelberg-Kulka, 2004). Non-functional roles proposed include noncoding DNA and chromosomal remnants from transposons and bacteriophages (Lehmann *et al.*, 2014). In *L. licerasiae*, 36% of the putative type II TASs was found to reside in putative genomic islands (Ricaldi *et al.*, 2012). Genomic islands (GI) are clusters of genes derived from horizontal gene transfers (Langille *et al.*, 2008) while GI that harbour virulence genes and present only in pathogenic strain or species are known as pathogenicity islands (PAI) (Hensel, 2006).

Transposons are transposable DNA segments within genome of organism (Wilson, 2006). Insertion sequence is the simplest type of transposon which only code for protein required for transposition and usually smaller than others (Lehmann *et al.*, 2014). At least 15 insertion elements were recognized in *Leptospira* and majority of them were found in pathogenic group (Bulach *et al.*, 2006a). While some insertion elements were found in intergenic regions and have no mutagenic effects (Nascimento *et al.*, 2004), others bring about some genetic changes. It was reported that the insertion and subsequent recombination of IS*1533* distort the single crc-like gene in *L. borgpetersenii* (Bulach *et al.*, 2006b).

Recent findings reported the presence of two circular plasmids in *Leptospira interrogans* Serogroup Canicola Serovar Canicola Strain Gui44 (Zhu *et al.*, 2014).

1.4 Epidemiology of Leptospirosis

Leptospirosis occurs in every part of the world except Antarctica (Pappas *et al.*, 2008; Adler *et al.*, 2011). It is one of the emerging infectious disease in the world as evidenced by large outbreaks in several countries such as Nicaragua, Brazil, the Asia-Pacific countries and the United States (Levett, 2001; Victoriano *et al.*, 2009). Classically, it has been classified as an occupational acquired disease in which vulnerable groups such as rice field workers, fish farmers, soldiers, veterinarians and sewer workers are infected through accidental contact with infected animals, their urine or contaminated environments (Levett, 2001; Tansuphasiri *et al.*, 2006; Stern *et al.*, 2010). In recent years, recreational exposures are also associated with the outbreak of this disease (Morgan *et al.*, 2002; James *et al.*, 2003; Sejvar *et al.*, 2003; Narita *et al.*, 2005; Stern *et al.*, 2010).

Although it is the global infectious disease, the warm climate regions were recorded with higher incidence of the disease compare to other parts of the world

(Bandara *et al.*, 2014). This could be related to the extended survival of the *Leptospira* spp. in warm and humid environment (Levett, 2001). Furthermore, majority of the countries in the regions are developing countries in which normal daily activities, over-crowding and poor sanitation expose people to the infection (Victoriano *et al.*, 2009). This zoonotic disease is not exclusive to human being but it also occurs in domestic animals such as cattle, buffaloes (Bahaman *et al.*, 1987; Bahaman *et al.*, 1988) and farmed deer (Ayanegui-Alcerreca *et al.*, 2007; Ayanegui-Alcerreca *et al.*, 2010).

Leptospirosis was removed from the United States (US) list of nationally reportable infectious diseases in 1995 and has been reinstated in 2012 because the incidence appears to be increasing; new risk groups have been identified; there is potential for outbreaks to go unrecognized; and the technology for detection, diagnosis and treatment of leptospirosis are no longer limited (Katz *et al.*, 2011; CSTE, 2012; García and Stobierski, 2012; Traxler *et al.*, 2014). Pet owners are at risks because the number of positive *Leptospira* microscopic agglutination tests for 23,005 dogs significantly increased from 2002 to 2004 (Moore *et al.*, 2006). It was reported that during 1998–2009 in the United States, the average annual rate of leptospirosis-associated hospitalizations was 0.6 hospitalizations per 1,000,000 populations (95% CI 0.5–0.6) (Traxler *et al.*, 2014). The incidence rates ranged from 0.02 to 0.05 per 100,000 population from 1974 through 1994 (Katz *et al.*, 2011). In Hawaii, the number of confirmed cases reported per year ranged from 11 to 27 (median 20), and the estimated mean annual incidence rate was 1.63 per 100,000 population (Katz *et al.*, 2011).

Leptospirosis cases in patient with initial diagnosis of dengue were reported in Mexico (Dircio Montes Sergio *et al.*, 2012). Furthermore, coexistence between

leptospirosis and dengue agents was reported in the state of Veracruz where 85% of individuals who were leptospira positive were also dengue positive (Navarrete-Espinosa *et al.*, 2006). Human leptospirosis is a reportable disease in Puerto Rico and approximately 15–100 cases of suspected leptospirosis were reported to the Puerto Rico Department of Health (PRDH) each year during 2000–2009 (Rivera *et al.*, 2012). In Trinidad and Tobago, veterinary students and other university students were exposed to *Leptospira* infection as shown by serological test (James *et al.*, 2013). In an outbreak among canyoning participant on 27 June 2011 on the Caribbean island of Martinique, two gendarmes were diagnosed with leptospirosis using quantitative real-time polymerase chain reaction (qPCR) (Hochedez *et al.*, 2013).

