EPIDEMIOLOGY OF HUMAN LEPTOSPIROSIS AND MOLECULAR CHARACTERIZATION OF *Leptospira* spp. ISOLATED FROM THE ENVIRONMENT AND ANIMAL HOSTS IN PENINSULAR

BENACER DOUADI

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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BENACER DOUADI

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INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

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ABSTRACT

Leptospirosis is a globally important zoonotic disease caused by spirochetes from the genus *Leptospira*. Transmission to humans occurs either directly from exposure to contaminated urine or infected tissues, or indirectly via contact with contaminated soil or water. In Malaysia, leptospirosis is an important emerging zoonotic disease with dramatic increase of reported cases over the last decade. However, there is a paucity of data on the epidemiology and genetic characteristics of *Leptopsira* in Malaysia. The first objective of this study was to provide an epidemiological description of human leptospirosis cases over a 9-year period (2004–2012) and disease relationship with meteorological, geographical, and demographical information. An upward trend of leptospirosis cases were reported between 2004 to 2012 with a total of 12,325 cases recorded. Three hundred thirty-eight deaths were reported with an overall case fatality rate of 2.74%, with higher incidence in males (9696; 78.7%) compared with female patients (2629; 21.3%). The average incidence was highest amongst Malays (10.97 per 100,000 population), followed by Indians (7.95 per 100,000 population). Stratification according to geographical distribution showed that the state of Malacca recorded the highest disease incidence average (11.12 per 100,000 population) followed by Pahang (10.08 per 100,000 population). In view of the recent rise in reported human cases, the next aim was to isolate and characterize *Leptospira* species from the environment (water and soils), and host reservoirs (rat, dog, cat, swine) from selected sites in different States in Malaysia. Positive isolates were obtained from urine and kidney samples of 59/167 rats, 11/50 dogs and 5/81 swine. Among 151 environmental samples collected from different sites, 35 samples (28 water, 7 soil) were positive with 8 (7 water, 1 soil) pure culture samples. Microscopic agglutination test (MAT) identified 4 *Leptospira* spp. serogroups (Javanica, Bataviae, Pomona and Canicola) in the zoonotic samples, while environmental samples were unidentified. Molecular characterization by PFGE showed

a high diversity among the environmental isolates (8 profiles), while only 5 different patterns were generated among zoonotic and clinical isolates. RAPD-PCR using primer pairs B11 and B12 subtyped 71 isolates into 32 RAPD profiles. A Multilocus sequence typing (MLST) using 7 seven house-keeping loci generated a phylogenity tree with 7 different MLST type (STs) among the 63 isolates, however no environmental strains were typed using this scheme. Antimicrobial susceptibilty analysis showed all 65 *Leptospira* spp. isolated from humans (n = 1), reservoir animals (rat, n = 60; dog, n = 1; swine, n = 1) and the environment (n = 2) were resistant to trimethoprim, chloramphenicol and sulfomethaxazole. Doxycycline, ampicillin and penicillin G were only effective against the clinical and zoonotic isolates. Finally, multiplex PCR (mPCR) was developed and proved to be a promising tool for the rapid early detection and differentiation of leptospirosis from various specimens using two primer sets, LG1/LG2 for genus confirmation, which targeted the 16SrRNA gene and species identification and pathogenicty determination using LP1/LP2 primers which targeted *ligB* gene. These results highlight the need for close partnerships between scientists, clinicians, veterinarians and public health officers in order to reduce the disease burden.

ABSTRAK

Leptospirosis adalah penyakit zoonotik yang penting di peringkat global yang disebabkan oleh spirochetes genus *Leptospira*. Transmisi kepada manusia berlaku sama ada secara langsung melalui pendedahan kepada air kencing yang tercemar atau tisu yang dijangkiti, atau secara tidak langsung melalui sentuhan dengan tanah atau air yang tercemar. Di Malaysia, leptospirosis adalah penyakit baru yang penting dengan peningkatan dramatik dalam laporan kes leptospirosis sepanjang dekad yang lalu. Walau bagaimanapun, terdapat kekurangan data mengenai epidemiologi dan genetik *Leptopsira* di Malaysia. Objektif pertama kajian ini adalah untuk menyediakan deskripsi tentang epidemiologi kes leptospirosis manusia dalam tempoh 9 tahun (2004-2012) dan perkaitan penyakit ini dengan maklumat cuaca, geografi, dan demografi. Kecenderungan kes leptospirosis telah dilaporkan di antara 2004 dan 2012 dengan sebanyak 12,325 kes direkodkan. Tiga ratus tiga puluh lapan kes kematian telah dilaporkan dengan kadar kematian kes keseluruhan sebanyak 2.74%, dengan kadar jangkitan yang lebih tinggi pada pesakit lelaki (9696; 78.7%) berbanding dengan pesakit wanita (2629; 21.3%). Purata jangkitan adalah paling tinggi di kalangan orang Melayu (10.97 bagi setiap 100,000 penduduk), diikuti oleh orang India (7.95 bagi setiap 100,000 penduduk). Stratifikasi mengikut taburan geografi menunjukkan bahawa negeri Melaka mencatatkan purata insidensi penyakit yang tertinggi (11.12 bagi setiap 100,000 penduduk) diikuti oleh Pahang (10.08 bagi setiap 100,000 penduduk). Memandangkan terdapat peningkatan dalam kes manusia yang dilaporkan di Malaysia baru-baru ini, tujuan kajian ini adalah untuk mengisolasi dan mengklasifikasikan ciri-ciri spesies *Leptospira* dalam persekitaran (air dan tanah) dan pembawa hos haiwan (tikus, anjing, kucing, babi) dari kawasan tertentu di negeri-negeri di Malaysia. Isolat positif diperoleh daripada sampel air kencing dan buah pinggang dari tikus (59/167), anjing (11/50) dan babi (5/81). Antara 151 sampel alam sekitar yang dikumpulkan dari kawasan

persekitaran yang berlainan, 35 sampel adalah positif (28 air, 7 tanah) dengan hanya 8 sampel (7 air, 1 tanah) adalah kultur asli. Ujian mikroskopik agglutinasi (MAT) mengenalpastikan 4 empat serogroup yang berlainan di kalangan sampel zoonotik, akan tetapi identiti bagi isolat dari hos alam sekitar tidak dapat dikenalpasti. Pencirian molekul isolat *Leptospira* menggunakan PFGE menunjukkan corak kepelbagaian yang tinggi di kalangan isolat alam sekitar (8 profil), manakala hanya 5 profil corak yang berbeza yang dikenalpastikan antara isolat zoonotik dan klinikal. RAPD-PCR menggunakan sepasang primers (B11, B12) subtaipkan 70 isolat kepada 32 profil RAPD. Multilocus urutan menaip (MLST) yang menggunakan 7 lokus penjagaan-dalaman menjanakan pokok filogenetik dengan 7 jenis MLST profil (STS). Walau bagaimanapun, strain alam sekitar tidak dapat diprofilkan menggunakan skim ini. Analisa kerintangan antimikrob menunjukkan 65 isolat *Leptospira* daripada manusia (n=1), haiwan (tikus, n=60; anjing, n=1; babi, n=1) dan alam sekitar (n=2) mempunyai kerintangan terhadap trimethoprim, chloramphenicol dan sulfomethaxazole. Doxycycline, ampicillin dan penisilin G menunjukkan keberkesanan terhadap semua isolat klinikal dan zoonotik sahaja. Akhir sekali, multiplek PCR telah dibangunkan dan dibuktikan menjadi alat yang menjanjikan pengesanan awal yang cepat dan pembezaan leptospirosis daripada pelbagai spesimen dengan menggunakan 2 set primer, LG1/LG2 untuk pengesahan genus *Leptospira* yang mensasarkan gen 16SrRNA dan primer LP1/LP2 yang mensasarkan gen *lig*B untuk mengesan status patogenik dan membezakan spesies strain. Keputusan kajian ini menyerlahkan keperluan kerjasama rapat antara saintis, doktor, doktor haiwan dan pegawai kesihatan awam bagi mengurangkan beban penyakit.

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LIST OF ABBREVIATIONS

>	Greater than
≥	Same or greater than
~	Approximately
=	equals to
ºC	Degree Celcius
DOXY	Doxycycline
µl	Microliter
µm	Micrometer
µg	Microgram
%	Percent
AMP	Ampicillin
AAC	Aminoglycoside acetyltransferase
ATCC	American Type Culture Collection
$BaCl_2$	Barium Chloride
bp	basepair
BSA	Bismuth Sulphite Agar
CAM	Chloramphenicol
CDC	Centers for Disease control and Prevention
CFU	Colony forming unit
D	Discriminatory Power
DFK	Dark field microscopy
dH_2O	Distilled water
ddH_2O	Double distilled water
DHFR	dihydrofolate reductase

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic
EMJH	Ellinghausen McCullough Johnson Harris (EMJH)
ELISA	Enyme-linked immunosorbent assay
EtBr	Ethidium Bromide
EtOH	Ethanol
Fig.	Figure
g	Gram
HCL	Hydrocloric acid
H_2S	Hydrogen sulphite
H_2SO_4	Sulphuric acid
KAc	Kalium Acetate
kb	Kilobase pair
LB	Luria-Bertani
LPS	Lipopolisacharide
M	Molar
MAT	Microscopic agglutination test
MIC	Minimum inhinitory concentration
MLST	Multi-locus sequence typing
MLVA	Multilocus variable number tandem repeat analysis
$MgCl_2$	Magnesium chloride
mM	Milimolar
mg	Miligram
ml	Mililiter
mm	Milimeter
N	Normality

NA	Nutrient agar
NARMS	National Antimicrobial Resistance Monitoring System
Na	Nalidixic Acid
NaCl	Natrium Chloride
NaOH	Natrium Hydroxide
NCCLS	National Committee for Clinical Standard
No.	Number
OD	Optical density
PCR	Polymerase Chain Reaction
PenG	Penicillin G
PFGE	Pulsed field gel electrophoresis
pmol	picomole
psi	Pound per square inch
R	Resistant
RAPD	Random amplified polymorphic DNA
Ref.	Reference
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
Spp.	Species
SMX	Sulfamethoxazole
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tmp	Trimthoprim
Tris	Tris (Hydroxymethyl) methylamine
UPGMA	Unweighted pair group arithmetic means methods

μl	Microlitre
UV	Ultraviolet
V	Volt
WHO	World Health Organization
w/v	Weight per unit volume
5'-CS	5 conserved segment
3'-CS	3 conserved segment
5 FLU	5- fluorouracil

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Leptospirosis is an important zoonotic disease with a worldwide distribution. It is caused by spirochetes from the genus of *Leptospira*. There are around 22 *Leptospira* species classified according to DNA-DNA hybridization and over 300 serovars based on agglutinating LPS antigens (Bourhy *et al*., 2014). These species can be further divided into pathogenic, non-pathogenic and opportunistic/possibly pathogenic groups. The main reservoirs for the pathogenic *Leptospira* are the rodents, including rats that may carry various types of pathogenic serovars and may continue shed leptospires in the urine life time (Wangroongsarb *et al*., 2002). Saprophytic species are naturally present in the environmental water and soil and do not usually cause disease (Mohammed *et al*., 2011). Humans can be infected via direct contact with contaminated urine of animal reservoirs or indirectly by contaminated water and soil (Bharti *et al*., 2003). The symptoms of leptospirosis may vary from flu-like illness to organ failure and the disease is often misdiagnosed as dengue fever or malaria. There are two phases of leptospirosis infection where they differ in signs and symptoms, that is anicteric and icteric leptospirosis (Levett, 2001). In the past, leptospirosis was known as an occupational disease, mainly occurring among the fishermen, abattoir workers, veterinarian and sewage workers (Ullmann & Langoni, 2011). However, recreational activities as water sports and travels to the endemic countries have also been recognized as risk factors for this disease (Victoriano *et al*., 2009).

Leptospirosis mainly occurred in the tropical and subtropical countries because of the climatic conditions such as high humidity and warm temperature which are ideal

for *Leptospira* to survive for long periods in the environment. In Malaysia, several outbreak cases have been reported (Koay *et al.* 2004, Sharma and Yadav, 2008). Early research on the isolation of *Leptospira* in Malaysia began in the 1920's (Fletcher, 1928). To date, more than 37 pathogenic serovars have been identified in Malaysia (Bahaman and Ibrahim, 1988). The first case of human leptospirosis in Malaysia was reported in 1925 (Fletcher, 1928). Following Fletcher, several investigations on human leptospirosis in Malaysia have disclosed a high prevalence of this infection. These investigations indicate that the disease was endemic in the country. The risk to leptospiral infection with respect to occupation, location, sex, race and age groups was demonstrated. Both civilians and military personnel were affected (El Jalii and Bahaman, 2004). In environment, the early research on the isolation of *Leptospira* from water and soil in Malaysia began in the 1970s (Baker and Baker, 1970; Alexander *et al.*, 1975). To date, 29 pathogenic serovars have been identified in the Malaysian environmental water and wet soil (Alexander *et al.*, 1975). In animals, Fletcher (1928) detected the first case of leptospirosis in black rat (*Rattus rattus*). Several studies reported the infection in wide variety of animals in Malaysia (Bahaman and Ibrahim, 1988).

Historically, the annual incidence of leptospirosis in Malaysia ranged between 1−10 per 100,000 population (Lim, 2011). Although there is a dramatic increase in the number of reported cases over the last decade, the incidence of human leptospirosis is still underestimated and not well documented due to misdiagnosis and underreporting, yielding the epidemiological status of this infection unclear.

The rapid urbanization of cities and in combination with inefficient garbage management has created favorable conditions for animal carriers to thrive and pose

health risk for leptospirosis as infected animals and carriers contaminate environmental waters and soils via their excretion and urine. In light that previous studies on leptospirosis in Malaysia were mainly diagnosed through serological testing such as Microscopic Agglutination Test (MAT). In serological study carried by Noor Rafizah *et al*. (2013) in the northeastern states of Malaysia and based on MAT results revealed that 8% (84/999) of patients were shown to be positive. Based on the cut off titer of $\geq 1: 400$, serogroup Sejroe was found to be the predominant. However, the cut-off value of the MAT usually depends on the baseline in the community in a particular geographical area and varies from laboratory to laboratory (Sanders *et al*., 1999). In addition, MAT is tedious and requires an extensive range of antisera. Additional sets of hyper immune sera are required to further identify the serogroups of the 33 strains isolated by Azali *et al*. (2016) from water and soil samples in the northern eastern of Malaysia. Therefore, a new and rapid diagnosis technique is now greatly needed.

A variety of methods have been developed for detection, identification and molecular characterization of *Leptospira* spp. These include both phenotypic and genotypic methods such as amplified fragment length polymorphism, PCR-based typing, pulsed field gel electrophoresis (PFGE), Multi-locus Sequence Typing (MLST) and Multi-locus Variable Number of Tandem Repeats Analysis (MLVA)**.**

The choice of these methods for subtyping depends on: (a) high degree of typeability, the method should be able to type the vast majority of strains encountered; (b) have good discrimination with the ability to recognize a reasonable number of types; (c) show good reproducibility over a long period of time and in different centers; (d) be readily applicable to natural isolates as opposed to laboratory collections of strains; (e)

should not be too complicated or expensive (Busch and Nitschko, 1999; Olive and Bean, 1999).

Locally, there are only few studies on the molecular characterization of this emergent spirochete. In a study carried by El Jalii & Bahaman (2007). RAPD- PCR showed to be a reproducible and useful technique in characterization of clinical and zoonotic strains. RAPD revealed a high genetic diversity among the isolates tested and could distinguish between the strains of the same serovar. Hence, combination of molecular methods such as RAPD-PCR, PFGE and MLST, are needed to obtain information on the strains circulating in the environment and the host reservoirs that contribute to the transmission to humans.

At present, studies on antimicrobials activity against *Leptospira* spp. are limited to a few agents and human studies. *In vitro* antimicrobial susceptibility testing has also only been used on a small scale to examine the activities of numerous drugs against *Leptospira* isolates (Murgia and Cinco, 2001, Ressner *et al*., 2008) using broth macrodilution methodologies to produce susceptibility results. In Malaysia, there is not much information on the antimicrobial susceptibilities of Malaysian *Leptospira* isolates. Therefore, there is a need to monitor and evaluate the susceptibility of the local isolates to different antimicrobial compounds use in the treatment of leptospirosis.

1.2 Objectives

The overall objective is to determine the epidemiology and the genomic diversity of the *Leptospira* spp. in human, host reservoir and environment in Peninsular Malaysia. Specifically, the aims are:

i. To understand the epidemiology of human leptospirosis in Malaysia by providing a baseline information for future epidemiological studies.

ii. To isolate and determine the *Leptospira* spp. from various sources, environment (water and soil), and host reservoir (rats, pigs, stray cats and dogs) in Peninsular Malaysia (North, East, West and South).

iii. To subtype the *Leptospira* spp. strains by molecular methods such as Randomly Amplified Polymorphic DNA (RAPD), Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST).

iv. To determine the antimicrobial resistance profiles of the *Leptospira* spp.

v. To develop a specific and rapid DNA- based detection of *Leptospira* spp.

Despite the dramatic increase of the leptospirosis cases in Malaysia since the last decade, there is still a paucity of scientific information about the status of leptospirosis in this country in terms of the reservoirs of *Leptospira* spp., pathogenicity, transmission route and the genetic diversity of the strains circulating locally (Publication 1). Therefore, the current research aims to bridge the gap in existing research literature by reviewing the history and epidemiology of human leptospirosis in Malaysia from 1925 to 2012 (Publication 2 and 3). Based on the human leptospirosis data provided by the Malaysian Ministry of Health between 2004 and 2012, a combination of statistical tools were used to correlate the influence of the demographical, geographical, and socio-environmental factors on the incidence of leptospirosis locally to obtain a better understanding of the disease trends (Publication 4).

As the majority of the human leptospirosis cases reported previously in Malaysia was related to the exposure of humans to an environment contaminated by *Leptospira* spp., a study was carried out to investigate the prevalence and pathogenic status of this fastidious organism in the urban Malaysian environment (Publication 5). Since domestic and wild animals are considered as the main maintenance hosts of these bacteria, a study was undertaken to isolate *Leptospira* spp. and characterise the serovars circulating in the urban rat, swine, stray dog and cat populations from selected areas in Peninsular Malaysia. This was followed by investigation on their genetic diversity using PFGE, RAPD-PCR and MLST (Publication 6, 7 and 8). Publication 8 reports the susceptibility of recent local isolates obtained from different hosts and sites to traditional antimicrobial compounds used for leptospirosis treatment. Finally, a rapid and sensitive mPCR was developed to determine the genus and pathogenic status of the *Leptospira* strains. This mPCR can be used to overcome some of the limitation of the dignastic tools used for detection of *Leptospira* spp. which mainly based on serological

techniques (Publication 9). The thesis concludes with a general conclusion (Chapter 4) of the study findings.

CHAPTER 2: LITERATURE REVIEW

2.1 Historical Perspectives of Leptospirosis

Leptospirosis is an infection disease caused by pathogenic species from the genus of *Leptospira*. It was first described by Adolf Weil in 1886 (Faine, 1999). The morphology description of this bacteria is an organism with end hooked and it was described by Stimson (1907) using silver staining on kidney sample of a yellow fever patient. The organism that resembles a question mark, was then named as *Spirochaeta interrogans* (Stimson, 1907). In 1914, Wolbach and Binger isolated spirochetes from a fresh water source and named *Spirochaeta biflexa*. Approximately a year later, Inada *et al*. (1916) concluded the spirochetes isolated from liver of a guinea pig inoculated with Weil's disease patients' blood as the etiologic agent of leptospirosis and proposed the name of *Spirochaeta icterohaemorrhagiae*. In 1917, Noguchi isolated spirochetes from wild rats which morphologically and immunologically similar to causative agent of leptospirosis and thus classified the organism under a new genus – *Leptospira*. Later, Noguchi (1918) concluded both *S. icterohaemorrhagiae* and *S. biflexa* are identical and classified under genus *Leptospira*.