A total of 526 confirmed cases of leptospirosis were reported in 2011 by 27 European countries, giving an overall case rate of 0.11 per 100 000 population (ECDC, 2013). These numbers show that leptospirosis remains a rare disease in Europe. The highest number of cases were notified from Romania but the number was decreasing from 0.84 per 100,000 inhabitants in 2010 to 0.46 per 100 000 inhabitants in 2011 (Pappas *et al.*, 2008; ECDC, 2013). Leptospirosis were found to be endemic in South-East Austria where an autochthonously acquired infection rate of 1.26 per 100,000 inhabitants per year was reported from 2004–2012 (Hoenigl *et al.*, 2014). In 2006, an outbreak occurred among triathlon participants in Germany and 5 confirmed cases were reported (Brockmann *et al.*, 2010).

A leptospirosis epidemic took place from 30th November 2006 through 3rd January 2007 in a rural area of Tala-Athmane, Algeria which led to the hospitalization of 48 patients due to the proximity of homes to two uncontrolled garbage dumps invaded by rodents (Afiri *et al.*, 2013). Study in urban areas in Kenya

showed that 41 (18.3%) of 224 rodents carried *L. interrogans* and *L. kirschneri* in their kidneys (Halliday *et al.*, 2013). It was reported that the overall incidence of leptospirosis estimated from 75-102 cases per 100,000 persons annually in Kilimanjaro Region of Tanzania (Biggs *et al.*, 2013). It was found that municipalities with lower socioeconomic status were identified with the highest leptospirosis rates in Nicaragua because of greater unsatisfied basic needs for quality of the household and for sanitary services, and higher extreme poverty and illiteracy rates (Bacallao *et al.*, 2014).

Leptospirosis is endemic in New Zealand and Australia (Vickery *et al.*, 2006). A study to determine association between the abundance and leptospiral status of rodents with colonies of fruit bats (*Pteropus conspicillatus* spp.) in Australia had found potential pathway for transmission of leptospires from fruit bats to rodents, via rodent contact with infectious fruit-bat urine (Tulsiani *et al.*, 2011). In molecular epidemiology study of *L. borgpetersenii* Serovar Arborea isolated in Queensland, 1998–2005, clonality among rodent isolates from geographically related areas was reported (Slack *et al.*, 2010). A summer of natural disasters in Queensland 2011 had caused the emergence of this species as the dominant infecting serovar in Australia (Wynwood *et al.*, 2014). In the Waikato region of New Zealand, it was reported that leptospiral infection was highly associated with people who had exposure to animals through their occupation (97%) and dry stock cattle farmers are at the highest risk (Cowie and Bell, 2012). A total of 207 hospitalisations were reported in New Zealand from 2003 to 2005 (Vickery *et al.*, 2006).

Norway rats (*Rattus norvegicus*) are the important reservoirs of leptospirosis in urban areas of Tokyo, Japan (Koizumi *et al.*, 2009a). Several cases of Weil's diseases were reported from those areas (Kokudo *et al.*, 2009; Inoue *et al.*, 2010).

Imported animals such as African dormice (*Graphiurus murinus*), raccoons (*Procyon lotor*) and southern flying squirrels (*Graucomys volans*) were also reported as new reservoirs for *Leptospira* in Japan (Masuzawa *et al.*, 2006; Yanagihara *et al.*, 2007; Koizumi *et al.*, 2009b). The number of leptospirosis cases in Japan decreased dramatically after 1960 because of the modernization of the agriculture, introduction of inactivated vaccine against *Leptospira* which was applied for humans and good maintenance of infrastructures such as water works and sewage systems (Yanagihara *et al.*, 2007). Some of the cases were imported from countries such as Vietnam, Thailand, Indonesia and Malaysia (Yanagihara *et al.*, 2007; Mishima *et al.*, 2013).

Leptospirosis in India is commonly associated with natural disasters such as flood following heavy rainfall during the monsoon season (Victoriano *et al.*, 2009). A cross sectional study in India showed that the average positive samples was high during the monsoon season (402 cases, 46.25%) with the common serovars were *L. australis* (36.76%), *L. canicola* (30%), *L. autumnalis* (14.57%), *L. icterohaemorrhagiae* (12%), *L. patoc* (4.68%) and *L. grippotyposa* (1.87%) (Arumugam *et al.*, 2011). A high prevalence of leptospirosis in rodents and dogs in Mumbai proves possible role of these animals in transmission of leptospires to humans (Patil *et al.*, 2014).

An outbreak of leptospirosis occurred in Metro Manila, the Philippines, following a typhoon in September 2009 where 471 patients were hospitalized and 51 (10.8%) died (Amilasan *et al.*, 2012). Patients were presented with fever (98.5%) myalgia (78.1%), malaise (74.9%), conjunctival suffusion (59.3%), oliguria (56.6%), diarrhea (39%), and jaundice (38%) while the severe form of disease identified were renal failure (82%), pulmonary hemorrhage (8%), meningitis (5%), and myocarditis (4%) (Mendoza *et al.*, 2013). *Leptospira* isolates from rats in the Philippines are

highly virulent, causing pulmonary haemorrhage, severe hepato-renal damage and death as shown in hamsters (Villanueva *et al.*, 2014b). Population are at risk because it was reported that the same serovars were isolated from both rats and humans suggesting the transmission of the serovars from rats to human (Villanueva *et al.*, 2014a). In Leyte, Philippines, *Leptospira* were isolated from coastal soil after a storm surge during Super Typhoon Haiyan (Yolanda) and these isolates were shown to survive in seawater for four days suggesting their tolerance to salinity (Saito *et al.*, 2014).

Two prospective-hospital based studies were conducted amongst adult patients admitted to Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima Province, Thailand between July 2001 to December 2002 and between July 2011 to December 2012 found that leptospirosis is one of the major causes of acute undifferentiated febrile illness (AUFI) (Thipmontree *et al.*, 2014). Low prevalence of pathogenic (0.9%) and intermediate (5.5%) *Leptospira* in floodwater during the 2011 floods in the Bangkok Metropolitan Region was reported and it was consistent with the low number of human leptospirosis cases reported to the Bureau of Epidemiology in Thailand (Thaipadungpanit *et al.*, 2013).

In a cross-sectional seroprevalence study conducted in rural villages in Khammouane Province, Lao People's Democratic Republic, in December 2006, 23.9 % out of 406 subjects were positive for antibodies against *Leptospira* (95% confidence interval [CI]: 19.7–28.1%) with agglutination titers ranging from 1:100 to 1:800 (Kawaguchi *et al.*, 2008).

L. interrogans and *L. borgpetersenii* species are widely distributed amongst rodent populations in Thailand, Lao PDR and Cambodia and the roles of the rodents

as reservoirs for human leptospirosis were confirmed through strain typing (Cosson *et al.*, 2014).

In Malaysia, leptospirosis has been described since the British Occupation with the earliest report in 1925 (El Jalii and Bahaman, 2004). Leptospirosis is an endemic disease in Malaysia with incidence and mortality rates of 12.49 and 0.16 in 100,000 population, respectively (Mun, 2013; MOH, 2014). As of 24 August 2013, a total of 2,925 leptospirosis cases have been detected and caused 28 deaths (Mun 2014). *Leptospira* spp. have been detected from humans (El Jalii and Bahaman, 2004), livestock (Bahaman *et al.*, 1987; Bahaman *et al.*, 1988), environmental samples (Slack *et al.*, 2009) and rodents (Mohamed-Hassan *et al.*, 2010) in Malaysia. It was reported that 37 out of 250 identified serovars worldwide were isolated from Malaysia (El Jalii and Bahaman, 2004). Among the earliest studies of environmental pathogenic leptospira in Malaysia was in 1974 where 13 serogroups comprising a total of 29 serovars were isolated from surface waters (Alexander *et al.*, 1975). Pathogenic species were also detected in Kelantan and Terengganu environments (Ridzlan *et al.*, 2010). Current study reported isolation of pathogenic *Leptospira* from urban sites (Benacer *et al.*, 2013). A novel species, *Leptospira kmetyi*, was also isolated successfully from the Malaysian soil (Slack *et al.*, 2009).

In Kelantan, the number of leptospirosis cases increased threefold following massive flood during December 2015 (Yahaya, 2015). The Raja Perempuan Zainab II Hospital (HRPZ II) handled 187 leptospirosis cases from 1 to 26 January 2015 (Hasni, 2015). There were also several cases reported upon visits to recreational areas contaminated with leptospires. A total of 10 patients acquired leptospirosis after visiting Jeram Pasu, Pasir Puteh and eight patients acquired the disease after visiting Jeram Linang, Machang (Hanim, 2014).