2.2 Taxonomy and Classification of Leptospires

The genus *Leptospira* is classified under the order of Spirochaetales, in the family Leptospiraceae (Mohammed *et al.,* 2011). Conventionally, *Leptospira* spp are classified into two distinct species, which are the pathogenic species – *Leptospira interrogans*, and the saprophytic species – *Leptospira biflexa* (Morey *et al.*, 2006). Both species were then further subdivided into different serovars based on expression of surface lipopolysaccharide O antigens (Adler & de la Peña Moctezuma, 2010) and antigenically associated serovars are formed into various serogroups (Bharti *et al.*, 2003). Each serovar can be identified and differentiated by using serological method (WHO, 2003).

A new genotypic classification system based on DNA relatedness was devised which classified *Leptospira* spp. into genomospecies (Brenner *et al.*, 1999). In addition, 16SrRNA sequence analysis revealed the presence of pathogenic, saprophytic and intermediate species (Cerqueira & Picardeau, 2009). Under the genotypic classification system, there are at least 22 species classified according to DNA-DNA hybridization analysis (Table 2.1) (Bourhy *et al*., 2014). *Leptospira* strains can be further classified based on antigen recognition to serovars and serogroups based on cross-agglutination absorption test (CAAT) (Faine, 1999). The latter can group the strains based on their expressed surface exposed epitopes within the lipopolysaccharide (LPS), specifically the sugar composition and protein orientation within the membrane (Adler & de la Pena Moctezuma, 2009). To date there are more than 300 serovars based on agglutinating LPS antigens (Victoriano *et al*., 2009).

Table 2.1: Pathogenic, Saprophytic and Intermediate *Leptospira* spp..

Pathogenic *Leptospira*	Intermediate *Leptospira*	Saprophytic *Leptospira*
Leptospira interrogans	*Leptospira inadai*	*Leptospira biflexa*
Leptospira kirschneri	*Leptospira broomii*	*Leptospira wolbachii*
Leptospira borgpetersenii	*Leptospira fainei*	*Leptospira meyeri*
Leptospira santarosai	*Leptospira wolffii*	*Leptospira vanthielii*
Leptospira noguchii	*Leptospira licerasiae*	*Leptospira terpstrae*
Leptospira weilii		*Leptospira idonii*
Leptospira alexanderi		*Leptospira yanagawae*
Leptospira kmetyi		
Leptospira alstonii		
Leptospira mayottensis sp. nov		

2.3 Morphology and Characteristics of Leptospires

Leptospires are tightly coiled spirochetes, usually 0.1 μm in diameter and 6 to 20 μm in length, but occasional cultures may contain much longer cells (Figure 2.1). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Faine *et al*., 1999). Leptospires have a typical double membrane structure as common found in other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and overlain by an outer membrane (Haak *et al*., 2000). Leptospiral lipopolysaccharide (LPS) has a composition similar to other Gram-negative bacteria (Mohammed *et al*., 2011). The two periplasmic flagella with polar insertions are located in the periplasmic space and are responsible for motility. The motile forms of *Leptospira* are generally classified into two types: the swimming (translating) and rotating groups. The translating cells swim smoothly whereas the rotating cells show no net displacement and spin at one position (Nakamura *et al*., 2014).
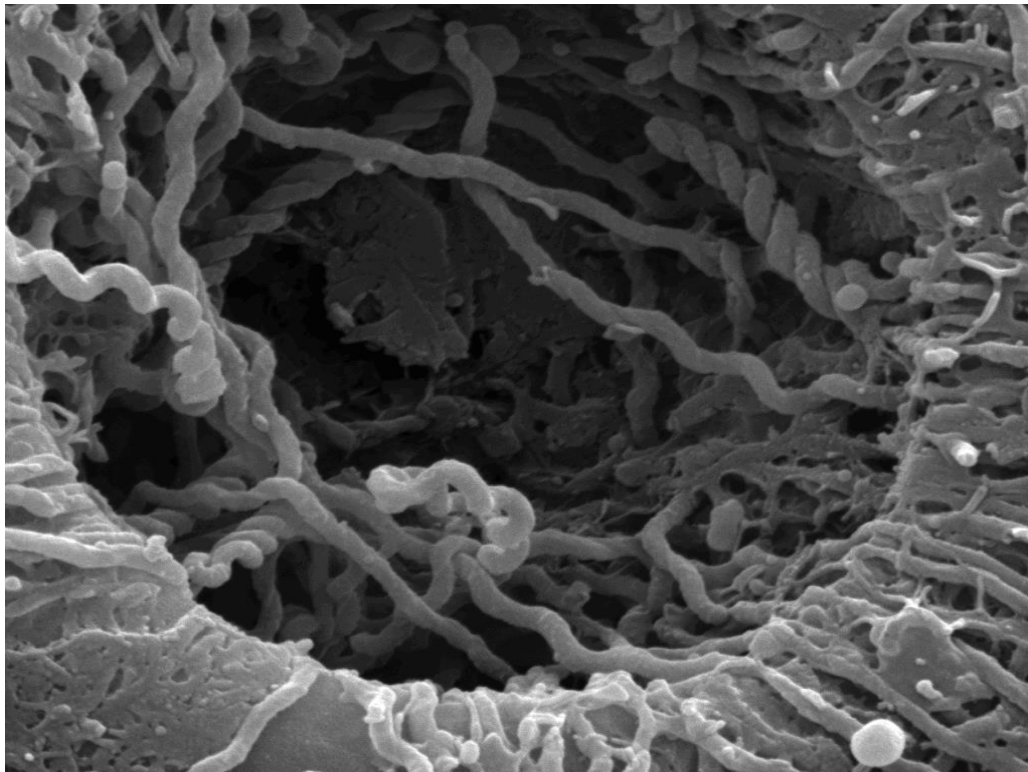
Figure 2.1: Electron microscope image of *Leptospira interrogans* (this study).

2.4 Epidemiology of Leptospirosis

Leptospirosis is an increasingly public health problem worldwide, as evidenced by the marked increasing incidence rates and multiple outbreaks in different countries (Hartskeerl *et al*., 2011). The epidemiology of leptospirosis is dynamic and an understanding of this is required to study the interaction between humans, animal and environment. The transmission of this disease may be influenced by climatic events, environmental factors, animal reservoirs, human demographic and social trends (Ullmann & Langoni, 2011). Wild and domestic animals are regarded as the principal reservoirs for this spirochete, which has been reported in at least 150 mammalian species (Sykes *et al*., 2011). Particular serovars have been related to specific animal reservoirs, however, all animals are susceptible to infection by any serovar (Bharti *et al*., 2003). Rodents, cattle and dogs are considered as the main important source of

human infection (Levett, 2001). Leptospires colonize the kidneys of the animal host and are shed in their urine and contaminate the environment. Human and animal infections are acquired via direct contact with urine, blood, or infected animal tissue or indirect contact with water (includes rivers, lakes, ponds) or soil and mud contaminated with the urine of reservoir animals (Bharti *et al*., 2003). In the past, many cases were documented and related to some activities such as livestock farms, veterinarians, miners, rodents control workers and abattoir and sewer workers (Okello *et al*., 2015). However, travel to endemic areas and recreational activities such as water sports have increasingly been reported as major risk factors for this infection (Victoriano *et al*., 2009). Floods and heavy rainfall were also been associated with numerous cases of leptospirosis around the world. Several outbreaks were reported during the floods that occurred in Johor in Malaysia between 2006 and 2007 (Badrul Hisham *et al*., 2009) and in other countries such as Nicaragua in 2007 (Schneider *et al*., 2012), Sri Lanka in 2008 (Agampodi *et al*. 2011) and Philippines in 2009 (McCurry, 2009).

2.5 Clinical Manifestations of Human Leptospirosis

Human leptospirosis may present a variable clinical manifestations that may range from subclinical disease to jaundice, renal failure, and potentially fatal lung disease (Bharti *et al*., 2003). Leptospirosis is typically biphasic illness (Figure 2.2). The initial acute or septicemic phase is characterized by bacteremia that typically lasts about one week. Most of the cases present with a febrile illness of sudden onset. Fever, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting, and prostration usually characterize acute leptospirosis (Dolhnikoff *et al*., 2007). A substantial proportion of people infected by *Leptospira* may have subclinical disease or very mild symptoms, and do not seek medical attention (Levett, 2001). In this

leptospiremic phase, leptospires may be found in the blood and cerebrospinal fluid (Martinez Garcia *et al.*, 2000). The resolution of symptoms may coincide with the second or immune phase, when circulating immunoglobulin M (IgM) antibodies begin to be produced, accompanied by excretion of spirochetes in the urine (Dolhnikoff *et al.*, 2007). However, fever may recur after a remission of 3 to 4 days, producing a biphasic illness (Dolhnikoff *et al.*, 2007). Weil's syndrome can develop after the acute phase as the second phase of a biphasic illness, or can simply present as a single, progressive illness. It is characterized by high fever, intense jaundice, bleeding, renal and lung dysfunction, neurologic alterations, and cardiovascular collapse with a variable clinical course (Vinetz *et al.*, 1996; Levett, 2001; Carvalho & Bethlem, 2002).
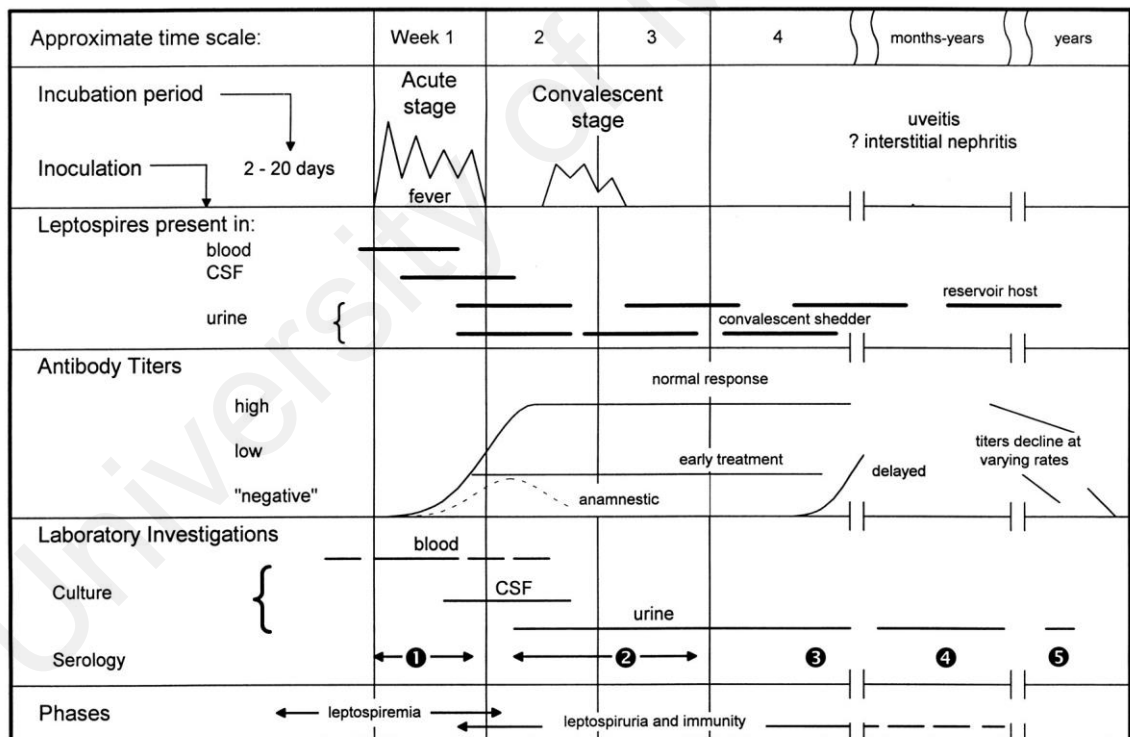
Figure 2.2: The biphasic nature of leptospirosis and relevant diagnostic investigations at different stages of disease (Levett, 2001).

2.6 Leptospirosis in Animals

Most mammals can be infected with *Leptospira* spp., however animals such as rats are natural reservoirs that do not show clinical signs. These animals excrete the bacteria in their urine and contaminate the surrounding environment such as soil, mud, ground waters, streams and rivers where the bacteria can survive for months to years. Rodents are the most important source of leptospirosis infection for humans and animals (WHO, 2003). The role of rodents as carriers and the main source of leptospiral infection in human have been investigated in different countries (Turk *et al*., 2003; Socolovshi *et al*., 2011; Kositanont *et al*., 2012; Mohamed-Hassan *et al*., 2012). Moreover, different species of rodents, such as *Rattus rattus*, *R. norvegicus*, *R. exulans*, *Mus musculus* and others are known to carry different pathogenic leptospiral serovars (Mathias & Levett, 2002; Mohamed-Hassan *et al*., 2012).

The clinical manifestations of leptospirosis in animals may vary according to current status of immunity of animal species and the virulence of the infecting serovars. Usually the clinical signs range from mild to severe depending of the age of the host. Normally the severe cases are common in the young animals (Faine *et al*., 1999). In cattle the most common cause of the infection is *L. borgpetersenii* serovar Hardjo (Chideroli *et al*., 2016). Infection in cattle may cause significant economic losses due to abortion, death of the embryo, death of the calves during the first days of the life and milk drop (Lucchese *et al*., 2015).

In sheep and goats, leptospirosis manifests as two distinct clinical syndromes: an acute and often fatal systemic disease, and reproductive problems such as infertility, abortion and stillbirth. The acute systemic disease is characterized by icterus, hemoglobinemia, hemoglobinuria, fever, and death and has been associated with

infections by the Pomona serogroup in particular. Besides serovars Hardjo and Pomona, other serovars such as Hebdomadis, Ballum, Grippotyphosa and Australis were isolated from sheep and goats (Martins &Lilenbaum, 2014; Sharma *et al*., 2014).

In horses, the clinical manifestations of leptospirosis is primarily associated with abortion, stillbirth and equine recurrent uveitis (Turk *et al*., 2013). The most infecting serovars for horses are Bratislava, Pomona and Icterohaemorrhagiae (Turk *et al*., 2013; Finger *et al*., 2014).

In pigs, leptospirosis shows similar symptoms to those in sheep and cattle. Primarily signs such as abortions, infertility and birth of premature and weak piglets are seen in newborn and young piglets. In adults, leptospirosis is predominantly a chronic, asymptomatic disease while pregnant sows, new born and young piglets are the most susceptible to acute leptospirosis. Serovar Pomona is the most important cause of porcine leptospirosis in the world. It has been associated with stillborn piglets and mummified fetuses (Ramos *et al*., 2006). Besides, serovar Pomona, other serovars such as Bratislava, Castellonis and Icterohaemorrhagiae have been isolated from swine in different countries (Cisneros Puebla *et al*., 2002; Brown *et al*., 2011).

In dogs, leptospirosis results in illness of varying severity, depending on the infecting strain, geographical location, and host immune response. Some dogs display mild or no signs of disease, whereas others develop severe illness or death, often as a result of renal injury (Sykes *et al*., 2011). Usually leptospirosis in dogs is associated with *L. interrogans* serovar Canicola (Andre-Fontaine, 2006). However, other serovars such as Australis, Pyrogenes, Autumnalis, Pomona, Bratislava and Grippotyphosa have also been isolated from dogs in different countries (Sonrier *et al*., 2000; Greenlee *et al*., 2005; Andre-Fontaine, 2006; Sykes *et al*., 2011).

In cats, leptospirosis clinical signs are rarely reported, but disease is generally mild. Experimental infection of cats results in leptospiremia and leptospiruria (Sykes *et al.*, 2011). Cats may be exposed as a result of rodent contact. In India, Natarajaseenivasan *et al.* (2002) reported that about 33.3% of cats were infected with serovar Autumnalis. The latter was also detected in rats from rice fields in the same region (Natarajaseenivasan *et al.*, 2002). Serovars Canicola, Grippotyphosa, and Pomona have been isolated from cats (Sykes *et al.*, 2011).

2.7 *Leptospira* in the Environment

Leptospires are able to survive in alkaline soil, mud, swamps, streams, rivers (Mohammed *et al.*, 2011). Several growth factors such as pH, temperature and the presence of inhibitory compound can influence the survival of pathogenic strains in environment. They are sensitive to dryness, heat, acids and basics condition (Mohammed *et al.*, 2011). The longer exposure of *Leptospira* in the environmental waters and soils thus increase the chances of infecting susceptible host (Monahan *et al.*, 2009). This increases the disease transmission from animal to humans indirectly. *Leptospira* species do not multiply outside the host. They favor alkaline condition for growth and survival (Monahan *et al.*, 2009). Under laboratory condition, leptospires remain viable for months at pH 7.2 to 8.0 in room temperature (Tangkanakul, 2000; Mohammed *et al.*, 2011). They require a moist environment for survival because dryness or temperature higher than 50ºC can kill them. They can remain viable in water for several months but not in natural river water (Mohammed *et al.*, 2011). Viscosity can affect the growth of leptospires in fresh water for 110 days in distilled water, increasing to 347 days in more viscous solution (Trueba *et al.*, 2004). A viscous environment enables leptospires aggregate and thus prolong their survival time period

by accumulating the nutrient and enzymes from lysing cells (Trueba *et al*., 2004). Leptospiral cells are highly motile microorganisms thus they show constant gyrations in water and liquid medium, whereas motility is occasional in semi-solid media. Therefore, it is possible that cells in agarose used less energy than cells in liquid environments (Trueba *et al*., 2004).

2.8 Laboratory Diagnosis of Leptospirosis

2.8.1 Direct Demonstration of Leptospires: Culture and Dark Field Microscopy

Leptospires can be visualized using dark field microscopy (Figure 2.3) in examination of the specimens such blood, urine and cerebro-spinal fluid and it is useful in situation when the laboratory has limited recources (Faine *et al*., 1999). However, this technique has limitations, such as the examination can be done only when the leptospiraemia occurs during the first few days of the acute phase (Levett, 2001). In addition, the false positive misdiagnosis has frequently occurred. Especially when a small number of leptospires present or the presence of other components such as fibrin threads in blood which easily mistaken for leptospires. Therefore a high degree of operator skill is required (WHO, 2003). Leptospires are slow-growing bacteria which usually take up to 4 months before being discarded (Levett, 2003). These microorganisms are cultivated in artificial media containing 10% rabbit serum or 1% bovine serum albumin plus long-chain fatty acids at pH 6·8–7·4. The most commonly used medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, an oleic acid-albumin medium (Ellinghausen & McCullough, 1965). This medium contains Tween 80 and bovine serum albumin with the addition of 5- fluorouracil to inhibit the growth of other microorganisms (Levett, 2001). Semi-solid media can be prepared by additional of minute quantity of agar. Leptospiral cultures should be checked for the

presence of contaminating bacteria after 3–4 days and maintained by repeated subculture or by storage in semisolid agar containing hemoglobin (Faine *et al*., 1999). Leptospires have longer survival time period in undisturbed liquid culture for months and sometimes even for years (Bharti *et al*., 2003). Cultures of leptospires from human and animal specimens may be influenced by many factors including the sample type and the condition of sample taken (Faine *et al*. 1999). The survival of the organism in the collected primary tissue or body fluid sample is dependent on the response of host's immune system (Smythe *et al*., 2002) and the exposure of the host to administered antibiotics (Picardeau, 2013).
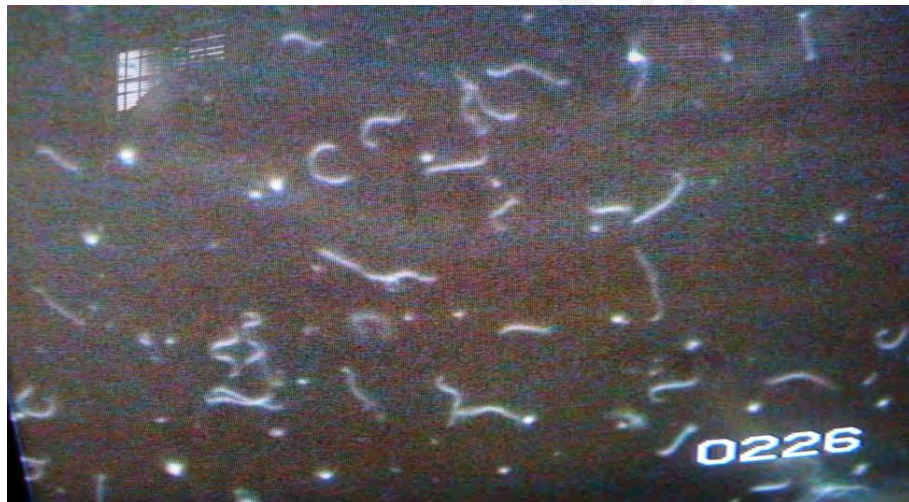
Figure 2.3: *Leptospira* spp. as seen under dark field microscope (this study).