1.5 Leptospirosis the Disease

1.5.1 Clinical presentations

Leptospirosis can take many forms in humans from mild, influenza-like illness to severe forms such as multiorgan failure (Evangelista and Coburn, 2010). The most frequent symptoms of mild leptospirosis are fever, headache, myalgia, arthralgia, vomiting, anorexia, gastrointestinal upset, cough and nausea (Jaureguiberry *et al.*, 2005; Lin *et al.*, 2008; Forbes *et al.*, 2012). These symptoms occur in the first phase of the disease and lasting about a week followed by immune phase (Levett, 2001). Most severe presentations were developed in the second phase of the disease or it may simply present as a single phase illness (Bharti *et al.*, 2003). Severe leptospirosis represented by jaundice, hepatomegaly, generalised vasculitis, meningoencephalities, pulmonary haemorrahgae, acute renal failure (or acute kidney injury (AKI)), liver failure and rhabdomyolysis (Bharti *et al.*, 2003; Forbes *et al.*, 2012). These symptoms are collectively known as Weil's disease (McBride *et al.*, 2005; Forbes *et al.*, 2012). Weil's disease occurs more often in adolescent than in children patients (Guerrier *et al.*, 2013). Severity of the disease is determined by several factors which include the infecting serovar, and the age, health and immunological competence of the patient (Adler and de la Peña Moctezuma, 2010). Mortality from leptospirosis was high in adults and adults with AKI (Daher *et al.*, 2014).

Leptospirosis changes several haematological parameters in infected patient. It was reported that serum nitrite levels were significantly higher in patients with acute leptospirosis [n=20, (0.359±0.229) μM] compared to controls [(n=13, (0.216±0.051) μM] (P=0.014) (Gunaratna *et al.*, 2012). Mean haemoglobin and haematocrit levels were significantly lower in patients with severe leptospirosis

compared to mild disease from day 3 to day 10 of illness ($p < 0.001$) while total white cell and neutrophil counts were significantly higher in patients with severe leptospirosis from day 3 to day 8 of illness ($p < 0.05$) (De Silva *et al.*, 2014). Lower gastrointestinal bleeding was reported in severe leptospirosis patient caused by coinfection with *Entamoeba histolytica* which was detected by histological examination (Legris *et al.*, 2014).

1.5.2 Pathology

A common pathological finding in leptospirosis is hemorrhagic voluminous lungs covering the heart almost completely (Chakurkar *et al.*, 2008). Haemorrhage in other organs like the heart, gastrointestinal tract, brain, pancreas and adrenals were also reported (Salkade *et al.*, 2005). A mottled appearance with a red and yellow colour of the liver, typical of "acute yellow liver atrophy", was observed at autopsy in patient with Weil's disease (Shintaku *et al.*, 2014).

1.5.3 Treatment

It was reported that doxycycline intake of 200 mg oral doxycycline/week is effective against *Leptospira* (Sejvar *et al.*, 2003). Lower dosage is inadequate to prevent leptospirosis in an endemic country such as Malaysia (Mortimer, 2005). Leptospirosis patient with multiple organ failure regain hepatic and liver function after plasma exchanges and high-volume hemofiltration (HVHF) (Bourquin *et al.*, 2011).

1.6 Pathogenesis

Leptospires gain entry into the body via cuts and abrasions or nasal, oral and conjunctiva mucosa during immersion in water for a long period of time (Lim *et al.*, 2011; Adler, 2014). Efficient dissemination of *L. interrogans* in bloodstream is

established through binding of extracellularly excreted enolase to plasminogen of the host (Nogueira *et al.*, 2013). Adhesions to plasminogen are also facilitated by proteins known as leptospiral surface adhesions 23, 26, and 36 (Lsa23, Lsa26, and Lsa36) (Siqueira *et al.*, 2013). Pathogenic strain may also adhere to macrophage to enhance their uptake in the host (Toma *et al.*, 2014). The macrophage are then induced to death by elevation of intracellular free Ca^{2+} ($[Ca^{2+}]$ i) (Zhao *et al.*, 2013). Leptospiral invasion and toxicity of the outer membrane lead to the generation of cytokines, chemokines, and inflammatory cell infiltrations in host which result in severe organ dysfunctions (Praditpornsilpa *et al.*, 2006). In case of jaundice, the invasion of the intercellular junctions of host hepatocytes disrupted the junction causing bile leaks from bile canaliculi (Miyahara *et al.*, 2014).

1.7 Virulence Genes

Leptospiral outer membranes are primarily building of lipopolysaccharide, surface-exposed lipoproteins and transmembrane proteins (Haake and Matsunaga, 2010). The arrival of the transposon mutagenesis systems has identified a small number of leptospiral genes encoding surface protein shown to be required for virulence such as *ligA, ligB, Loa22* and *lipL32* (Adler, 2014).