2.8.2 Serological Methods of Diagnosis

2.8.2.1 Microscopic Agglutination Test

Microscopic agglutination test (MAT) is considered the gold standard serological test for the diagnosis of leptospirosis. This test remains the reference test and is used to detect antibodies and determine their titer. It may give an indication of the serogroup to which the infective serovar belongs but only rarely identifies it (WHO,

2003). This technique is based on detection of IgM- and IgG-class antibodies but it is difficult to standardize as the live antigens are often used and various factors, such as the age and density of the antigen culture, can influence the agglutination titer (WHO, 2003). The method is simple and consists of mixing the test serum with a culture of leptospires and then evaluating the degree of agglutination using a dark-field microscope. The result can be determined as the end-point which is defined as that dilution of serum which shows 50% agglutination, leaving 50% free cells when compared with a control culture diluted 1:2 in phosphate-buffered saline (Levett, 2001). The sensitivity and specificity of MAT are very high (Alder *et al.*, 2011). A study showed that MAT sensitivity ranged from 91.1% to 100.0% and specificity from 94.3% to 100% (McBride *et al.*, 2007). However, MAT has a number of disadvantages, as it is laborious and time consuming. In addition MAT requires a large panel of antigens to maximize the chances of detecting an immune response to one of the pathogenic serogroups (Levett, 2003). Other difficulties such as cross contamination of the culture and culture medium condition will also affect the reading and intereptation.

2.8.2.2 Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assay **(**ELISA) was developped to ovecome the difficulties of the MAT. This test can be performed with commercial kits or with antigen produced "in house". A broadly reactive so-called genus-specific antigen is generally used to detect IgM, and sometimes also IgG antibodies (WHO, 2003). The presence of IgM antibodies may indicate current or recent leptospirosis, but it should be remembered that IgM-class antibodies may remain detectable for several years. The advantages of ELISA is that it can be strandardised, easy to peform and less expensive compared to MAT. However, ELISA is less specific as weak reaction due to the

presence of other diseases. In addition, ELISA is unable to indictae the infecting serovar as it based on genus-specific antigen, thereforce additional test such as MAT is still needed.

2.8.3. Molecular Methods

2.8.3.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR)-based methods are used for bacterial genotyping identification and discrimination of bacterial strains. The hallmark of PCR is the ability to produce literally millions copies of a particular DNA segment with high fidelity within 3 to 4 hours time (Tenover *et al*., 1997). The PCR technique is based on repeated cycles of high temperature for denaturation of the DNA, oligonucleotide (primer) annealing and an extension step, which is mediated by a heat stable polymerase. In each cycle of the PCR, the number of copies of the chosen sequence is doubled so that the amount of the target DNA is exponentially increasing. Then the target DNA is separated by agarose gel electrophoresis. PCR has been used for detection of *Leptospira* in different specimens since 1990s (Mérien *et al*., 1992; Gravekamp *et al*., 1993). The PCR developed by Merien *et al*. (1992) claimed to detect as few as 10 leptopires/ml in urine, CSF and blood when combined with the DNA hybridization technique (Mérien *et al*., 1992). However, this assay amplified DNA from both pathogenic and non-pathogenic serovars. The second mixed primer sets G1/G2 and B64-I/ B64-II are used in the approach described by Gravekamp *et al*. (1993) to detect all seven pathogenic species and differentiate them from non-pathogenic species. Besides these two widely used PCRs, assays with primers targeted at other genes were also developed, including *flaB* (Kawabata *et al*., 2001), *ompL1* (Reitstetter, 2006),

*LipL*32 (Bomfim et al., 2008), 23s rDNA (Harkin *et al*., 2003) and *lig* genes (Palaniappan *et al*., 2005).

PCR assay had a higher sensitivity in detecting leptospires in different specimens from both humans and animals (Mérien *et al*., 1992; Heinemann *et al*., 2000; Wagenaar *et al*., 2000) compared to culture technique. The high sensitivity of PCR assays may be due to the fact that these assays detect both viable and dead bacteria, while culture requires sufficient numbers of viable bacteria. PCR assays also provide a considerable time advantage, compared with long incubation time for culture.

Recently, real-time PCRs (qPCRs) were introduced for diagnosing leptospirosis. This further improved the conventional PCRs with it rapidity and sensitivity by taking less time and reducing false positive results caused by carry-over contaminations (Ahmed *et al*., 2009; Bedir *et al*., 2010).

2.8.3.2 Randomly Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) assay was first described by Williams *et al*. (1990) and Welsh and McClelland (1990). This type of assay is based on the use of short random sequence primers, 6 to 10 base in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures in order to initiate amplification of regions of the bacterial genome. A number of studies have reported success in using RAPD assays for epidemiological investigations of leptospirosis in regions of high endemicity (Roy *et al*., 2004, 2005; Natarajaseenivasan *et al*., 2005). This technique was applied successfully for *Leptospira* serovar identification (Ramdass *et al*., 1997; Sharma *et al*., 2014) which could be useful in molecular epidemiological studies of leptospirosis. RAPD is simple to perform, widely

applicable and does not require previous knowledge of the genome. However, this technique is not suitable for large-scale studies, reproducibility is poor and comparisons of data between laboratories is difficult (Cerqueira & Picardeau, 2009).

2.8.3.3 Multi-locus Sequence Typing

Some pathogenic strains are genetically very homogeneous and making strain discrimination difficult. Since the last decade, multi-locus sequence typing (MLST) (Maiden *et al*., 1998) has been increasingly recognized as markers of choice for genotyping a number of pathogens including *Leptospira* spp. This technique is based on determination of the DNA sequence of a series of selected housekeeping, ribosomal, and/or virulence-associated genes (Enright & Spratt, 1999; Urwin & Maiden, 2003). MLST is a highly robust and efficient method in identifying ancestral relationships and segregating outbreak associated strains according to their genome species status. To date; three MLST schemes are available for *Leptospira*. The first one published employed six loci (*adk, icdA, lipL32, lipL41, rrs,* and *secY*). This scheme comprises all pathogenic *Leptospira* spp. and has made a database with sequences of nearly 120 strains available for off-line (Ahmed *et al*., 2006). The second scheme was updated to incorporate a total of seven pathogenic species which were developed earlier by Thaipadungpanit *et al*. (2007). By switching the *fadD* locus for *caiB* and introducing degenerate bases into the primers, it is now possible to identify an additional five species to improve the accuracy and potential for the technique (Boonsilp *et al*., 2013). The third scheme was Introduced by Varni and colleagues (Varni *et al*., 2013) who re-assessed *Leptospira* MLST data from studies published earlier, together with new sequence sets from regional isolates. Interestingly enough, they noted a unidirectional correlation between sequence types and serogroup, where sequence types contained

isolates belong to a single (or at most two) serogroup. The tested isolates exhibited congruence between allelic profile and serogroup, providing an alternative to serological methods. MLST is a simple molecular technique that does not require large quantities of purified DNA. It is therefore possible to use this method worldwide as a routine technique for generating reliable, reproducible and easy-to-interpret results that are widely exchangeable (Cerqueira & Picardeau, 2009).

2.8.3.4 Multilocus Variable Number Tandem Repeat Analysis

Many demands are placed on new typing methods, including high discriminatory power so that unrelated and related strains can be identified (Struelens, 1996) and should be easy to perform, interpret and it should be possible to standardize, so that results can be exchanged between laboratories and be effective for local, national, and international surveillance (Struelens, 1996).

MLVA involves amplifying the target loci by polymerase chain reaction and then measuring (either by gel or capillary electrophoresis) the lengths of the amplified DNA segments. The number of repeats for each locus is inferred by subtracting the known length of the flanking sequence from the total amplicon length and dividing the result by the known length of each repeat sequence. The MLVA result or strain-specific profile is a series of numbers, each of which represents the number of repeats at one of the loci in a standard order (Gilbert, 2008). Majed *et al.* (2005) and Slack *et al.* (2005) described a MLVA typing scheme for *L. interrogans* srains. These researchers highlighted the value of using VNTR as a typing scheme in identifying serovars types and also in discriminating the strains within same serovar (Majed *et al.*, 2005; Slack *et al.*, 2005).

Salaun *et al.* (2006) extended multilocus VNTR analysis (MLVA) identification of strains to species other than *L. interrogans*, including *L. kirschneri*, and *L. borgpetersenii*. They showed that this simple PCR-based MLVA typing technique is a powerful methodology for the epidemiology of leptospirosis. However, this technique has some limitations such as inaccuracy of agarose gel electrophoresis and problems associated with the transfer of the data between different laboratories. Instead of agarose gel electrophoresis, most laboratories now use capillary gel electrophoresis.

2.8.3.5 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Schwartz & Cantor, 1984). PFGE is often considered the "gold standard" of molecular typing methods. Bacterial strains grown either in broth or on solid media are combined with molten agarose and poured into wells of PFGE plug molds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are then subjected to in situ detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme (Olive & Bean, 1999). Then the digested bacterial plugs are inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of very-large-molecular-length DNA fragments ranging from 10 to 800 kb (Schwartz & Cantor, 1984). After staining the gel in ethidium bromide and destaining, the gel can be photographed and analyse using commercially available software. Several studies proved PFGE as a useful technique allowing determination of *Leptospira* strains at the serovar level through the analysis of the whole genome DNA (Galloway & Levett,

2008; Galloway & Levett, 2010, Kositanont *et al*., 2012; Moreno *et al*., 2016). Hermann *et al*. (1992) reported good concordance between the results of PFGE and serotyping. However, discrepancies between PFGE and serological methods have also been described. As PFGE has been found to be unable to discriminate between *L. interrogans* serovars Icterohaemorrhagiae and Copenhageni. Nonetheless, almost 90% can be identified on the basis of their unique PFGE patterns (Galloway & Levett, 2008). The main advantages of this technique are the high level of reproducibility and discrimination. The discriminatory power can be increased further by the use of more than one restriction enzymes. The major difficulties associated with PFGE relate to the technical demands of expensive equipment and time-consuming process varying from 1 – 3 days dependent upon individual protocols.

2.8.4 Antimicrobial Susceptibility Test

The clinical presentation of leptospirosis is unspecific, which frequently leads to misdiagnosis (Bruce *et al*., 2005). Misdiagnosis of this infection has become a critical issue, where dengue, malaria and other infectious diseases shared a similar clinical manifestations. Treatment normally follows an empirical chemotherapy route, which requires information regarding the susceptibilities of *Leptospira* isolates to various antimicrobial agents. Effective and appropriate antibiotic selection for treatment is essential in order to prevent complications. Several studies have been carried out on antimicrobial susceptibility of *Leptospira* isolates (Chakraborty *et al*., 2010; Miraglia *et al*., 2013). However, there is variability in susceptibility to some antimicrobial agents among different strains, suggesting that more extensive testing to look for geographic variability is needed. In general, *Leptospira* is sensitive to certain antibiotics such trimethoprim, sulfamethoxazole, ampicillin, penicillin G and doxycycline. The later,

has been widely recommended and utilized for the prophylaxis and treatment of mild leptospirosis (Suputtamongkol *et al.*, 2004; Ressner *et al.*, 2008; Chakraborty *et al.*, 2010). Most of the previous studies were carried using minimum inhibitory concentration (MIC), as *Leptospira* is slow growing in solid media. Recently, Wuthiekanun *et al.* (2013, 2015) introduced a solid culture medium (*Leptospira* Vanaporn Wuthiekanun [LVW] agar) led to the description of susceptibility testing of *Leptospira* spp. using the Etest method. This method showed to be easy to perform and could become useful in initial screening test for the epidemiological surveillance of antimicrobial resistance of *Leptospira* spp. However, the technique of antimicrobial susceptibility testing has some shortcomings such as the result is not available until 2 days after the chemotherapy has started, different species have different susceptibilities to the same antibiotics, and breakpoints of different values must be tested. There is also no international agreement for the interpretation of breakpoints in antibiotic susceptibility tests. Finally, several of the presently performed susceptibility tests are highly dependent on experimental conditions (Brown-Elliott *et al.*, 2012).

2.9 Genome

Recently, the leptospiral genome has been sequenced and some of their genetic features were studied in detail providing valuable information of leptospiral virulence factors and helped in understanding of the pathogenesis and immunity-induced mechanisms (Ahmed *et al.*, 2012). The size of the genome differs from species to species. For example there is big difference between pathogenic and saprophytic. In saprophytic *L. biflexa* one third of the genes within the genome are not present in pathogenic species. These relate to processes developed to aid survival under more diverse, potentially extreme, environmental conditions such as a greater number of

nutrient acquisition and sensing mechanisms (Adler *et al*., 2011). Both pathogenic and saprophytic strains require different pathways for survival. However, there is also a differentiation within individual pathogenic species. The genomes of *L. borgpetersenii* showed approximately 700 kb reduction compared to *L. interrogans* and revealed substantial differences in genetic content and organization. It also suggested that *L. borgpetersenii* is undergoing a process of insertion sequence (IS)-mediated genome reduction and might be evolving towards dependence on a strict host-to host transmission cycle (Bulach *et al*., 2006).

The complete genomes that have been published include of *L. interrogans* serovar Lai (Ren *et al*., 2003), *L. interrogans* serovar Copenhageni (Nascimento *et al*., 2004), the genome sequencing of two serovar Hardjo type Bovis strains L550 and JB197 of *L. borgpetersenii* (Bulach *et al*., 2006) and the genome sequence of *Leptospira biflexa* (Picardeau *et al*., 2008).

CHAPTER 3: PUBLISHED WORKS

In this chapter, nine original works in which the candidature is the principal author are included. Each publication is in the original published format of the respective journals except for the paper accepted in the journal of Tropical Biomedicine which still in process for publication. Each publication is preceded by declaration of the contribution of the co-authors.

3.1 Publication 1

Human Leptospirosis in Malaysia: Reviewing the Challenges After 8 Decades (1925-2012). Benacer D, Thong KL, Verasahib KB, Galloway RL, Hartskeerl RA, Lewis JW, Mohd Zain SN.

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3.1.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D
Analyses of data	Benacer D
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Review

Human Leptospirosis in Malaysia: Reviewing the Challenges After 8 Decades (1925-2012)

**Douadi Benacer, Msc[1], Kwai Lin Thong, PhD[1],
Khebir Bin Verasahib, PhD[2], Renee L. Galloway, MPH[3],
Rudy A. Hartskeerl, PhD[4], John W. Lewis, PhD[5],
and Siti Nursheena Mohd Zain, PhD[1]**

Abstract

The history and epidemiology of human leptospirosis in Malaysia from 1925 to 2012 are described. Previous studies have demonstrated that leptospirosis is an endemic disease in Malaysia occurring in both urban and rural locations. The number of cases has risen dramatically since the Ministry of Health Malaysia highlighted leptospirosis as a notifiable disease in 2010, with reported cases increasing from 248 cases in 2004 to 3604 in 2012. The incidence of infection among the population suggests that occupation, sex, age, ethnic background, water recreational activities, and sporting events are risk factors. A robust surveillance system is now in place to monitor temporal and spatial changes in the incidence and prevalence of infection and to identify risk areas and disease behavior. Despite extensive studies over the past decade, there is a still a need to describe local serovars in host carriers and the human population, with the view to develop an effective vaccine against leptospirosis.

Keywords

Leptospira, leptospirosis, outbreaks, rodents, risk factors, prevention

Introduction

Leptospirosis is an emerging zoonotic disease with more than 1 million cases per year globally.[1] It has been recognized as a reemerging infectious disease, particularly in tropical and subtropical countries.[2] A striking rise in the number of reported cases and frequency of outbreaks have been reported in Southeast Asia (Thailand, India, Malaysia, and Indonesia) and also Central and South America.[3,4] The number of human cases worldwide is not well documented, and as a

[1]University of Malaya, Kuala Lumpur, Malaysia
[2]Ministry of Health, Putrajaya, Malaysia
[3]Centers for Disease Control and Prevention, Atlanta, Georgia, USA
[4]WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam, Netherlands
[5]University of London, Surrey, TW, UK

Corresponding Author:
Siti Nursheena Mohd Zain, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Email: nsheena@um.edu.my

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consequence of underreporting in many areas of the world, the prevalence of leptospirosis in the tropics varies from 0.1 to 10 per 100 000 population, with an average case fatality rate (CFR) of 10%.[5] It is possible that 100 or more of 100 000 may become infected during outbreaks or in high-risk areas.[6] However, these figures are likely to be an underestimate because of misdiagnosis and inadequate surveillance systems in many countries.[7]

Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*, with at least 22 species classified according to DNA-DNA hybridization analysis and more than 300 serovars based on agglutinating LPS antigens.[8] Rodents, dogs, cattle, and swine are regarded as the principal reservoirs for this spirochete, which has been reported in at least 150 mammalian species.[9] Human and animal infections are acquired via direct contact with urine, blood, or infected animal tissue or indirect contact with water or soil contaminated with the urine of reservoir animals.[2] Although particular serovars have been related to specific animal reservoirs, all animals are susceptible to infection by any serovar.[2] In the past, leptospirosis was recognized as an occupational disease, infecting mainly farmers, veterinarians, miners, fishermen, and abattoir and sewer workers.[10] However, travel to endemic areas and recreational activities such as water sports have increasingly been reported as major risk factors for this infection.[3]

In Malaysia, leptospirosis is a reemerging disease, and the high humidity and warm temperatures allow *Leptospira* to survive for long periods in the environment. Heavy rainfall and frequent flooding, especially during the monsoon season, also increase the incidence of leptospirosis. Several outbreaks of leptospirosis associated with rainfall have been documented.[11,12] Historically, the annual incidence of leptospirosis in Malaysia ranged between 1 and 10 per 100 000 population.[13] Despite the dramatic increase in cases reported over the past 9 years (Figure 1), the human incidence is still likely to be underestimated because of misdiagnosis and underreporting, and the epidemiology of this infection remains unclear.

The present review describes the historical and epidemiological features of leptospirosis in Malaysia, and preventive measures are discussed with the view to reduce the possible risk of disease transmission.

Methods

MEDLINE, PubMed, and Scopus databases were used to search for peer-reviewed articles on the current status of leptospirosis as well as risk factors, control, and prevention. Studies on the history and current status of the disease in Malaysia were obtained by searching local university libraries and websites of the local government health office. Data on the status of leptospirosis in Asia-Pacific countries were also presented.

Historical Aspects of Human Leptospirosis in Malaysia

Inada et al[14] isolated *Leptospira* for the first time from human patient blood in Japan, in 1915,[14] whereas in 1928, Fletcher was not only the first to isolate this pathogen from the blood, liver, and kidneys of 21 patients in Malaysia,[15] but also identified 3 different serovars—namely, *Leptospira interrogans* serovar Icterohaemorrhagiae, *L interrogans* serovar Hebdomadis, and *L interrogans* serovar Pyrogenes. Fletcher also introduced a new medium for the isolation of leptospires, and this is still used in many laboratories in Malaysia and worldwide.

Earlier work in Malaysia primarily focused on those individuals serving in the military. In 1957, McCrumb et al[16] reported that of 614 military personnel and 238 civilian patients suffering from fever who were admitted to the military hospital, 34.7% and 13%, respectively, were confirmed to be infected with *Leptospira*, based on serology tests.[16] Tan and Lopes[17] carried out a survey on 2 categories of Malaysian soldiers. The first category included those in service between 6 months to 20 years, whereas the second category included the new recruits (2 to 3 weeks) in

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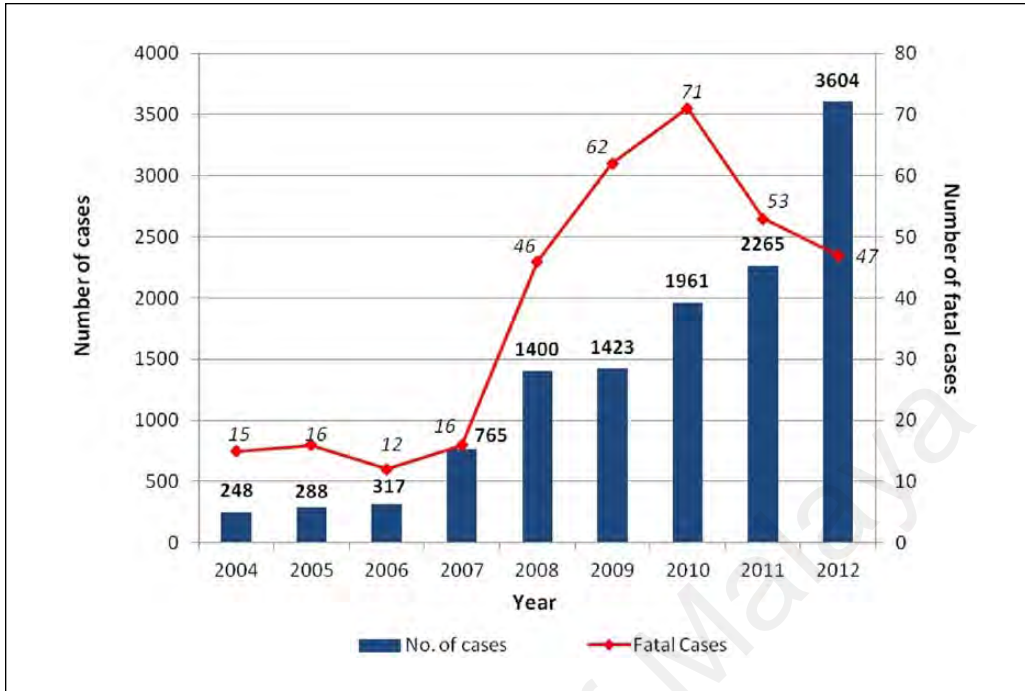
Figure 1. Reported leptospirosis cases in Ministry of Health hospitals, Malaysia, between 2004 and 2012.

training. Based on serological results, the prevalence values among newly recruited soldiers (22.0%) was almost double compared with those who had been in service longer (12.1%). In another survey, Supramaniam[18] reviewed the status of leptospirosis among army personnel and confirmed through serological testing that 2 fatal cases in 1969 and 1978 were a result of leptospirosis. In this survey, only 4.6% of soldiers presented with fever symptoms, although a higher incidence of 30 % was recorded for British soldiers.

Several studies were also extended to include civilians in order to link occupation, intrinsic factors (sex and age), and ethnicity. Tan,[19-21] Ungku Omer,[22] and Shafei et al[23] noted that rubber plantations laborers and those dealing with sewage, drainage, forestry, and town cleaning as well as antimalaria workers were at high risk of infection, whereas laborers, military personnel, tin miners, farmers, and paddy planters were at moderate risk. Shop owners, policemen, and veterinary staff also fell in the moderate-risk group, whereas school teachers, housewives, and office workers had the least risk.

Relative to host age, the highest incidence occurred among patients between the ages of 20 and 40 years, with a higher rate of infection among male patients.[24,25] Based on ethnicity, infection among the Malays was the highest, followed by the Indians and Chinese. The general clinical features recorded included fever, chills, jaundice, abdominal pain, cough, and hepatomegaly.[24,25]

Chronology of Leptospirosis Outbreaks in Malaysia

Since the first reported case in the early 1920s, subsequent outbreaks have been reported, with an increasing number of cases over the years. The chronology (Table 1) and location (Figure 2) of leptospirosis outbreaks in Malaysia since 1984 are given below.

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Table 1. Chronology of Leptospirosis Outbreaks in Malaysia.

| Outbreaks	Year	Description	Reference
Mulu caves, Sarawak	1984	After exploration of the Mulu caves in Gunung Mulu National Park, Sarawak, 16 British cave explorers return to Britain ill; 5 patients had fever of unknown origin and hepatomegaly with no renal failure. Leptospirosis was suspected and later confirmed by serology	Waitkins (1986)[26]; Self et al (1987)[27]
Sarawak	1985	A group of British tourists visited the Sarawak chamber and 2 contracted leptospirosis	Sheena and Buchan (1987)[28]
Beaufort, Sabah	1999	After swimming in a creek near an oil palm plantation in Kampung Kebatu, Beaufort, Sabah, 46 locals fell ill. One fatality was reported when a 15-year-old boy died from hemorrhagic shock secondary to pulmonary hemorrhage. Investigations revealed creek water contaminated with urine tainted with leptospirosis of animal origin (cattle, pigs, dogs, rodents, and wild animals), with prior flooding facilitating the spread of the organism	Koay et al (2004)[11]
The Eco-Challenge, Segama River, Sabah	2000	Athletes kayaking and swimming in the Segama River were diagnosed with leptospirosis. This outbreak was recognized as the first international outbreak associated with outdoor adventure. Experts pinpointed the river water as the source of outbreak. Athletes who took doxycycline prior to the challenge were spared from infection	Sejvar et al (2003)[29]
Johor	2006-2007	Following floods that affected all 8 districts in Johor between December 2006 and January 2007, 20 cases of leptospirosis, with 2 deaths, were reported	Badrul Hisham et al, 2009[12]
Juru, Penang	2009	There were 26 leptospirosis cases, with 2 deaths, reported at the illegal migrant detention center in Juru, Penang. The 2 who died were Burmese migrants, and drinking water contaminated with animal urine, potentially rats, was suspected to be the cause	International Detention Coalition (2009)[30]
Maran, Pahang	2010	A total of 8 deaths were reported among the 83 people involved in the rescue operation of a drowned victim. The investigations disclosed that the river water was contaminated with urine of rats or other animal carriers. The infections occurred while rescuers used river water for their daily chores. On outbreak confirmation, the recreational park was temporarily closed to the public	Sapian et al (2012)[31]
The Bukit Jugra Royal Malaysian Air Force base	2011	A total of 24 air force trainee commandos were infected; it was confirmed serologically as caused by *Leptospira.*. Investigation showed that the infection occurred while training in water contaminated with urine of rats or other animals	Malay Mail Online (2011)[32]
Kangar, Perlis	2012	A family of 8 of 28 men who went fishing at a swamp developed symptoms and were hospitalized in Hospital Tuanku Fauziah, Kangar, Perlis. Serological tests for leptospiral IgM confirmed that 6 of the 8 men tested positive. Water samples from the swamp were screened and confirmed by PCR as being tainted with *Leptospira*	Baharudin et al (2012)[33]

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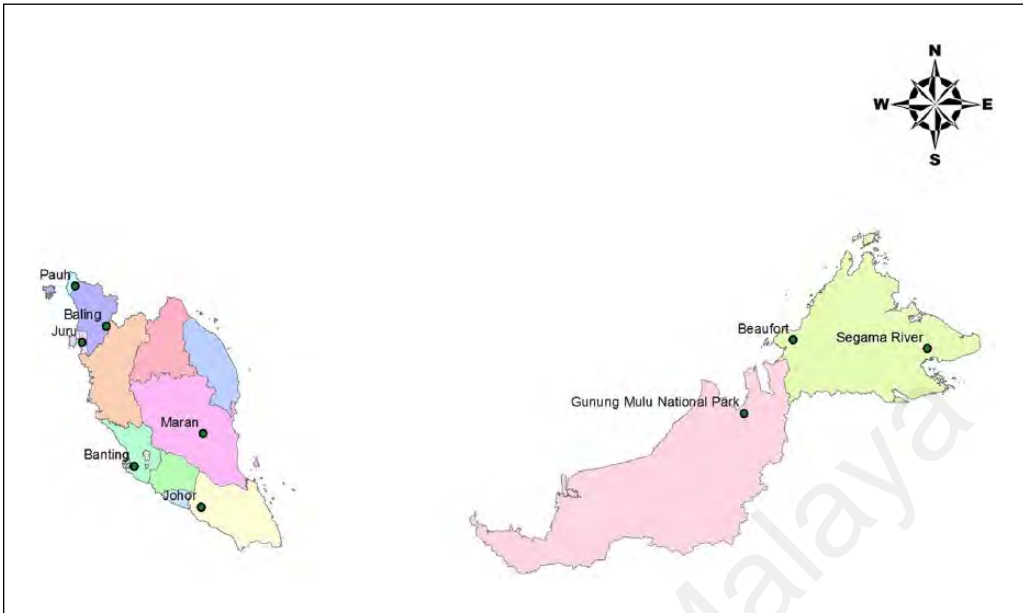
Figure 2. The map shows the sites of reported human leptospirosis outbreaks in Malaysia.

Current Status and Future Challenges

Despite extensive studies over the past 8 decades, there are still gaps in our knowledge related to the epidemiology of human infections. Misdiagnosis and underreporting are the main obstacles in estimating the incidence not only in Malaysia but also in many Asian countries because of the similarity in clinical symptoms with other tropical diseases such as dengue fever, malaria, or hemorrhagic fever.

The present method of diagnosis includes the microscopic agglutination test (MAT), which is undertaken by a small number of public health laboratories, which focus on the detection of positive cases without first determining the circulation of serovars locally. However, with MAT false-negative results do occur, and an extensive range of antisera is required. Tests in Malaysia are run within such a limited range of 25 antisera that new serovars have not been identified domestically.[34] Positive samples need to be processed further at overseas reference laboratories, but this is not always followed through. Without this crucial information, there is no evidence to link the modes of transmission between humans and potential host reservoirs to provide a further understanding of the epidemiology of this disease. More important, the development and commercialization of a vaccine is not possible.

Prior to 2010, the number of reported cases and outbreaks increased significantly, resulting in a high number of deaths. This added pressure on local health authorities made them recommend that leptospirosis be classed as a mandatory notifiable disease from December 2010. Following the directive, between 2011 and 2012, 5869 cases were reported with more than 100 fatalities (Figure 1). The annual incidence rates ranged from 7.83 to 12.49 cases per 100 000 population, and the CFR decreased from 2.42% in 2011 to 1.30% in 2012. The highest incidences occurred in the west-coast states of Peninsular Malaysia—namely, Selangor, Perak, Kelantan, and Pahang (Figure 3), with most cases being reported during heavy rainfall and flooding during the monsoon season. This demonstrated that extrinsic factors such as climatic conditions played a major role in disease transmission.[35] Intrinsic factors such as host sex were also shown to influence

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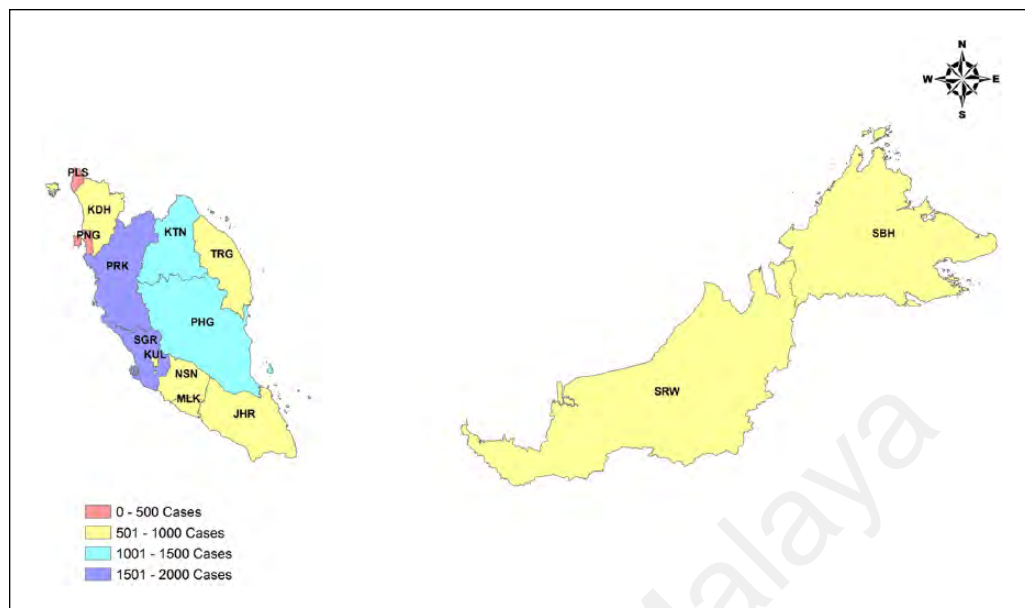
Figure 3. The map shows the number of reported human leptospirosis cases from 2004 through 2012 in the 13 states.
Abbreviations: PLS, Perlis; KDH, Kedah; PNG, Penang; PRK, Perak; KTN, Kelantan; PHG, Pahang; SGR, Selangor; KUL, Kuala Lumpur; NSN, Negri Simbilan; MLK, Melaka; JHR, Johor; SRW, Sarawak; SBH, Sabah.

infection, with men at a higher risk of contracting leptospirosis than women (by 4:1) because of the nature of their occupations and more extensive outdoor activities.

The epidemiology of leptospirosis is dynamic. New risk groups may be formed as a result of changes in agricultural or social practices or new reservoir hosts may emerge. Continuous surveillance, improved management systems, and the establishment of a monitoring system are key components in regulating the impact of this disease. Because of its sporadic nature, leptospirosis is not given the same priority as dengue fever and malaria to the extent that high-impact research activity, disease monitoring, and long-term control measures, especially of rodent populations, have been overlooked by government bodies and organizations.

Leptospirosis in the Asia-Pacific Region

Similar to the scenario seen in Malaysia, other countries in this region underestimate the disease burden of leptospirosis because of underreporting and the unavailability of laboratory diagnostics. The South East Asia region, in particular, is noted to have the highest incidence, often influenced by sociocultural, occupational, behavioral, and environmental factors. Rural areas are at higher risk because the population is mainly involved in agriculture or animal husbandry, and animals are present in large numbers.[36] The annual incidence in this region varied from low (<1 per 100 000), to moderate (1-10 per 100 000), to high (>10 per 100 000),[3] with mortality between 5% and 40%.[37]

Thailand recorded the highest number of cases, which occurred primarily during the rainy season. Disease notification reports indicated a drastic increase in the number of cases, with an incidence of 0.3 per 100 000 in 1995, which peaked in 2000 to an incidence of 23.7 per 100.000 population.[36] The outbreaks corresponded with the wet season in August and declined by November.

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In the past decade, the number of reported cases stabilized to between 2800 and 5500 cases annually.[38] As of December 1, 2008, reported cases totaled 3350, with 59 deaths in 71 of 76 provinces in 48 weeks. The overall incidence was 5.3 per 100 000 population, with a male to female ratio of 1:0.25.[36] The predominant serovar circulating in the population was *L interrogans* serovar Autumnalis.[39]

In the Philippines, both urban and rural areas recorded outbreaks usually during the typhoon season (July-October).[40] The most prevalent *Leptospira* serovars isolated were Pyrogenes, Bataviae, Pomona, Grippotyphosa, Manilae, and Javanica mainly among workers involved with animals, such as abattoir and dog pound employees as well as fish inspectors.[3] During a typhoon that occurred between September and mid-November 2009, Metro Manila reported 2299 patients, including 178 who died (CFR of 8%), in 15 hospitals with suspected signs and symptoms of this illness, as determined by the Department of Health.[41]

In Indonesia, leptospirosis is often linked to the disease being clinically unapparent, too mild for definitive diagnosis, or misdiagnosed as dengue fever or other endemic diseases; laboratory facilities to perform confirmatory tests are also lacking.[42]

The annual number of reported cases from 2007 to 2011 were 664, 426, 335, 409, and 857, respectively; however, not all provinces routinely report leptospirosis cases to the authorities. The annual case fatalities recorded for 2007 to 2011 were 8.28%, 5.16%, 6.87%, 10.51%, and 9.57%, respectively.[43] However, the actual number was possibly higher. The predominant serovar circulating in the population was identified as *L interrogans* serovar Bataviae.[44]

Leptospirosis has been endemic in India since the early 20th century and related both to monsoon and poor sanitary conditions, with multiple epidemics reported in recent years.[45,46] The Andaman and Nicobar Islands reported the highest incidence, where serovars Ratnapura, Valbuzzi, and Grippotyphosa were reported as causes of severe epidemics.[46] Based on data published in 2013, the prevalence was 38.1% in Calicut, 52.7% in the Andaman and Nicobar islands, and 32.9% in Chennai.[47]

Leptospirosis has been gazetted a notifiable disease in all territories of Australia since 1988.[48] Queensland and Victoria recorded the highest incidences,[49] with the average annual incidence in humans being 1.0 per 100 000 population[50] and prevalent among agriculture sector workers, especially banana farmers and dairy workers.[51] In 2009, 149 cases were reported nationally, with more than 75% of the cases in Queensland.[52] A total of 24 serovars were isolated from domestic, native, and feral animals and included the following species: *L interrogans, L borgpetersenii, L santarosai, L weilii*, and *L meyeri*.[50]

Mode of Transmission and Maintenance Hosts

Leptospirosis is caused by pathogenic organisms belonging to the genus *Leptospira*, and human infections are the result of direct or indirect exposure to urine of infected animals. Other modes of transmission include handling infected animal tissues and ingestion of contaminated food and water.[53] Presence of moisture is an important factor to the survival of this pathogen in the environment.

In other regions, different serovars are prevalent and associated with one or more maintenance hosts, which are most often wildlife species and, sometimes, domestic animals and livestock.[54] Although the rodent was recognized as the primary carrier, especially in South East Asia, farm animals and dogs have also been incriminated as common reservoirs that can shed leptospires throughout their life span without clinical manifestations.[53]

In Malaysia, *Leptospira* was first isolated from the kidneys and urine of the black rat (*Rattus rattus*).[15] Other studies incriminating the rodent followed suit in the South East Asia region,[3,55,56] isolating primarily the Icterohaemorrhagiae serovar, however other serovars have also been isolated.