The expression of both *ligA* and *ligB* genes of the *L. interrogans* in the saprophyte *L. biflexa* serovar Patoc significantly enhanced the ability of transformed *L. biflexa* to adhere in vitro to extracellular matrix components and cultured cells (Figueira *et al.*, 2011). Mutation of *ligB* alone in *L. interrogans* does not affect leptospiral virulence in hamster model (Croda *et al.*, 2008). However, the roles of these proteins in pathogenesis are not easy to understand because *ligA* is unique to *L. interrogans* and *L.kirsheneri* while *ligB* is found in all pathogenic strains (McBride *et al.*, 2009).

The gene encoding Loa22 is the only gene found to date that fulfils Koch's molecular postulates for a virulence factor (Ko *et al.*, 2009). The expression of *Loa22* gene is found to be significantly increased during acute infection in guinea pigs (Nally *et al.*, 2007). Disruption of this gene also resulted in loss of virulence (Ristow *et al.*, 2007). However, this protein is not specific for pathogenic strains. It had an ortholog in *L. biflexa* (Picardeau *et al.*, 2008). Even though the gene coding *Loa22* is not expressed in nonpathogenic species (Ristow *et al.*, 2007), it is not suitable to use this gene as PCR marker.

LipL32 is the major leptospiral outer membrane protein. It makes up 75% of the outer membrane proteome (Cullen *et al.*, 2002). The *lipL32* gene consists of 816 bases and it encodes a protein of 272 amino acids (Haake *et al.*, 2000). This gene is highly conserved among the main pathogenic species with an average DNA sequence identity of 96.4% (Haake *et al.*, 2000). This feature makes the gene coding for this protein suitable candidates for PCR marker though the role of this outer membrane protein remains unclear because leptospiral virulence is retained in LipL32-mutant strain (Murray *et al.*, 2009).

1.8 Laboratory Diagnosis

1.8.1 Isolation of *Leptospira* spp.

Leptospires are isolated from clinical samples such as blood, serum, urine, kidney, tissues and fluids from aborted fetus (Balamurugan *et al.*, 2013). Water samples usually collected from pools of water in places such as markets, roadsides, living areas and agriculture sites (Ganoza *et al.*, 2006; Saito *et al.*, 2013). Other studies also reported the collection of water from drains, lakes, swamps and rivers (Benacer *et al.*, 2013; Saito *et al.*, 2013). For soil samples, normally, about 15 - 20 g of topsoil is

collected from wet and shaded areas where the presences of rodents were suspected (MOH, 2011; Benacer *et al.*, 2013; Saito *et al.*, 2013). Water samples and soil samples mixed with distilled water are passed through 0.45 µm, 0.22 µm or 0.2 µm filters (Ridzlan *et al.*, 2010; Benacer *et al.*, 2013; Saito *et al.*, 2013).

1.8.2 Culture methods

Long chain fatty acid is the only carbon sources utilize by leptospires (Levett, 2001; Adler and de la Peña Moctezuma, 2010). They are slow-growing obligate aerobes bacteria with an optimal growth temperature of 28 - 30°C (Levett, 2001; Ko *et al.*, 2009; Adler and de la Peña Moctezuma, 2010; Evangelista and Coburn, 2010). The growth of leptospires in synthetic media is supported by vitamins B1 and B12, long-chain fatty acids, and ammonium salts (Levett, 2001). Currently, many laboratories use oleic acid-albumin medium EMJH because of greater yield and faster growth (Budihal and Perwez, 2014). Leptospires have been cultured successfully in liquid, solid and semisolid media. It is cultured in liquid media containing rabbit serum as described by Fletcher, Korthoff, Noguchi and Stuart (Adler and de la Peña Moctezuma, 2010). Media solidified with 0.8 - 1% agar will support subsurface colonies whilst media with 1.2- 2% agar support surface colonies (Adler and Faine, 2006). Agar may also be added at lower concentration, 0.1 - 0.2%, to make semisolid media in which leptospires form Dinger's disk (Levett, 2001; Zuerner, 2005).

1.8.3 Serological and genotypic tests

ELISA is widely used for the diagnosis of leptospirosis because of its minimal training (Picardeau *et al.*, 2014). This test uses antigen preparation from leptospiral sonicates or recombinant protein such as LipL32, LigA, or OmpL1 (Adler and de la Peña Moctezuma, 2010). ELISA detects IgM antibodies which occurred during the

first week of illness as compared to IgG which is detectable after 7 days and this allowed the treatment to be initiated at the most effective stage (Levett, 2001).

Microscopic Agglutination Test (MAT) which is previously known as the agglutination-lysis test remains as the gold standard for the detection of the infecting serovars (Levett, 2001). Extensive modifications on the earlier methods lead to the MAT method which can be performed and read in microtiter trays (Cole *et al.*, 1973). The end point of titre varies with geographical areas. A low titre (>1/100) in areas where leptospirosis is not endemic may indicate that the patient has leptospirosis while in endemic areas, a high titre (>400-800) is required for suspecting leptospirosis (Cerqueira and Picardeau, 2009).

It has been reported that primers (G1/G2) enabled the amplification of DNA from *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai* and *L. meyeri*, whereas the primers (B64-I/B64-II) enabled the amplification of *L. kirschneri* (Bal *et al.*, 1994), both by conventional PCR. Current studies involved the use of quantitative real-time PCR (qPCR) assays targeting the 16S rRNA (Thaipadungpanit *et al.*, 2011), *secY*, *lfb1* and *lipL32* (Bourhy *et al.*, 2011), *lipL21* and *lipL41* (Chandan *et al.*, 2010), and *gyrase subunit B (gyrB)* (Subharat *et al.*, 2011) genes. Other genotypic test for leptospires includes loop-mediated isothermal amplification (LAMP). LAMP produces stem-loop DNA structures by using loop primer (Lin et al., 2009). It was first applied to leptospires in 2008 using a set of 5 primers targeting *lipL41* gene, a gene encoding the outer membrane protein LipL41, which is conserved in pathogenic species (Shang *et al.*, 1996; Lin *et al.*, 2009). Other studies reported the use of primers targeting leptospiral *rrs*, a 16S rRNA gene (Sonthayanon *et al.*, 2011). However, this assay has low specificity as compared to *lipL41* LAMP (Koizumi *et al.*, 2012).

Every test has its own limitation. For example, the antigens used in serological tests may not cover all of the circulating strains (Picardeau *et al.*, 2014). Specificity issue with MAT was also reported (Chirathaworn *et al.*, 2014). PCR is highly sensitive particularly during initial stage of infection but it lacks the ability to identify the infecting serovar (Babic-Erceg *et al.*, 2014). Therefore, serological and genotypic tests should be combined for laboratory confirmation.

1.8.4 Molecular characterization of *Leptospira* spp.

A move away from phenotype-based typing methods towards genotype-based methods such as pulsed-field gel electrophoresis (PFGE) and PCR-based methods began in the 1990s (Cerqueira *et al.*, 2010).

Restriction endonuclease analysis (REA) is a non-sequenced method that involves the comparison of restriction profiles of either total genomic DNA or PCR products, in standard or acrylamide gels (Cerqueira *et al.*, 2010). REA using EcoRI and HaeIII enzymes gave unique restriction patterns for serovars belonging to *L. interrogans* and *L. biflexa* (Venkatesha and Ramadass, 2001). Even though the reliability of REA of total genomic DNA has been proved, there are some problems associated with this technique such as labor-intensive, the need for large volume of cultures as well as the difficulties of interpretation and interlaboratory comparisons (Cerqueira and Picardeau, 2009).

Ribotyping is established through determination of the restriction fragment length profiles of digested chromosomal DNA probed with rRNA (Cerqueira and Picardeau, 2009). Ribotyping was also combined with Southern blot (Kositanont *et al.*, 2007) and denaturing high pressure liquid chromatography (D-HPLC) (Fenner *et al.*, 2010) for typing of *Leptospira* species. This typing method is not so

discriminative because of the small number of rRNA genes (Cerqueira and Picardeau, 2009).

Pulsed-field gel electrophoresis (PFGE) is considered a powerful and reliable molecular typing method for classifying *Leptospira* strains because of its interlaboratory reproducibility, enabling standardization and data sharing between laboratories (Cerqueira and Picardeau, 2009; Mende *et al.*, 2013). Molecular serovar characterization of *Leptospira* isolates from animals and water in Colombia using pulsed-field gel electrophoresis (PFGE) identified two molecular serotypes which were not related (< 73.7% DC) to any of the 200 reference serovars (Romero-Vivas *et al.*, 2013). Even though PFGE is considered to be the gold standard for the molecular typing of *Leptospira* serovars, this method is labour-intensive and not available in most laboratories in the tropical and sub-tropical countries in which the incidence of the disease is highest (Cerqueira and Picardeau, 2009).