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Historically, leptospirosis was recognized as a disease of dogs prior to any other animals, including humans.[57] Dogs are primarily carriers of Canicola serovars; however, other serovars have also been reported: Icterohaemorrhagiae, Pomona, Bratislava, and Grippotyphosa.[58] Similar studies in highlighting dogs as carriers were also conducted in India and Thailand.[55,59] Farm animals such cattle, sheep, and pigs are noted to be maintenance hosts for several *Leptospira* serovars, and therefore, pose a high risk of infection, especially to farmers, abattoir workers, and livestock handlers. In the Asia-Pacific region, several serovars were reported from cattle, sheep, and pig.[60,61] Generally, cattle are the natural host of serovars Hardjo, Pomona, and Grippotyphosa, whereas pigs harbor Pomona, Tarassovi, and Bratislava and sheep harbor Hardjo and Pomona.[62] Among wild animals, *Leptospira* sp have been isolated from monkeys, bats, squirrels, and mongooses.[63]

Risk Factors

Several factors contribute to the spread of leptospirosis, especially in tropical and subtropical regions, where the warm weather and heavy rainfall make it favorable for the survival of leptospires in the environment. Natural disasters, such as cyclones and subsequent flooding, have been noted as high-risk factors because of the leaching of leptospires from the environment. Other factors include types of agricultural and social practices, inadequate housing, and poor sanitation[64] in addition to occupational and recreational activities.[3] Information regarding these vital factors is grossly missing, and therefore, it is imperative that future studies are able to relate the risk factors to disease incidence in order to predict disease outbreaks.

Recreational Activities

In recent years, recreational activities have emerged as the primary risk factor for leptospirosis in Malaysia,[31,33] especially after 2000,[29,31] particularly outdoor and leisure activities, including gardening and water sports (canoeing, swimming, and fishing). Those at risk include international visitors, particularly those travelling to endemic areas and engaging in jungle activities, camping, hiking, kayaking and water skiing,[6] and cave exploration.[65] In a recent study among febrile patients (with no other associated symptoms for inclusion criteria) in the northeastern states of Malaysia, serological (ELISA, MAT) results revealed that 16.2% (33/204) of patients became positive after a recent recreational activity.[66]

Occupational Exposure

Leptospirosis has been recognized as an occupational disease for many decades, occurring mainly among sewer workers, miners, and fish farmers. These workers are at a higher risk of contracting the disease because of their constant contact with water polluted with leptospires via contaminated urine of reservoir hosts. Nevertheless, workers in other occupations, such as garbage collectors, abattoir workers, farmers, livestock handlers, rodent control workers, pet shop workers, rice field workers, rubber and palm oil collectors, and military personnel have recently also been identified as at risk of contracting the infection.[3,23,37,66]

Natural Disasters

Several leptospirosis outbreaks have been linked to flooding, typhoons, and cyclones.[3,12,67] Floods damage water and sanitation networks, displace populations, destroy homes, and increase environmental exposure to pathogens. Floods also flush drains, leading to the contamination of water bodies with the urine of infected dead animals, which become a source of contagion.[67] In

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2007, serological investigations carried out after a major flood hit in Johor confirmed 20 leptospirosis cases, with 2 deaths, among 2000 cases of waterborne diseases reported.[12]

Other Factors

Rapid urbanization and the high population density in the cities of developing countries have attracted the primary host reservoir of this disease—rodents. A recent study identified 2 pathogenic serovars infecting the population—predominantly *L borgpetersenii* serovar Javanica (85%) and *L interrogans* serovar Bataviae (15%).[34] The great abundance of rodents has been linked to increased human exposure to the pathogen; however, it is also dependent on human susceptibility, which varies according to time and place. Overcrowding of cities, with inadequate living facilities, poor sanitary systems, improper garbage management, and the boundless presence of stray animals in the environment increase the risk of leptospirosis in humans.[68]

Control and Prevention

The control and prevention of leptospiral infection is a complex process because it requires an understanding of the epidemiology of the disease before proper intervention can be put in place to reduce the risk of infection. The primary method of prevention can be achieved by controlling the infection source (reservoir), the transmission route between infection source and human host, and the infection in the human host.

Reservoirs. Rodents are known to be natural reservoirs and potential lifelong carriers of leptospires,[69] not only for humans but also for domestic animals. Several strains are directly linked to rodents such as *L interrogans* serovar Copenhageni, *L interrogans* serovar Icterohaemorrhagiae, *L kirschneri* serovar Bim, and *L borgpetersenii* serovar Ballum.[2,70] Mohamed-Hassan et al[71] also noted leptospiral antibodies to serogroups Canicola, Pyrogenes, and Hebdomadis from *L interrogans* species in rats. Despite some positive reports on the use of antileptospiral vaccines in humans, long-term studies have not been carried out.[2] Rodent control is essentially the most effective method of preventing human infection, particularly in urban areas. Currently, the conventional use of rodenticides and continuous trapping in urban cities have reduced the rodent population, thus reducing the risk of infection. Good sanitation and proper garbage disposal are important factors to reduce the rodent population in urban areas. Immunization of livestock and pets can reduce the risk of human transmission; however, the existence of wildlife reservoirs has complicated prevention. Therefore, it is advisable for owners to minimize contact between their animals and rodents and other wild animals.

Water and Soil in the Environment. Pathogenic leptospires colonize the renal tubules of mammals and are shed in their urine, contaminating surface water and soil, where leptospires can survive for long periods. Early research on the isolation of *Leptospira* from water and soil began in the 1970s in Malaysia after Alexander et al[72] successfully isolated 29 serovars from natural waters and wet soils. Several pathogenic *Leptospira* species were successfully isolated—namely, *L interrogans*,[72] *L Borgpetersenii*,[73] and *L kmetyi*.[74] More recently, Benacer et al[75] recovered 1 pathogenic species (*L alstonii*) and 1 intermediate species (*L wolffii*) from water and soils in urban sites. The removal of leptospires from the surrounding water and soil is certainly impractical. However, drainage of wet areas and minimizing indirect contact with urine-contaminated soil and water remain the most effective preventive measures. It is advisable to cover blisters, cuts, or broken skin with waterproof plasters, especially while swimming in fresh water. Wearing protective clothes during recreational and occupational activities may additionally decrease the risk of infection.

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Public Health. Based on the cases reported, more than 60% were the result of occupational or recreational activities. Therefore, creating awareness among high-risk groups about disease transmission and taking preventive measures is the key to prevention. Public health authorities can take the initiative to provide information packs to be distributed to high-risk groups, which includes those serving with the military and adventure travelers. Information should include advice on drinking only purified and boiled water, proper water and food storage, avoiding prolonged immersion in and consumption of river water, and finally, wearing waterproof clothing such as rubber boots and gloves.[76] Tour companies and adventure sport organizers could also assist in distributing the information packs to travelers.

Finally, preexposure chemoprophylaxis could become increasingly important as more people engage in adventure travel and ecotourism. The use of doxycycline as a prophylactic measure has been reported.[29,77] Sejvar et al[29] noted that taking doxycycline prior to engaging in water activities was effective as a prophylaxis for leptospirosis in individuals with identifiable, short-term exposure to high-risk activities and environments. However, such a prophylactic method is only suitable when exposure can be accurately predicted and only for a short period of time. Therefore, the use of doxycycline is unlikely to be applicable as a general public health measure unless convenience-based clinical trials are undertaken in the longer term.

Conclusion

Eight decades after it was first discovered in Malaysia, leptospirosis is still a widely prevalent disease in the country, with an increasing number of cases over the years, especially after leptospirosis was gazetted as a notifiable disease in 2010. Therefore, there is an urgent need to formulate and institute better disease control and prevention strategies. Presently, local authorities play a major role by providing better garbage management and establishing good rodent control programs around human habitation and recreational areas. However, the involvement of more health workers is necessary to educate the public about the dangers of the disease, its mode of transmission among the population, and who is most at risk, so that the disease can be recognized and treated immediately. The limitation of the present study not only relates to the underreporting and misdiagnosis of the disease, but also to the lack of information about the type of serovars that infect the human population in Malaysia. Hence, the method of transmission of leptospires between potential reservoir hosts to humans requires further investigation in order to contribute to our understanding of the epidemiology of the disease. Only when such data are available can an effective veterinary and human vaccine be developed and commercialized to reduce mortality.

Declaration of Conflicting Interests

Funding

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3.2 Publication 2

Epidemiology of human leptospirosis in Malaysia, 2004–2012.

Benacer D, Thong KL, Min NC, Verasahib KB, Galloway RL, Hartskeerl RA, Souris M, Mohd Zain SN.

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3.2.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Thong KL, Mohd Zain SN
Analyses of data	Benacer D, Min NC, Souris M
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Epidemiology of human leptospirosis in Malaysia, 2004–2012

Douadi Benacer [a], Kwai Lin Thong [a], Ng Choung Min [b], Khebir Bin Verasahib [c], Renee L. Galloway [d], Rudy A. Hartskeerl [e], Marc Souris [f], Siti Nursheena Mohd Zain [a],*

[a] Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia
[b] Institute of Mathematical Sciences, Faculty of Science, University of Malaya, Malaysia
[c] Disease Control Division, Ministry of Health, 62590 Putrajaya, Malaysia
[d] Bacterial Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA
[e] Royal Tropical Institute (KIT), Amsterdam, The Netherlands
[f] Institut de Recherche pour le Développement, UR 178 Marseille, France

ABSTRACT

Leptospirosis is an emerging disease, especially in countries with a tropical climate such as Malaysia. A dramatic increase in the number of cases has been reported over the last decade; however, information on the epidemiological trends of this disease is lacking. The objective of this study is to provide an epidemiological description of human leptospirosis cases over a 9-year period (2004–2012) and disease relationship with meteorological, geographical, and demographical information.

A retrospective study was undertaken to describe the patterns of human leptospirosis cases and their association with intrinsic (sex, age, and ethnicity) and extrinsic (location, rainfall, and temperature) factors. Data was grouped according to age, sex, ethnicity, seasonality and geographical distribution, and analyzed using statistical tools to understand the influence of all the different factors on disease incidence.

A total of 12,325 cases of leptospirosis were reported between 2004 and 2012 with an upward trend in disease incidence, with the highest in 2012. Three hundred thirty-eight deaths were reported with an overall case fatality rate of 2.74%, with higher incidence in males (9696; 78.7%) compared with female patients (2629; 21.3%), and overall male to female ratio of 3.69:1. Patients aged cohorts between 30–39 years old (16.22 per 100,000 population) had the highest disease incidence while the lowest incidence occurred between <1 to 9 years old (3.44 per 100,000 population). The average incidence was highest amongst Malays (10.97 per 100,000 population), followed by Indians (7.95 per 100,000 population). Stratification according to geographical distribution showed that the state of Malacca had the highest average disease incidence (11.12 per 100,000 population) followed by Pahang (10.08 per 100,000 population). The states of Terengganu, Kelantan, and Perak recorded similar rates of incidence ($\approx$8.00 per 100,000 population), while Johor with the least number of reported cases (1.80 per 100,000 population). Positive relationships were recorded between the number of reported cases with the number of raining days per month and monthly average temperature (p-value < 0.05). However, no significant association was noted between rainfall volume and number of reported Leptospirosis cases.

This collaborative efforts between medical, academic and governmental institutions has enabled the construction of this comprehensive database that is essential to understand the disease trends in Malaysia and add insights into the prevention and control of this disease.

1. Introduction

Leptospirosis is an important emerging global zoonotic disease, which is caused by the pathogenic spirochete belonging to the genus *Leptospira*. Twenty-two different species have been classified according to DNA–DNA hybridization and phylogenetic analysis (Bourhy et al., 2014; Saito et al., 2013), and over 300 serovars are based on agglutinating lipopolysaccharide antigens

* Corresponding author at: Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
   E-mail address: nsheena@um.edu.my (S.N.M. Zain).

(Victoriano et al., 2009). Domestic and wild mammals, including rodents, have been implicated as reservoirs for these organisms (Levett, 2001). Human infection is transmitted via direct contact with the pathogenic species through contaminated urine, blood, or infected tissue from animal reservoirs or indirectly from contaminated water and soil (Bharti et al., 2003). The clinical manifestations of leptospirosis vary from asymptomatic to a severe potentially fatal form (Levett, 2001). Leptospirosis is often poorly recognized and may be confused with a variety of other febrile diseases or systemic illness (Lau et al., 2010a) because symptoms are nonspecific and often lead to misdiagnosis.

Leptospirosis is recognized as a reemerging infectious disease with an estimate of more than one million cases per year globally (Cosson et al., 2014). Human infection is centered mainly in the tropical and subtropical regions, with marked incidence in Southeast Asia (Amilasan et al., 2012; Bahaman and Ibrahim, 1988; Hinjoy, 2014; Kawaguchi et al., 2008) as well as the Central and South America regions (Costa et al., 2012; Schneider et al., 2012). The warm and humid weather in these regions allow leptospires to thrive longer in the environment and increases the risk of exposure.

Previous studies have highlighted the influence of ethnicity, gender, season and age on the distribution of leptospirosis in Malaysia (El Jalii et al., 2000; Tan, 1979). Weather conditions, especially in tropical countries, have a significant effect on the occurrence of the disease (Levett, 2001), particularly during the rainy season. The monsoon season, normally accompanied by heavy rainfall and floods, increases the risk of leptospirosis. Numerous outbreaks of leptospirosis have been reported following these extreme weather events in many countries (Amilasan et al., 2012; Kawaguchi et al., 2008; Pappachan et al., 2004).

Leptospirosis is endemic in Malaysia. The first human study was reported in 1925, when Fletcher diagnosed 32 cases from rubber plantation workers and rural inhabitants (Fletcher, 1928). Historically, the annual incidence of leptospirosis in the Malaysian population ranged between 1 to 10 per 100,000 population (Lim, 2011). However, in the previous decade, there has been a dramatic increase in reported cases, with several disease outbreaks primarily related to recreational activities and natural disasters (Badrul Hisham et al., 2009; Sapian et al., 2012).

The emergence of leptospirosis in Malaysia has become a significant public health concern and highlights the putative roles of increased surveillance, including the assessment of local socioeconomic and environmental factors, on the transmission of leptospirosis. In this study, we discuss the epidemiology of leptospirosis over a 9-year period (2004–2012) to obtain a better understanding of the disease trends using a combination of statistical tools to correlate the influence of the demographical, geographical, and socio-environmental factors on the incidence of leptospirosis locally.

2. Methods

2.1. Study area

Malaysia consists of Peninsular Malaysia, which covers the southern most point of Eurasia, and Malaysian Borneo (East Malaysia), which is on the island of Borneo located in the South China Sea. Malaysia borders Thailand in the north of the peninsula, and Indonesia and Brunei on the island of Borneo with both parts separated by the South China Sea. Malaysia is composed of 14 states and covers an area of 329,847 square kilometers (land, 328,657 km^2; water, 1190 km^2) close to the equator. The average temperature is 27 °C and the climate is humid throughout the year, with an annual rainfall exceeding 2000 mm.

The climate on the east coast of Peninsular Malaysia and the coastal parts of Sabah and Sarawak is very much influenced by the monsoon season, particularly from November until March. During this season, the heavy monsoon rain sweeps across these areas. Meanwhile, the western coast of Peninsular Malaysia is less affected by the monsoon because of the protection afforded by the mountain ranges in the central part of the peninsula.

2.2. Data collection

Reported cases of leptospirosis during 2004–2012 from hospitals and private health care facilities from 14 states were provided by the Disease Control Division, Ministry of Health Malaysia. The clinical cases were confirmed based on serological tests (MAT ≥ 400) and PCR. Data were stratified according to age, sex, ethnicity, time of onset (time of year) and geographic location (states).

2.3. Meteorological data

Meteorological data was provided by the Malaysian Meteorological Department (Malaysian Meteorological Department, 2014) and corresponded to the main meteorological station of each state. The information given included the monthly accumulated rainfall, monthly number of raining days, monthly average temperature, monthly average of daily minimum temperature and monthly average of daily maximum temperature for the 9 years of the study (2004–2012).

2.4. Demographical data

Population statistics based on sex and ethnic groups by state for the 9-year period (2004–2012) were obtained from the Malaysian Department of Statistics (Malaysian Department of Statistics, 2014). Data based on ethnicity was available only for a 3-year period (2010–2012). Detailed population data by age was obtained for 2012 and was analyzed thoroughly to determine the incidence of disease according to 7 age groups: <1–9, 10–19, 20–29, 30–39, 40–49, 50–59, and ≥60 years.

2.5. Statistical analysis

Data were analyzed using R software version 2.15.0 (http://www.r-project.org) and Microsoft Excel. Incidences were calculated based on the number of reported cases in the population per 100,000 population. Case fatality rates (CFRs) were obtained by dividing the number of deaths by the number of reported cases of leptospirosis. Analysis of variance (ANOVA) is used to infer about the equality of averages for incidence as well as case fatality rates between the states. Pearson's chi-square (χ^2) test was used to test the difference in counts between groups. The negative binomial regression (Cameron and Trivedi, 2013) was used to estimate the effect of factors (state, gender, ethnicity, and season) with the number of reported leptospirosis cases from 2004 to 2012, where the factors are linked through a log link function to the means of the data. For count data, this approach is commonly used instead of the Poisson regression for situations where overdispersion (variance is greater than the mean) is observed. A p-value < 0.05 was used to indicate statistical significance of the results obtained.

3. Results

3.1. General

Between January 2004 and December 2012, there were 12,325 leptospirosis cases recorded resulting 338 deaths. This gives the
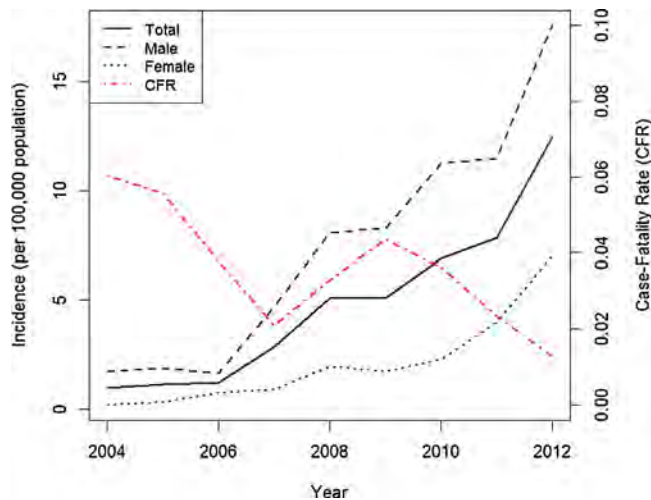
Fig. 1. Trends of incidence and case fatality rate (CFR) of reported cases of leptospirosis for all states in Malaysia during the years 2004–2012.

overall CFR of 2.74%. The annual incidence rate ranged from 0.97 to 12.47 cases per 100,000 population over the 9-year period with an average annual incidence of 4.83 cases per 100,000 population. The CFR and incidence are plotted against time (in reported years) to examine the trends across years (Fig. 1). The incidence markedly increased from 2004 to 2012; however, the CFR declined between those periods apart from a peak from 2007 to 2009.

3.2. Infection according to sex and age groups

Significantly more males were infected than females, with male subjects accounting for 9696 (78.67%) cases compared with 2629 (21.33%) female cases (χ^2 test, *p*-value = 2.2×10^{-16} < 0.01). This corresponded to an overall male to female ratio of 3.69:1, and an average annual incidence of 7.40 and 2.11 per 100,000 population for males and females, respectively. According to state, Sabah (15.62) recorded the highest male-to-female disease incidence ratio in 2010 (Table 1).