Leptospires were also typed based on insertion sequences (IS). IS*1500* and IS*1502* were initially found in *L. interrogans* while IS*1533* were identified in *L. borgpetersenii* (Cerqueira and Picardeau, 2009). This typing method was better than REA since it was reported that IS*1533* can differentiate between serovars and even closely serovars that were almost indistinguishable by REA (Zuerner *et al.*, 1995). Whole genome sequencing of several *Leptospira* species allow identification of more IS elements from a wide range of IS families (Cerqueira and Picardeau, 2009).

Randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) are the two methods which use low-stringency PCR with arbitrary primers to generate strain-specific fingerprints (Cerqueira and Picardeau, 2009). RAPD was used to determine the clonality of the isolates in case of laboratory-acquired leptospirosis (Sugunan *et al.*, 2004). It was reported that a 22-mer primer enhances

discriminatory power of AP-PCR fingerprinting technique in characterization of leptospires by differentiating between strains of different genospecies and strains of the same genospecies belonging to different serovars (Roy *et al.*, 2004). These techniques are simple and rapid but need an absolute standardization of experimental procedure for good reproducibility and interlaboratory comparisons (Cerqueira and Picardeau, 2009).

Whole genomes sequencing of several *Leptospira* species revealed that leptospiral genomes contain numerous repeated sequences including short repetitive DNA sequences with a structure typical of tandem repeats called Variable Number of Tandem Repeats (VNTR) (Cerqueira and Picardeau, 2009). VNTR analysis was able to determine the serogroup but not the serovar of the isolates (Forster *et al.*, 2013). Multi Locus VNTR Analysis (MLVA) can distinguish between the serovars but it requires primers specific for each species except for the phylogenetically closely related *L. interrogans* and *L. kirschneri* (Cerqueira and Picardeau, 2009).

Multispacer sequence typing (MST) was used for typing of various pathogens. The availability of genomic sequences of pathogenic strains has provided a way for MST to be applied for the typing of leptospires. Currently, MST has been done based on the sequence of several intergenic region in *L. interrogans* (Zilber *et al.*, 2014).

Other molecular typing includes Multiple Locus Sequence Typing (MLST). This method utilise two different set of housekeeping genes, *adk, icdA, lipL32, lipL41, rrs, and secY* on the one hand and *pntA, sucA, pfkB, tpiA, mreA, glmU, fadD* on the other hand (Ahmed *et al.*, 2006; Thaipadungpanit *et al.*, 2007). Combination of VNTR and MLST lead to intra-serovar discrimination, surmounted microscopic

agglutination test discrepancies and increased the discriminatory power of each technique applied separately (Caimi *et al.*, 2012).

1.8.5 Phylogenetic Analysis

Sequencing of ribosomal RNA gene was established as a standard for bacterial identification and phylogenetic relationship determination because the gene is highly conserved and hence the alignment is straightforward (Clarridge, 2004; Cerqueira and Picardeau, 2009). Even though this gene code for the 5S, 16S and the 23S rRNA, only 16S rRNA is used widely for taxonomic purposes until now (Clarridge, 2004; Morey *et al.*, 2006). Numerous 16S rRNA sequences of *Leptospira* are available in GenBank and these sequences are compared with sequences recovered from cultured organisms or environmental samples. Phylogentic analyses are carried out to determine relationship between those sequences and presented in the form of an evolutionary tree.

DNA sequences are aligned to screen for nucleotide changes among homologous sequences as a result of mutation at a specific position in a form of substitution, insertion and deletion (Röling and Head, 2004; Bromham, 2008). Insertion and deletion are collectively known as indel and they are represented as gaps in the alignment (Bromham, 2008). There are several ways to treat gaps. Complete-deletion eliminates all gaps during alignment, partial-deletion remove gap when it has a higher percentage of ambiguous and for pairwise-deletion only the gaps involved in the comparison are ignored (Tamura *et al*., 2015).

For a timely manner, searching homology of query sequences against large database such as Genbank requires a fast method. This was achieved by BLAST

alignment tool. It finds similar region between the query sequences with each sequence in the database and give scores for it.

Mulitple Sequence Alignment (MSA) is very useful in phylogenetic analysis for identifying evolutionary relationship among sequences and inferring phylogenetic trees. Multiple Sequence Comparison by Log-Expectation (MUSCLE) is one of the most common MSA used in molecular biology with an average of seven citations per day (MUSCLE, 2014). This MSA was found to be better than CLUSTALW in terms of speed and accuracy as the benchmark (Edgar, 2004).