Based on the detailed patient demographic description in 2012 (Table 2), analyses showed the mean (±standard deviation) age of the infected patients was 33.79 (±17.55) years with a median of 31 years. The highest incidence occurred in the 30–39-year-old age group (16.21 per 100,000 population) while the lowest incidence occurred in the <1–9 year old (3.4) and 10–19 year old (11.5) age groups. Malacca recorded an unusually high incidence of leptospirosis across all age groups compared with the other states, while Johor recorded the lowest incidence across all age groups compared with the rest (Table 2). Fig. 1 illustrates the incidence trends and CFR for all states from the period of 2004–2012.

3.3. Ethnicity

Malays, the predominant ethnic group in Malaysia, were found to be the highest infected compared with other ethnic groups. The average incidence over the period of 2010–2012 for Malays was 10.97 per 100,000 Malays population, rising from 9.12 in 2010 to 14.18 in 2012. Malays were followed by Indians, the third largest ethnic group, with an increasing incidence from 4.78 in 2010 to 11.94 in 2012, giving an average incidence of 7.95 per 100,000 Indians population. Chinese showed the lowest average incidence of 2.22 per 100,000 Chinese population compared with the other two ethnic groups. Over the 3 year (2010–2012) period for which ethnicity data was available, Malacca, showed a dramatic increase in the disease rate in the Malay community (Tables 3 and 4).

3.4. Geographical distribution

Between January 2004 and December 2012, all the states in Malaysia showed an increase in recorded cases. Malacca recorded the highest confirmed leptospirosis cases with an average incidence rate of 11.12 per 100,000 population (Table 5), followed by Pahang (10.90 per 100,000 population). Terengganu, Kelantan, and Perak recorded similar incidence rates of confirmed leptospirosis cases over the 9-year period (about 8.00 per 100,000 population). Johor recorded the lowest average disease incidence (1.79 per 100,000 population) in 2005, however accounted the highest number of fatal cases with a CFR of 0.20. Overall, the CFR declined throughout the 9-year period. The geographic distribution of the reported human cases is summarized in Table 5 (ANOVA, *p*-values = 0.03 (incidence) and 0.28 (CFR)).

3.5. Season

It is observed that more cases in the peninsular were reported during the wet season than the dry, particularly between the months of October to March and between October to February in east Malaysia. From the negative binomial regression analyses, the number of rain days in a month and the average monthly temperature were significant factors associated with the number of reported leptospirosis cases (Table 6). However, no significant association was observed between the average rainfall with number of reported cases (Figs. 2 and 3).

4. Discussion

Leptospirosis is a long-standing disease in Malaysia. To date more than 37 *Leptospira* serovars from 13 different serogroups have been identified in Malaysia with more than half carried by rodents (Bahaman and Ibrahim, 1988). Since the first human case reported in the early 1920s, the number of cases increased subsequently, particularly over the 9-year study period showing a clear increase in annual incidence rising from 0.97 in 2004 to 12.47 per 100,000 population in 2012, with an average incidence of 4.83 cases per 100,000 population.

The disease trend showed that the number of the cases were linked to frequency of outbreaks (Badrul Hisham et al., 2009; Sapian et al., 2012) especially in the last four years of the study particularly after a major flood during the monsoon seasons (Thayaparan et al., 2013) and in combination of other such as socio-demographic, occupation types, recreational activities, environment and climatic changes. However, the overall disease incidence was still much lower compared our country neighbor, Thailand (6.6 per 100,000 population) (Hinjoy, 2014).

Similarly, the average case fatality rate over the 9-year period (0.03) in this country was also lower compared to Thailand and the Philippines (Hinjoy, 2014; Yanagihara et al., 2007). Mortality rates declined in the last 3 years of this study and despite lower fatality, disease incidence continued to rise, indicating improvements in monitoring and surveillance by the authorities, increased awareness among the population, as well as an improvement in early diagnosis and initiation of supportive therapy and antimicrobial drugs.

Higher number of cases in neighboring countries such as Thailand and Philippines were as a result of natural disasters such as flooding. In Thailand, cases were mainly in the underdeveloped northern-east region at the Thai-Myanmar and mostly occurring amongst agricultural workers primarily to those working in the rice fields.

Similarly, the Philippines is situated in a cyclone zone and infection outbreaks primarily affects urban slum inhabitants of the city

Table 1
The male to female disease incidence ratio, according to all states in Malaysia from 2004 to 2012.

Year	2004	2005	2006	2007	2008	2009	2010	2011	2012
State									
Johor	12.81	12.30	3.81	6.26	9.31	4.40	5.67	3.58	1.17
Kedah	13.96	13.91	–	8.56	5.78	4.79	6.14	4.19	3.37
Kelantan	13.97	8.72	1.82	8.94	3.28	9.73	5.45	2.26	4.55
Malacca	5.92	–	1.01	3.52	11.33	1.83	2.30	1.87	2.13
Negeri Sembilan	7.89	–	–	6.26	4.09	3.08	2.90	2.78	2.03
Pahang	6.28	8.51	3.67	5.92	5.17	5.38	4.09	3.30	2.61
Perak	6.15	9.66	2.29	2.55	3.89	4.84	5.24	2.60	2.53
Perlis	–	–	–	4.13	6.20	–	2.59	3.83	5.81
Penang	8.01	6.00	5.99	10.63	7.30	4.31	6.95	3.11	3.26
Sabah	11.12	2.77	2.77	11.68	7.14	5.52	15.62	3.88	2.11
Sarawak	9.18	4.14	6.12	13.46	1.42	10.43	4.00	4.69	2.96
Selangor	4.71	4.23	3.51	5.14	5.60	4.80	6.20	2.87	2.63
Terengganu	–	3.12	4.08	2.81	2.99	3.72	5.71	3.89	3.66
W.P. Kuala Lumpur	8.98	3.84	2.28	3.99	3.83	2.49	3.48	3.32	1.92
Mean Ratio	8.89	6.45	2.28	5.29	4.12	4.79	4.99	2.89	2.51

Table 2
Incidence per 100,000 population according to age groups and states in the year 2012.

Age Group	Age Groups (Years)							All Age Groups
	<1–9	19–20	20–29	30–39	40–49	50–59	≥60	
State								
Johor	0.34	1.76	2.55	1.88	2.82	3.15	2.31	2.00
Kedah	2.56	17.07	18.32	14.96	13.20	15.13	13.07	13.39
Kelantan	0.86	8.93	16.00	16.74	15.39	11.62	9.62	10.19
Malacca	25.84	44.25	61.59	76.29	39.84	69.60	60.82	52.34
Negeri Sembilan	1.12	10.34	17.21	15.48	17.26	26.78	20.77	14.28
Pahang	4.12	9.51	15.16	16.84	12.59	9.35	5.24	10.51
Perak	5.92	14.70	15.91	13.88	11.78	11.10	5.63	11.54
Perlis	4.95	12.24	15.49	11.95	21.90	0.00	10.99	11.30
Penang	0.87	6.46	5.63	5.43	7.93	13.24	21.89	7.87
Sabah	1.45	7.44	11.14	16.53	18.29	22.32	34.72	12.00
Sarawak	1.52	6.58	16.08	19.95	16.04	12.05	11.74	11.37
Selangor	5.82	16.59	19.11	17.08	14.82	14.11	15.83	14.97
Terengganu	2.98	9.80	15.74	20.54	12.00	11.85	14.29	11.42
W.P. Kuala Lumpur	1.99	16.54	22.76	22.53	13.53	22.74	10.04	16.49
Mean Incidence	3.44	11.48	15.74	16.22	13.63	15.21	14.33	12.47

Table 3
Incidence per 100,000 population based on ethnicity for years 2010–2012.

Year	2010			2011			2012		
Ethnicity	Malay	Chinese	Indian	Malay	Chinese	Indian	Malay	Chinese	Indian
State									
Johor	2.66	0.38	0.92	1.79	0.48	0.91	2.09	1.23	1.35
Kedah	7.31	0.39	5.85	6.35	0.39	2.19	15.13	2.34	7.98
Kelantan	10.16	5.73	22.22	14.00	15.15	0.00	9.61	7.53	0.00
Malacca	3.09	0.00	2.02	28.77	6.17	25.10	64.61	15.57	60.74
Negeri Sembilan	9.56	0.44	3.37	16.43	3.97	11.26	17.32	3.51	10.43
Pahang	19.96	4.76	14.26	5.71	2.15	4.69	7.47	1.28	4.64
Perak	22.77	2.86	11.94	11.47	2.57	5.60	11.46	1.85	9.08
Perlis	3.47	0.00	0.00	8.39	5.29	33.33	9.81	0.00	225.81
Penang	4.24	0.60	3.83	2.62	0.74	0.63	11.33	4.56	7.45
Sabah	0.89	2.37	0.00	0.43	1.01	0.00	4.56	9.66	0.00
Sarawak	0.70	1.20	0.00	3.11	1.19	0.00	11.05	2.52	42.86
Selangor	5.93	1.24	2.50	9.51	1.37	8.89	18.88	3.87	14.62
Terengganu	15.80	0.00	0.00	11.08	0.00	0.00	10.47	7.41	0.00
Kuala Lumpur	9.33	0.30	5.70	23.25	2.12	13.81	24.14	4.67	14.34
Mean Incidence	9.12	1.22	4.78	9.60	1.69	7.12	14.18	3.77	11.94

NB: The value 0.00 indicates there is no reported case of leptospirosis for that particular State during that period.

(Metro-Manila) which is characterized by the high population density and poor sanitation.

In contrast, Singapore is a small developed country and reports annually of 26–70 cases only. It has developed an electronic death or infectious disease notification system (Communicable Diseases Live & Enhanced Surveillance System or CDLENS), that facilitates good communication among healthcare professionals and authorities to communicate updates.

Gender played a big role in the disease distribution with incidence in males was three and a half times higher than in women. Similarly, several published works have shown that leptospirosis was gender-biased with more males infected (Chou et al., 2008; Goris et al., 2013) and is thought to be influenced by occupa-

Table 4
The average incidence rate for each ethnic group per 100,000 population for all 14 states over the years 2010–2012.

Year	Average over Years 2010–2012		
Ethnicity	Malay	Chinese	Indian
State			
Johor	2.18	0.70	1.06
Kedah	9.60	1.04	5.34
Kelantan	11.26	9.47	7.41
Malacca	32.16	7.25	29.29
Negeri Sembilan	14.44	2.64	8.35
Pahang	11.05	2.73	7.86
Perak	15.23	2.42	8.87
Perlis	7.22	1.76	86.38
Penang	6.06	1.97	3.97
Sabah	1.96	4.34	0.00
Sarawak	4.96	1.64	14.29
Selangor	11.44	2.16	8.67
Terengganu	12.45	2.47	0.00
Kuala Lumpur	18.91	2.37	11.28
Average Incidence	10.97	2.22	7.95

Table 5
The average incidence (per 100,000 population) and CFRs for all states from 2004 to 2012.

Average over years 2004–2012		
State	Incidence	CFR
Johor	1.79	0.05
Kedah	4.17	0.03
Kelantan	8.01	0.03
Malacca	11.12	0.04
Negeri Sembilan	6.57	0.01[a]
Pahang	10.09	0.02
Perak	7.99	0.06
Perlis	3.21	0.09[a]
Penang	2.28	0.01
Sabah	2.33	0.04
Sarawak	3.61	0.05
Selangor	3.90	0.02
Terengganu	8.07	0.06
Kuala Lumpur	5.57	0.01
Overall Average	4.83	0.03

[a] Average over all years except years 2005 and 2006, where no cases were reported.

Table 6
The negative binomial regression analyses between number of rain days and temperature with the number of reported leptospirosis cases from 2004 to 2012.

Weather	Coefficient	95% Confidence Interval
Rain days		
(Constant)	7.28[*]	(5.79, 9.19)
Number of Rain Days	1.02[*]	(1.00, 1.03)
Temperature		
(Constant)	0.04[*]	(0.004, 0.33)
Average Temperature	1.23[*]	(1.13, 1.34)

[*] Estimate significantly different from 1.0 (p-value < 0.05).

tional exposure and activities that put men at a higher risk (Hinjoy, 2014; Mohan et al., 2009). Previous investigations in Malaysia have shown high leptospirosis incidence especially among male-dominated occupations such as paddy planters, rubber tappers, and palm oil workers, laborers dealing with sewage, draining, town cleaning, forestry, those involved in antimalarial programs, and military personnel (El Jalii et al., 2000; Shafei et al., 2012; Tan, 1973).

Unfortunately, it was not possible to analyze the impact of occupation on disease incidence as this information was grossly lacking. In essence, gender may have a compounding effect on occupation.

Males were also known to be more involved in outdoor activities such as water sports and recreational activities compared with
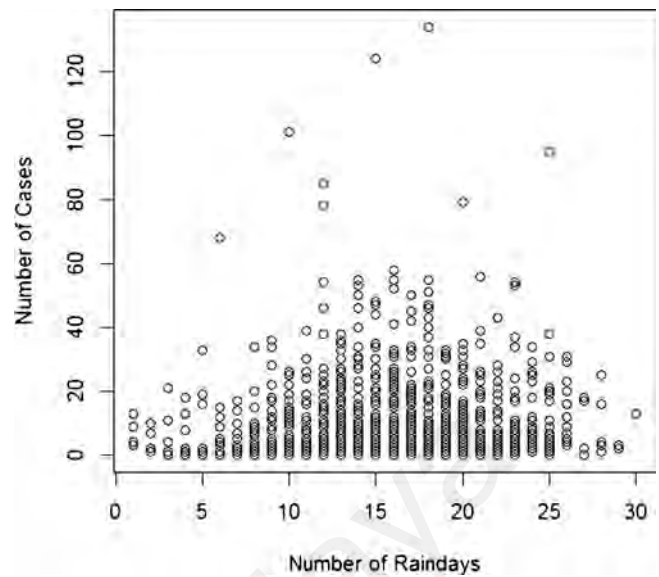
Fig. 2. Number of leptospirosis cases according to the monthly number of raining days from 2004 to 2012.

females. These activities are known risk factors for leptospirosis (Lau et al., 2010a) and have been linked to several outbreaks in this country (Koay et al., 2004; Sapian et al., 2012).

Leptospirosis affects a vast range of age groups, with a higher number of cases occurring amongst the working class ranging from 20 to 49 years old, with the highest amongst the 30–39 years old. This was in agreement with previous studies (Goris et al., 2013; Jansen et al., 2007) as the middle age groups are generally more mobile thus at higher risk of exposure compared to the young and school-going children. Despite known association between leptospirosis with occupation and recreational activities (Bharti et al., 2003; Jansen et al., 2007) as previously reported, we were unable to conclude this information, as data on occupation was not available.

Based on the patient's ethnicity, infections were highest among Malays (10.97 per 100,000 Malays population), followed by Indians (7.95 per 100,000 Indians population). This is not surprising because Malays make up 50% of the total population (Malaysian Department of Statistics, 2014) although, infections in the Indian community exceeded Malays in the two states of Perlis and Sarawak in 2012. The low Indian population in Sabah and Terengganu also corresponded with the low number of cases among Indians in both states.

In a previous serological study, Tan (1979) correlated the ethnic distribution among 18 occupations though serological testing of 4646 febrile patients with leptospirosis. Indians recorded the highest infection (44.1%), followed by Malays (33.9%) and Chinese (22.0%). Indians employed as oil palm plantation workers, rubber tappers, miners, and farmers were at higher risk for infection (Tan, 1979) compared with the rest. Chinese recorded the lowest infection (2.22 per 100,000 population) even in population densities where the Chinese were higher or equal to the Malay population as observed in Pulau Pinang, Kuala Lumpur and Perak.

In relation to geographical distribution, Malacca noted a record high of 50 times more cases in the 9-year study. Based on the national statistics, more than 85% of the population resides in the urban districts of Jasin, Alor Gajah, and central Malacca. The impact of rural-to-urban demographic migration has often led to rapid urbanization and creation of urban slums and this factor has been associated with the emergence of leptospirosis in many countries (Lau et al., 2010b). Urban slums are typically associated with bad sanitation infrastructure, poor waste management and
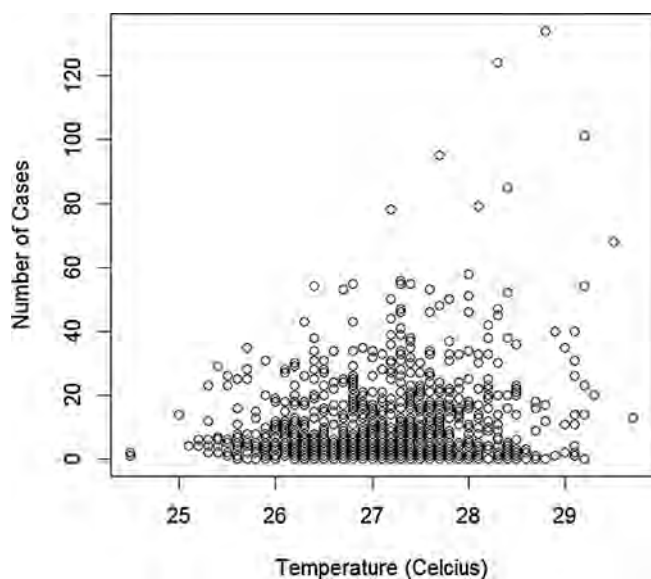
Fig. 3. Number of cases by average monthly temperature from 2004 to 2012.

where humans typically inhabit in close proximity with animal and environmental reservoirs of infectious agents. It is also reported the emergence of *Leptospira* transmission are associated with proximity of residence to open sewers and accumulated refuse, flood-risk areas and areas with high rat infestation (Barcellos, 2000; Felzemburgh et al., 2014). In addition to environmental features, low socioeconomic status among slum residents was also reported to contribute to the risk of leptospirosis (Barcellos, 2000; Felzemburgh et al., 2014; Oliveira et al., 2009). The impact of season can be excluded as, the state of Malacca is on the west coast of Peninsular Malaysia and protected by the mountain ranges and least prone to the effects of flooding by the northeast monsoon.

It is estimated that urban population growth in Malaysia was expected to rise from 54.7% in 1995 to approximately 73% by 2020 (Osman et al., 2004). This rapid growth if not managed with proper would indirectly create the growth of slums with poor irrigation and flash-flood management, all of which create favorable conditions for the survival of the host reservoir and subsequently the contamination of environmental waters and soils via their excreta and urine (Benacer et al., 2013a).

In Malaysia, the majority of leptospirosis cases are caused by exposure to an environment contaminated by *Leptospira* spp. (Benacer et al., 2013b). To date, more than 29 pathogenic *Leptospira* serovars have been isolated from natural water and soil locally (Alexander et al., 1975; Benacer et al., 2013b). The climatic conditions such as high humidity (70–90%) and warm weather provided an appropriate niche for this organism to survive longer in the environment. Thus, the association between the disease frequency and meteorological parameters were explored. The monthly number of reported cases of leptospirosis all year round showed a seasonality pattern. The reported cases were significantly higher during the wet season than the dry season, especially between October to March in the peninsula and between October to February in east Malaysia. This result agrees with other investigations in the region such as Indonesia, Laos, and Thailand (Chadsuthi et al., 2012; Kawaguchi et al., 2008; Victoriano et al., 2009). A significant association was also shown between the number of rain days and infection incidence as observed in Malacca, Pahang, Perak, Terengganu, and Kelantan.

Continuous rain over several days or weeks keeps soil moist and forms small lakes, muddy ponds, and streams that allow leptospires to survive longer in the environment. This ultimately leads to an increase in human and animal exposure to the bacteria (Ullmann and Langoni, 2011), especially near forests and farms. Heavy rain can also unclog drains, flush out urine or infected dead animals, which contaminates water bodies and becomes a source of contagion. Flooding often destroys properties and damages the water and sanitation networks, and contaminates drinking water with pathogens. Interestingly, the association between the number of cases in relation to rainfall volume was not significant in the current study despite being recognized as a risk factor associated with leptospirosis (Bharti et al., 2003; Levett, 2001). When compared with neighboring countries such as Thailand and the Philippines, only a handful of flooding incidents in Malaysia were known to be associated with a leptospirosis outbreak. During the period of this study, only one incident occurred between 2006 and 2007, in Johor, after a major flood caused more than 20 confirmed leptospirosis cases with two deaths among 2000 cases of waterborne diseases reported (Badrul Hisham et al., 2009).

This study found significant association between environmental temperature and the number of leptospirosis cases, with most cases being recorded between 27 to 28 °C (27.5 °C). This finding was in agreement with previously published studies (Desvars et al., 2011; Ullmann and Langoni, 2011) that explained the warm climate relationship to leptospirosis, where the pathogen survives and thrives at an average temperature of about 23.5 °C (Chadsuthi et al., 2012).

The high temperatures in this region encourage water-based activities such as swimming, bathing, and drinking, which promotes contact between humans, livestock, pets, and wildlife through more intense sharing of shrinking surface-water sources (Lau et al., 2010b). Furthermore, prolonged immersion can make the skin and mucous membranes more penetrable and allow the entrance of leptospires (Ullmann and Langoni, 2011). In addition, recreational water activities such as fishing, kayaking, and water skiing in contaminated water have been reported as risk factors for infection, especially when ingestion of water occurred (WHO, 2003). In Perlis, a family of 8 men developed symptoms and were hospitalized after fishing at a nearby swamp. Investigation revealed that the swamp was the source of the infection (Baharudin et al., 2012).

Leptospirosis is an endemic disease in Malaysia. A better understanding of the epidemiology of leptospirosis in this country is necessary to link factors that contribute to the emergence of this disease, such as climatic events, environmental factors, animal reservoirs, human demographic and social trends, as well as cultural and anthropological factors.

The involvement of more health workers is necessary to educate the public about the dangers of the disease, its mode of transmission among the population and those most at risk, so that the disease can be recognized and treated immediately. In addition, implementation of a robust surveillance system to monitor temporal and spatial changes in incidence and prevalence is indispensable to identify risk areas and risk behavior and, in turn, design adequate control and prevention measures.

Acknowledgments

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3.3 Publication 3

Pathogenic and Saprophytic *Leptospira* Species in Water and Soils from Selected Urban Sites in Peninsular Malaysia.

Benacer D, Woh PY, Mohd Zain SN, Amran F, Thong KL.

Microbes and Environments Journal, 2013, Vol. 28, 135–140.

3.3.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Woh PY
Drafting of manuscript	Benacer D, Woh PY
Revising manuscript for intellectual content	All authors

Pathogenic and Saprophytic *Leptospira* Species in Water and Soils from Selected Urban Sites in Peninsular Malaysia

DOUADI BENACER[1,2], PEI YEE WOH[1,2], SITI NURSHEENA MOHD ZAIN[1], FAIRUZ AMRAN[3], and KWAI LIN THONG[1,2]*

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; [2]Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia; and [3]Bacteriology Unit, Institute for Medical Research, 50588 Kuala Lumpur, Malaysia

Leptospira species were studied in water and soils from selected urban sites in Malaysia. A total of 151 water (*n*=121) and soil (*n*=30) samples were collected from 12 recreational lakes and wet markets. All samples were filtered and inoculated into semi-solid Ellinghausen and McCullough modified by Johnson and Harris (EMJH) media supplemented with additional 5-fluorouracil. The cultures were then incubated at 30°C and observed under a dark field microscope with intervals of 10 days. A PCR assay targeting the *rrs* gene was used to confirm the genus *Leptospira* among the isolates. Subsequently, the pathogenic status of the isolates was determined using primer sets G1/G2 and Sapro1/Sapro2, which target the *secY* and *rrs* genes, respectively. The isolates were identified at serogroup level using the microscopic agglutination test (MAT) while their genetic diversity was assessed by pulsed field gel electrophoresis (PFGE). Based on dark field microscopy, 23.1% (28/121) water and 23.3% (7/30) soil cultures were positive for *Leptospira* spp. Of the 35 positive cultures, only 8 were pure and confirmed as *Leptospira* genus by PCR assay. Two out of 8 isolates were confirmed as pathogenic, 5 were saprophytic and one was intermediate. These 8 isolates were negative for the 25 reference hyperimmune rabbit sera tested in the MAT. PFGE showed that all 8 of these environmental *Leptospira* spp. were genetically diverse. In conclusion, the presence of pathogenic *Leptospira* spp. in the urban Malaysian environment may indicate and highlight the importance of water screening, especially in recreational lakes, in order to minimize any chance of *Leptospira* infection.

Key words: *Leptospira*, soil, water, MAT, PCR

Leptospirosis is an important global zoonotic disease and is caused by spirochetes from the genus of *Leptospira*. Two major species, including pathogenic strains of *Leptospira interrogans* and non-pathogenic or saprophytic strains of *Leptospira biflexa*, have been identified (19). The main reservoirs for pathogenic *Leptospira* are the rodents, including rats that may carry pathogenic serovars (37). Saprophytic species are naturally present in environmental water and soil and do not usually cause disease (22). Leptospirosis occurs when pathogenic species are transmitted into the bloodstream of humans via direct contact with contaminated urine of animal reservoirs or indirectly by contaminated water and soil (5). The symptoms of leptospirosis may vary from asymptomatic to fatal, according the phase of the infection. There are two phases of leptospirosis infection where they differ in signs and symptoms, that is anicteric and icteric leptospirosis (19). Leptospirosis is known to be an occupational disease, commonly occurring among farmers, veterinarian, abattoir workers and fishermen (28, 33); however, recreational activities such as water sports and travel to endemic countries have also been recognized as risk factors for this disease (35).

The high humidity and warm temperature of tropical and subtropical countries are ideal for *Leptospira* to survive for long periods in the environment. In Malaysia, several outbreak cases have been reported (18, 27). A recent outbreak of leptospirosis associated with a public recreational lake in Hutan Lipur Lubuk Yu, Maran, Malaysia has raised public concerns as it involved three fatalities (26).

Fletcher (8) reported the first fatal case of Malaysian human leptospirosis in 1925 due to *Leptospira* serogroup Icterohemorrhagiae. Subsequently, he identified other serovars, Icterohemorrhagiae, Hebdomadis and Pyrogenes. Between 1970 and 1986, Tan and co-workers reported nine different *Leptospira* serovars, including Pyrogenes, Autumnalis, Canicola, Hebdomadis, Icterohemorrhagiae, Pomona, Grippotyphosa, Celledoni and Sejroe from cases of leptospirosis in clinics and hospitals all over Malaysia (31, 32). In a recent study, the seroprevalence of leptospirosis among municipal workers in Kota Bharu, Kelantan, Malaysia showed that serovars Bataviae, Javanica and Patoc 1 were the 3 predominant *Leptospira* (29).

The isolation of zoonotic *Leptospira* was initiated by Gordon-Smith and co-workers who described 13 different *Leptospira* serogroups from rats (11). Apart from rats, other animal hosts included pigs, horses, dogs and cattle. The *Leptospira* serovars commonly involved in leptospirosis in animals are Pomona, Hebdomadis, Tarrasovi, Canicola and Hardjo (2).

Early research on the isolation of *Leptospira* from water and soil in Malaysia began in the 1970s (1, 3). To date, 29 pathogenic serovars have been identified in the Malaysian environmental water and wet soil (1). Ridzlan *et al.* (25) detected serovar Hebdomadis in water and soil samples collected from selected National Service Training Centres in

* Corresponding author. E-mail: thongkl@um.edu.my;
  Tel: +60–3–7967–4437; Fax: +60–3–7967–5908.

Kelantan and Terengganu, Malaysia.

Culturing is used to isolate and maintain live cultures of different isolated *Leptospira* spp. The microscopic agglutination test (MAT) is widely used as the standard serology method to demonstrate the types of leptospiral serogroups based on the antibody-antigen reaction (19). A positive MAT is determined based on the agglutination of leptospiral cells with the reference hyperimmuned antisera tested. Rapid detection of *Leptospira* by polymerase chain reaction (PCR) has also been established (12, 21) as a useful tool in the detection of leptospiral DNA from human, animal and environmental samples (24, 34, 36). PFGE has proven to be a discriminative tool in the characterization of *Leptospira* strains (10). With its reliability, reproducibility and easy interpretation, PFGE is able to overcome some limitations of the culture and serological methods and is the method of choice for molecular characterization of *Leptospira* spp. (14).

The rapid urbanization of cities and improper garbage management system in urban areas probably created favorable conditions for animal carriers. This may pose a health risk for leptospirosis as infected animals and carriers might contaminate environmental waters and soils via their excreta and urine. The aim of this study was to detect and characterize *Leptospira* species in water and soils from selected urban sites. PCR assay was used for detection and differentiation between pathogenic and saprophytic species. Determination of different serogroups among the positive isolates was carried out by MAT, and the genetic relatedness among these Malaysian isolates was determined by PFGE.

Materials and Methods

Study sites

In the present study, the sites were chosen based on places frequented by the public, such as recreational parks, and drain effluents from high density residential homes. A total of 151 water (*n*=121) and soil (*n*=30) samples were collected from 12 selected sites in 3 different states (Kuala Lumpur, Selangor and Johor) in Peninsular Malaysia. One hundred and twenty-one water samples were collected from lakes, swamps and effluent drain waters, while 30 soil samples were collected from roadsides near housing areas, wet and night markets. Two types of soils, sand and loam, were recognized (Table 1). Samples were collected for 36 sessions during a period of 6 months (October 2011 to March 2012). The average temperatures in these 3 states ranged from 23°C to 34°C and rainfall averaged 240 cm year^{-1}.

Sample collection

Water and soil sample collections were carried out as described by Henry and Johnson (13), with some modifications. All the samples were collected in early morning. Approximately 100 mL water from four selected marked points of lakes and swamps were collected, poured into a 500 mL sterile glass bottle, and mixed thoroughly. Aliquots of 250 mL of well-mixed water samples were transported to the laboratory. From the street drain water, 100 mL surface water from each drain was collected and transferred into sterile glass bottles. Approximately 20 g topsoil (15 cm by 5 cm) was collected from wet and shaded areas. The soil was immediately placed in a sterile plastic bag. The temperatures of water and soil were recorded in the field and pH was recorded upon return to the laboratory. All the samples were transported to the laboratory and processed within 12 hours. The summary of water and soil sample collection is shown in Table 1.

Leptospira *isolation and dark field microscopy examination*

Water samples (100 mL) were filtered through a sterile membrane

Table 1. Summary of water and soil sample collections and positive results

Sampling sites	Coordinates	Water				Soil			
		No. of samples	Average temperature	Average pH	No. of positive samples	No. of samples	Average temperature	Average pH	No. of positive samples
Kuala Lumpur:									
Drain effluent water									
1. Cheras	3°11'53"N 101°40'27"E	10	28°C	7.27	4	—	—	—	—
2. Pantai Dalam	3°6'49"N 101°39'45"E	10	28°C	6.47	1	10	28°C	6.54	3
3. Setapak	3°11'32"N 101°43'1"E	11	28°C	7.49	5	—	—	—	—
Lake water									
4. Taman Tasik Titiwangsa	3°10'42"N 101°42'25"E	10	30°C	7.55	1	—	—	—	—
5. Taman Setapak Jaya	3°11'27"N 101°43'41"E	10	29°C	7.01	0	—	—	—	—
6. Taman Tasik Perdana	3°8'30"N 101°41'4"E	10	30°C	7.54	0	—	—	—	—
7. UM Lake	3°7'9"N 101°39'26"E	10	29°C	7.76	1	—	—	—	—
Selangor:									
Drain effluent water									
1. SS2	3°7'6"N 101°37'17"E	10	28°C	7.57	6	10	28°C	6.56	3
2. Section 17	3°7'10"N 101°38'14"E	10	28°C	7.45	3	—	—	—	—
Lake water									
3. Taman Jaya	3°6'18"N 101°38'54"E	10	29°C	7.91	5	—	—	—	—
4. Taman Paramount	3°6'10"N 101°37'30"E	10	30°C	5.77	0	—	—	—	—
Johor:									
Swamp water									
1. Gemas Baru	2°34'46"N 102°36'43"E	10	28°C	6.63	2	10	28°C	6.38	1
Total	—	121	—	—	28	30	—	—	7

UM: University of Malaya

filter with 0.45 μm pore size. One milliliter of filtered water was inoculated into modified semi-solid Ellinghausen and McCullough modified by Johnson and Harris (EMJH) medium. Soil samples (20 g) in a plastic bag were soaked in sterile phosphate-buffered saline (PBS) solution at approximately three times the volume of the samples. They were mixed by vigorously shaking and allowed to settle for 15 to 20 min. The suspension was pre-filtered through sterile filter paper (Whatman no.1) and then through a sterile 0.45 μm membrane filter. The filtered water (1 mL) was inoculated into EMJH culture media. All the inoculated media were incubated aerobically at 30°C for seven days.

Two types of culture media for *Leptospira* spp. were prepared: liquid and semi-solid EMJH media. The enrichment media contained 1.0% of rabbit serum and bovine serum albumin. Semi-solid EMJH media was prepared by adding 0.13% of Bacto agar to the liquid media. Both media were supplemented with 5-fluorouracil (Merck, Darmstadt, Germany) at a concentration of 400 μg mL^{-1} to minimize bacterial contamination. All the cultures were incubated at 30°C for 30 days and examined under a dark field microscope for the presence of *Leptospira* at intervals of 10 days. *Leptospira* were identified by their characteristic motility as well as morphology. *Leptospira* are white, very thin, long, and rotate rapidly on their longitudinal axis, moving backward and forward. The samples were considered negative if the bacteria were not present in the culture. The positive samples were subcultured into liquid medium and used for further analysis (25).

PCR detection and confirmation of Leptospira *spp.*

DNA template preparation

Genomic DNA was extracted from 7 days' fresh culture using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The quantity of DNA was measured by Biophotometer (Eppendorf, Germany).

PCR confirmation of *Leptospira* spp.

To confirm the genus *Leptospira* and to determine the pathogenic status of the isolates, 3 published primers sets were used (12, 21, 23). In all PCRs, the reactions were performed in a final volume of 25 μL containing 1× PCR buffer, 1.5 mM MgCl$_2$, 200 μM each of dNTPs, 0.3 μM of each primer, 1 U of *Taq* DNA polymerase (Intron Biotechnology, South Korea) and 100 ng template DNA. The PCR products were analyzed by electrophoresis through a 1% agarose gel (Promega).

Primers LA/LB were used to target the 16S rRNA gene as described by Merien *et al.* (21). The cycling conditions consisted of initial denaturation at 94°C for 3 min, 35 cycles each of 94°C for 1 min, 57°C for 1 min, 72°C for 2 min, and further extension at 72°C for 10 min. Primers G1/G2, which target the *secY* gene, were used to detect pathogenic *Leptospira* except for *Leptospira kirschneri* (12). The cycling conditions consisted of initial denaturation at 94°C for 10 min, 35 cycles each of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and further extension at 72°C for 5 min. To detect saprophytic *Leptospira* among the isolates, Sapro1/Sapro2 primers were used (23). The cycling conditions consisted of initial denaturation at 94°C 10 min, 35 cycles each of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and further extension at 72°C for 5 min.

DNA sequencing

Amplified DNA products from representative isolates were verified by DNA sequencing. The amplicons were purified using a DNA purification kit (Qiagen, Hilden, Germany) and submitted to a commercial facility for sequencing (First BASE, Pte., Singapore). The resulting DNA sequence data were compared with the GenBank database using the BLAST algorithm available on the web site (http://www.ncbi.nih.gov).

Microscopic agglutination test

Serological identification of *Leptospira* isolates was performed using the microscopic agglutination test (MAT) as described by the World Health Organization (38). A set of 25 reference hyperimmune antisera representing the major *Leptospira* serovars in Malaysia was provided by the Institute for Medical Research (IMR), Malaysia. The antisera used in this study were: Patoc (Patoc I), Ballum (Mus 127), Sejroe (M84), Javanica (Veldrat Batavia 46), Tarassovi (Perepelicin), Bratislava (Jez Bratislava), Canicola (Hond Ultrecht IV), Hebdomadis (Hebdomadis), Pomona (Pomona), Hardjo (Hardjoprajitno), Australis (Ballico), Bataviae (Swart), Pyrogenes (Salinem), Icterohaemorrhagiae (RGA), Paidjan (Paidjan), Gurungi (Gurung), Djasiman (Djasiman), Bangkinang (Bangkinang I), Autumnalis (Akiyami A), Samaranga (Veldrat Sem 173), Pro-echimys (1161 U), Grippotyphosa (Mandemakers), Grippotyphosa (Moskva V), Cynopteri (3522 C) and Celledoni (Celledoni). The leptospiral isolates were cultured in liquid medium with an additional 1.0% rabbit serum to increase bacteria density. Agglutination of anti-leptospiral antibodies with live *Leptospira* was viewed under a dark field microscope. A positive MAT was scored when there was 50% agglutination, leaving 50% free cells as compared with the negative control (culture diluted 1:2 in phosphate-buffered saline only). Four known positive reference leptospiral cultures (Canicola, Pomona, Bataviae and Javanica) were included to test the viability of the antisera.

Pulsed-field gel electrophoresis analysis

PFGE analysis was carried out according to a previous protocol (10) with minor modifications. DNA was digested with 10 U of restriction enzyme *Not* I (Promega) at 37°C. The restricted fragments were separated by PFGE in 0.5× TBE buffer, for 24 h at 14°C in a CHEF Mapper system (Bio-Rad, Hercules, CA, USA) using pulsed times of 2.2 to 35 s. *Xba*I-digested *Salmonella* Braenderup H9812 was used as the DNA size marker. PFGE data were analyzed using BioNumerics Version 6.0 (Applied Maths, Belgium) software. Clustering was based on the unweighted pair group average method (UPGMA) with position tolerance of 1.0.

Results

Dark field microscopic examination showed that 35 (23.2%) of 151 samples (121 water, 30 soil) contained *Leptospira* isolates. The positive samples showed the typical morphology and characteristic motility of *Leptospira* genus; however, only 8 (22.9%) of these were pure, and the others (77.1%) were contaminated with a higher number of natural bacteria than the numbers of *Leptospira*. More *Leptospira* were found in the drain effluents compared to lake waters. From the 121 water samples, 28 (23.1%) positives were from drain effluents (*n*=21) and lake waters (*n*=7). Among the 30 soil samples, 7 (23.3%) showed positive isolates. The numbers of the positive leptospiral samples associated with different sampling sites are summarized in Table 1.