A phylogenetic tree is built of root, branches and nodes. Branches are lines connecting internal nodes together (Bromham, 2008). The unconnected nodes are called terminal nodes or tips and they correspond to taxa (e.g species) (Rogers, 2011). The phylogenetic tree may be rooted or unrooted (Röling and Head, 2004). Root represents the most ancient nodes (Rogers, 2011). A rooted phylogenetic tree shows ancestor-descendent relationship whereas an unrooted tree shows the topology of relationship but not the pattern of descent (Lesk, 2007).

The common method used to calculate evolutionary distances is Neighbour Joining (NJ) algorithm because it was relatively simple but powerful (Silva *et al.*, 2009; Rogers, 2011; Saito *et al.*, 2013). General steps for distance methods involve estimating distances and describing these distances as a branching diagram (Bromham, 2008). Unweighted pair group method with arithmetic mean (UPGMA) is another distance method that is rarely used. The difference between these two methods is that while NJ also calculates distances based on pairs of sequence differences, it does not average the branch lengths but considers the distances between the two nodes and the next closest nodes (Rogers, 2011).

Another popular method for the construction of phylogenetic tree is maximum likelihood. This method is specific for sequence data, starting from multiple sequence alignment, an evolutionary model and an initial tree which is commonly a Neighbour Joining tree (Röling and Head, 2004; Lesk, 2007). Maximum likelihood reconstructs ancestors at all nodes by assigning branch lengths based on the probabilities of mutational evetns postulated (Lesk, 2007). Each possible tree would be compared with the initial tree to find an optimum tree which is the one with the highest likelihood of generating the observed data (Röling and Head, 2004; Lesk, 2007). Maximum likelihood is suitable for small datasets because it requires a great deal of computational power (Rogers, 2011).

The statistical validity of a tree can be tested by bootstrapping. In brief, bootstrap operated by creating a new data set of the same size from the original data matrix with replacement and constructed a tree (Röling and Head, 2004; Rogers, 2011). These processes are repeated from 500 to 1,000 times as determined by the user and the score of each branch is determined by the frequency of its occurrence in total resamplings (Rogers, 2011). High bootstrap values indicate the significance of the grouping to the right of the node (Röling and Head, 2004). Usually, bootstrap value greater than 95% is acceptable (Bromham, 2008).

MEGA is a software developed for estimating evolutionary distances, reconstructing phylogenetic trees and performing various statistical methods from DNA or protein sequences. Its first version was released in 1993 and till now six versions have been released (Kumar *et al.*, 1994; Tamura *et al.*, 2013). Mega enable users to mine sequences from the Genbank and aligned them with the DNA sequences in questions and then inferring phylogenetic trees from it. Furthermore,

users can conduct various statistical analyses to test the reliability of the trees. This software is commonly used because it is free and user friendly.

Phylogenetic analysis reveals that the spirochetes represent a monophylectic bacterial phylum with leptospires forming most deeply branching group (Paster *et al.*, 1991). In contrast to the conventional taxonomy, phylogenetic analysis classified leptospires into one more group that is intermediate between pathogens and nonpathogens (Morey *et al.*, 2006). Leptospires that fall into this group include *L. broomii, L. fainei, L. licerasiae, L. inadai and L. wolffii* (Slack *et al.*, 2008). Interestingly, *L. kmetyi* is grouped together with pathogenic species and *L. terpstrae* is classified into nonpathogenic species (Lehmann *et al.*, 2014).

1.9 Rationale of Study

Malaysia is a tropical country with high seasonal rainfall, warm temperature, wet and humid climate. These conditions lengthen the survival of *Leptospira* in the environment. It is common that floods occur following heavy rainfall during monsoon season in the east coast state of Malaysia. The presence of leptospires in the environment during flooding may potentially cause outbreak of leptospirosis. Kelantan is among the affected states where many people were involved in agricultural activities. Only a few prevalence studies that were conducted in Kelantan were published. Moreover, little attention was given to the detailed aspects of the isolates especially on the molecular characteristics. Therefore, the incidence of leptospirosis in Kelantan may be underestimated.

This study utilised several techniques which are available in the literatures as part of contribution to the epidemiological data in this state. The Centers for Disease Control and Prevention (CDC; Atlanta, GA) has established standard pulsed-field gel electrophoresis (PFGE) protocol for characterisation of *Leptospira* isolates. While this method is excellently reproducible among laboratories, this method requires special apparatus which are not available in some laboratories in the developing countries. Moreover, most molecular typing methods do not include intermediate pathogenic species. Even though leptospiral 16S rRNA gene sequencing just utilise one gene and only discriminate *Leptospira* strains to the species level, it has long been used as a typing method for molecular characterization of isolates because it is relatively cheap and available (Cerqueira *et al.*, 2010). This study has the potential to be developed as a standard method for the characterization of leptospiral isolates because it requires only basic equipment for molecular works.