PCR for confirmation of *Leptospira* genus for the 8 positive pure cultures using LA/LB primers showed that all 8 isolates were *Leptospira* genus. Only 2 of these were pathogenic species as indicated by the presence of 240 bp amplicon. These 2 pathogenic isolates were isolated from drain effluents from Setapak (EW31) and Section 17 (EW77). DNA sequence analyses showed that EW31 and EW77 were closely related to *Leptospira alstonii* species (99% identity). One intermediate isolate (EW1) isolated from University Malaya Lake was 99% identical to *Leptospira wolffii*. Five isolates were confirmed as saprophytic using Sapro1/Sapro2 primers. These saprophytic isolates were isolated from drain effluents in Cheras (EW8), Pantai Dalam (EW107) and SS2 (EW49), and from lakes in Taman Jaya (EW42) and Taman Tasik
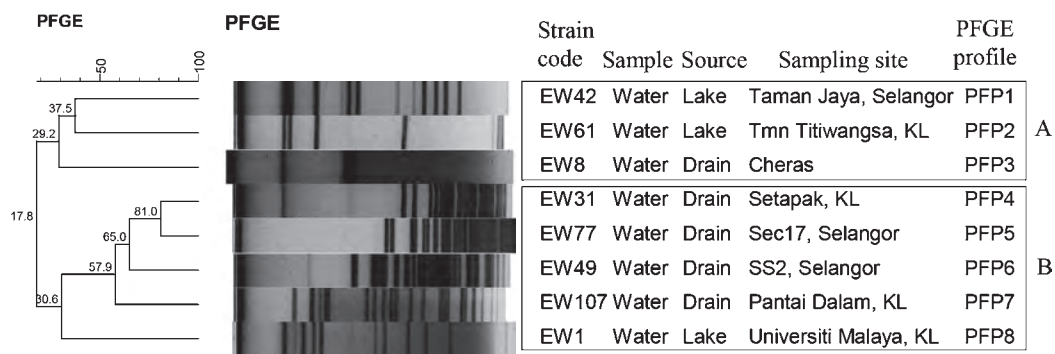
Fig. 1. Dendrogram based on cluster analysis of the PFGE profiles of environmental *Leptospira* strains generated using Bionumerics Version 6.0 (Applied Maths, Belgium) software and unweighted pair group arithmetic means methods (UPGMA) 240×133 mm (96×96 DPI)

Titiwangrsa (EW61). DNA sequencing analyses showed that EW8, EW42 and EW61 were 98% identical to *L. biflexa* and EW49 and EW107 were 99% identical to *Leptospira meyeri* species (GenBank accession nos. FJ812170, DQ991480 and HQ709385).

MAT analysis of the 8 confirmed *Leptospira* spp. using the 25 different hyperimmune antisera showed that none of the isolates was positive for the antisera used; however, a low titer toward serovar Patoc from *L. biflexa* species was observed in 3 saprophytic isolates (titer <1:40).

PFGE of *Not* I-digested chromosomal DNA subtyped the 8 isolates into 8 unique PFGE profiles (PFPs). The number of DNA fragments generated ranged from 2 to 23, with sizes ranging from 21 kb to 705 kb. Wide genetic diversity was found among the strains, as evidenced by F-values (F=0.2 to 0.8). The dendrogram showed 2 clusters, A and B (Fig. 1). Cluster A consisted of 3 isolates (EW42, EW61 and EW8), comprising 3 PFPs and cluster B consisted of 5 isolates (EW31, EW 77, EW49, EW107 and EW1), comprising 5 PFPs.

Discussion

The majority of the leptospirosis cases reported in Malaysia was related to the exposure of humans to an environment contaminated by *Leptospira* spp. In 2000, an outbreak of leptospirosis occurred during the Eco-Challenge in Sabah, Malaysia. Eighty out of 189 competitors (42%) contracted leptospirosis. Twenty-nine people were hospitalized but there were no fatalities (27). The climatic conditions in this country, such as warm weather, heavy rainfall and high humidity, provide an appropriate niche for this organism to survive in the environment. In the past, leptospirosis was associated with occupational activities in rural areas, such as farming, rice harvesting, forestry and livestock farming (5, 19); however, leptospirosis has now become a public health problem in urban areas in many developing countries (15, 17). In Malaysia, there is a paucity of information about leptospirosis in the urban environment in terms of prevalence and the circulating species. Therefore, a study was carried out to investigate the prevalence and pathogenic status of this fastidious organism in the urban Malaysian environment. Samples were collected between October 2011 and March 2012 by sampling 12 randomly selected sites from 3 different states. The present study showed that the rate of positive

samples (23%) collected from these sites was relatively high compared to another study by Ridzlan *et al.* (25), where they only found 10% (15/145) of positive water and soil samples in rural areas of Kelantan and Terengganu.

The presence of more *Leptospira* in drain effluent waters from night and wet markets compared to lake waters could be related to improper waste disposal. Rubbish not disposed of properly then becomes a food source for rats, cats, dogs and birds, which may be carriers of *Leptospira*. Several studies have reported that wild and domestic animals are maintenance hosts of *Leptospira* in Malaysia (2, 16). Three of five selected recreation lakes in Taman Jaya, Taman Tasik Titiwangsa and the University of Malaya were positive for *Leptospira*. Water in these lakes were nearly stagnant or showed a slow flow. Thus, there is a possibility that the lake waters were contaminated by the urine of domestic animals in the vicinity. The University of Malaya Lake is basically a man-made reservoir to meet the recreational and sporting needs of the students. The rubbish and garbage generated by the cafeteria near this lake provide an ample food source for rodents and cats that may carry this organism. In the recent outbreak in the Recreational Park in Maran, three fatal cases were reported. Water and soil samples from the outbreak site were found to be positive for *Leptospira* (26). The authorities suspected that water and soil were contaminated with urine of infected rats, as evidenced by the presence of dead rats and rat droppings near the food stalls in that area (26).

Leptospira is known to be sensitive to dryness; therefore, the soil samples had lower positivity because of their low water retention capacity and the sandy soil was relatively dry. Similarly, Khairani *et al.* (16) showed that serovar Hardjo has a higher survival rate in moist soils and at pH 6.9–pH 7.4. In Malaysia, the rainy season is between October and March. Several studies have reported the association between rainfall and the incidence of leptospirosis cases (5, 19). This may explain the high number of positive samples in our study. Between December 2006 and January 2007, an outbreak of leptospirosis involving 20 cases with 2 deaths occurred in Johor, Malaysia following a flood episode (20). Besides the rainfall factor, several other factors can affect the isolation of *Leptospira* from water and soil, such as pH, temperature, characteristics of water and soil and the presence of animals that are considered as reservoirs of *Leptospira* (25).

Among the 35 positive samples seen under the dark field microscope, only 8 (22.8%) were pure. The high contamina-

tion rate (77.2%) that we observed in the cultures made the isolation steps very difficult. Fast-growing contaminating microorganisms displaced slow-growing *Leptospira* in the enrichment medium such that this fastidious organism failed to establish itself in the medium (16). In this study, a pre-filter technique was applied using Whatman filter paper before filtration through a 0.45 μm syringe filter. This method helped to minimize bacterial contamination. In addition, the concentration of 5-fluorouracil selective antibiotics was gradually increased to inhibit the growth of other bacteria in culture media; however, this extra precautionary step did not remove all the microbial contamination. A recent study reported that the use of a combination of 5 selective antibiotics may help to prevent the growth of 16 microorganisms that are considered as possible contaminants during the isolation of *Leptospira* (7).

In the present study, specific PCR was able to confirm all 8 pure positive samples using *Leptospira* genus primers that target the *rrs* gene. Similarly, the two sets of primers that target the *secY* and *rrs* genes were useful and could differentiate the pathogenic and saprophytic isolates. The number of saprophytic isolates (*n*=5) was slightly higher than the number of pathogenic isolates (*n*=2). Pathogenic species of *Leptopsira* are probably less adapted to the environmental conditions than saprophytic species (4). The ability and usefulness of the PCR in this study proved its value in the detection and differentiation of *Leptospira* spp. In many studies, PCR is used for the early detection of *Leptospira* spp in clinical (9), animal (6) and environment (23) samples as it is specific and rapid. MAT is a serological test that is widely used to identify serogroups of *Leptospira*. In Malaysia, MAT with titer of >1:400 is considered positive. Although 25 different hyperimmune sera were used in this study, the serogroup of the 8 confirmed *Leptospira* isolates could not be identified. Additional sets of hyperimmune sera are required to further identify the serogroup of the isolates. Besides the requirement of a large number of antisera, MAT is tedious and time consuming (19). Due to these limitations, new molecular tools such as PFGE have been developed. In this study, PFGE analysis showed that the *Leptospira* isolates had distinct profiles. Two pathogenic isolates, EW31 and EW77, which were isolated from effluent drain waters, had close genetic relatedness (81% identity). According to Galloway and Levett (10), *Leptospira* isolates that shared ≥75% identity or with ≤3 band differences could be from the same species. This result concurred with the sequencing results, which showed that these 2 isolates had high identity (99%) to *Leptospira alstonii* species. Both isolates were from effluent drain water near wet markets where many rats were observed. Hence, we postulated that these 2 isolates were probably from the same origin. In previous studies, pathogenic *Leptospira* species isolated from water and soil belonged to *L. interrogans*, *Leptospira borgpetersenii* and *Leptospira kmetyi* (1, 25, 30). This is the first study to isolate *L. alstonii* and *L. wolffii* from water and soil in Malaysia.

In conclusion, this study has proven the presence of genetically diverse *Leptospira* in the Malaysian urban environment. Detection of pathogenic *Leptospira* in water samples especially in recreational lakes, may pose a health risk, especially to those who come into contact with contaminated water during sports activities. Necessary precautions should be taken by the authorities to monitor water bodies and to alert the public of contaminated water bodies in view of this. The presence of these pathogenic strains in effluent drains is also a concern for better control of the host reservoir population and appropriate garbage management.

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3.4 Publication 4

Isolation and Molecular Characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* Isolates from the Urban Rat Populations of Kuala Lumpur, Malaysia.

Benacer D, Mohd Zain SN, Amran F, Galloway RL, Thong KL.

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3.4.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Isolation and Molecular Characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* Isolates from the Urban Rat Populations of Kuala Lumpur, Malaysia

Douadi Benacer, Siti Nursheena Mohd Zain, Fairuz Amran, Renee L. Galloway, and Kwai Lin Thong*

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia; Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Kuala Lumpur, Malaysia; Bacteriology Unit, Institute for Medical Research, Ministry of Health, Kuala Lumpur, Malaysia; Bacterial Zoonoses Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract. Rats are considered the principal maintenance hosts of *Leptospira*. The objectives of this study were isolation and identification of *Leptospira* serovars circulating among urban rat populations in Kuala Lumpur. Three hundred urban rats (73% *Rattus rattus* and 27% *R. norvegicus*) from three different sites were trapped. Twenty cultures were positive for *Leptospira* using dark-field microscopy. *R. rattus* was the dominant carrier (70%). Polymerase chain reaction (PCR) confirmed that all isolates were pathogenic *Leptospira* species. Two *Leptospira* serogroups, Javanica and Bataviae, were identified using microscopic agglutination test (MAT). Pulsed-field gel electrophoresis (PFGE) identified two serovars in the urban rat populations: *L. borgpetersenii* serovar Javanica (85%) and *L. interrogans* serovar Bataviae (15%). We conclude that these two serovars are the major serovars circulating among the urban rat populations in Kuala Lumpur. Despite the low infection rate reported, the high pathogenicity of these serovars raises concern of public health risks caused by rodent transmission of leptospirosis.

INTRODUCTION

Leptospirosis, an infectious disease that affects both humans and animals, is recognized as one of the most widespread zoonoses worldwide.[1] Annually, an estimated one-half million cases of severe leptospirosis are reported globally.[2] However, this number is probably underestimated because of the lack of reported cases and the misdiagnosis of this disease in many countries.[3]

Leptospirosis is caused by the pathogenic strains of the bacterium *Leptospira*. Currently, there are nearly 300 known serovars,[4] and most of them have their primary reservoirs in wild and domestic animals, of which rodents and rats are the most common source worldwide.[5] Infected rats shed *Leptospira* spp. in their urine over an extended period of time,[6] and humans and animals get infected through direct or indirect contact with urine, water, or soil contaminated by *Leptospira* spp.[7] Approximately one-half of the pathogenic serovars belong to *L. interrogans* or *L. borgpetersenii*.[8]

Classically, the diagnosis of leptospirosis is based on serological tests, such as the microscopic agglutination test (MAT). In this test, reaction takes place between a leptospiral isolate and reference hyperimmune rabbit antisera.[9] However, this method is laborious and time-consuming, and it requires extensive collection of reference strains and their corresponding rabbit antisera.[10] Various molecular approaches have been developed, such as polymerase chain reaction (PCR)-based methods, to improve the diagnosis of leptospirosis. PCR has been successfully applied as a rapid, sensitive, and specific tool for the detection of several microorganisms, including *Leptospira*,[11] in a variety of specimens from different hosts.[12–14] The rapidity and reproducibility of pulsed-field gel electrophoresis (PFGE) makes it a very useful technique for typing *Leptospira* strains.[15] PFGE is able to discriminate between closely related serovars of the *Leptospira* spp., which may aid in the identification of the different strains at serovar level,[16] and this identification is important in understanding the epidemiology of leptospirosis in both humans and animals in any geographic region.[17]

Presently, leptospirosis is recognized as a globally re-emerging disease, with a marked increase in the number of cases in Latin America and Southeast Asia, including Malaysia.[18] According to the Ministry of Health Malaysia, the numbers of reported cases of leptospirosis have increased from 263 in 2004 to 1,418 in 2009.[19] Previous studies on leptospirosis in Malaysia focused mainly on humans and domestic animals in the vicinity of outbreak sites.[20,21] However, there is very little information about this disease in Malaysian urban rat populations. Therefore, the purpose of this study was to isolate and detect the *Leptospira* spp. in the urban rats of Kuala Lumpur using MAT and PCR. In addition, the serovar identification of these isolates was determined by PFGE.

MATERIALS AND METHODS

Choice of the study sites. Kuala Lumpur, the capital city of Malaysia, is characterized by a tropical climate of high temperature and high humidity year round, with temperatures ranging between 30°C and 36°C; rainfall is fairly even throughout most of the year but typically heavier between October and February during the monsoon season. The choice of the study sites was based on the suitability of the habitat for rodents to forage and breed and the possibility of spreading the diseases. The first site, Chow Kit market (03°09′53.75″ N, 101°41′56.84″ E), is the largest fresh food wet market, commonly found in Asian countries, in Kuala Lumpur. Here, water is used extensively to wash the floors, keep the fruits and vegetables fresh, and keep fish and shellfish alive. This market provides an extensive range of raw foods, including fruits, vegetables, seafood, and meat to the public. In turn, tons of rubbish are deposited into several steel containers. Excess garbage falls to the ground, forming temporary grounds for rats to forage. In contrast, Bangsar (3°6′49″ N 101°39′45″ E) is an affluent residential suburb on the outskirts of Kuala Lumpur with mixed residential sites. The hawker centers, restaurants, and roadside stalls sell cooked food, and rodents found here thrive on leftovers.

* Address correspondence to Kwai Lin Thong, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia. E-mail: thongkl@um.edu.my

The third site, Setapak (3°11′32″ N 101°43′1″ E), is a highly dense residential site for the lower income bracket. Nearby, a small wet open market provides fresh produce to the community. Similarly, because of poor garbage management, this site also attracts rodents, stray dogs, and cats to breed and source for food.

Trapping and host identification. Trapping was conducted over a period of 6 days and 5 nights for each session, with a total of 13 trapping sessions conducted over a 6-month period (October of 2011 to March of 2012).

A total of 30 steel wire traps ($18 \times 12 \times 28$ cm) were placed in each site with baits such as dry fish, breads, and peanut butter. The traps were placed in the evening and collected in the early morning before the market was opened to the public. The captured rats were placed in black plastic bags to reduce stress and transported to the laboratory for examination. The rats were then euthanized with chloroform, and morphometric measurements were recorded.

Age and species identifications were carried out according to Medway[22] based on the phenotypic characteristics, such as fur color (ventral and dorsal), body weight, hind foot, and head–body length.

Isolation of *Leptospira* and dark-field microscopy examination. Euthanized rats were immediately killed, and selected organs, such as blood, livers, and kidneys, were collected. Skin, liver, and kidney lesions were recorded to investigate the relationship of these lesions with the transmissibility of *Leptospira* spp. Two drops of blood collected by cardiac puncture were inoculated into the modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. The urine was collected by direct puncture of the bladder and then cultured into the medium. Samples of liver and kidney tissues were removed using a sterile blade, and a small piece of tissue was placed in a sterile syringe without a needle and squeezed into the EMJH medium. All inoculated media were incubated aerobically at 30°C and examined under a dark-field microscope for the presence of *Leptospira* at 10-day intervals for a period of 3 months. Samples that failed to show any evidence of growth after 3 months were considered negative.

Two media types were used in this study: liquid and semisolid, in which 0.13% Bacto agar was added. Both media were enriched with 1.0% rabbit serum and bovine serum albumin and supplemented with 5-fluorouracil (Merck, Darmstadt, Germany) at a concentration of 400 μg/mL to minimize bacterial contamination.

Detection and determination of the pathogenic status of the leptospiral isolates by PCR. *DNA template preparation.* Genomic DNA was extracted from 7-day-old culture media using Wizard Genomic DNA Purification Kit (Promega, Madison, WI) using the manufacturer's instructions. The quantity of DNA was measured by Biophotometer (Eppendorf, Hamburg, Germany).

PCR detection and confirmation of Leptospira *isolates.* PCR primers LA/LB ([5′-GGC GGC GCG TCT TAA ACA TG-3′] and [5′-TTC CCC CCA TTG AGC AAG ATT-3′]), which target the 16S rDNA gene, were used to confirm the genus *Leptospira*.[23] The cycling conditions consisted of an initial denaturation at 94°C for 3 minutes, 35 cycles each of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 2 minute, and additional extension at 72°C for 10 minutes. To determine the pathogenic status of the isolates, G1/G2 primers ([5′-CTG AAT CGC TGT ATA AAA GT-3′] and [5′-GGA AAA CAA ATG GTC GGA AG-3′]), which target the secY gene among the isolates except for *L. kirschneri*, were used.[24] The cycling conditions consisted of an initial denaturation at 94°C for 10 minutes, 35 cycles each of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and additional extension at 72°C for 5 minutes. In both PCRs, the reactions were done in a final volume of 25 μL containing $1 \times$ PCR buffer, 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphates (dNTPs), 0.3 μM each primer, 1 U *Taq* DNA polymerase (Intron Biotechnology, Sungnam, Kyungki-Do, South Korea), and 100 ng DNA template. The PCR products were analyzed by electrophoresis through a 1% TBE agarose gel (Promega, Madison, WI).

DNA sequencing. Amplified DNA products from representative isolates were verified by DNA sequencing. The amplicons were purified using a DNA purification kit (Qiagen, Hilden, Germany) and sent to a commercial facility for sequencing (First BASE, Pte. Ltd., Singapore). The resulting DNA sequence data were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm available at the website (http://www.ncbi.nih.gov).

MAT. MAT was performed as described by the World Health Organization.[25] A set of 25 reference hyperimmune antisera representing the major Leptospira serovars in Malaysia was provided by the Institute of Medical Research (IMR), Malaysia and used to determine the serogroup level of the isolates. The leptospiral isolates were cultured into liquid medium with additional 1.0% rabbit serum to increase bacterial density. A positive MAT was scored when there was 50% agglutination, leaving 50% free cells compared with negative control (culture diluted 1:2 in phosphate buffer saline only). Four known positive reference leptospiral cultures (Canicola, Pomona, Bataviae, and Javanica) were included to test the reactivity of the antisera. The antisera used in this study were raised against serovars Patoc (Patoc I), Ballum (Mus 127), Sejroe (M84), Javanica (Veldrat Batavia 46), Tarassovi (Perepelicin), Bratislava (Jez Bratislava), Canicola (Hond Ultrecht IV), Hebdomadis (Hebdomadis), Pomona (Pomona), Hardjo (Hardjoprajitno), Australis (Ballico), Bataviae (Swart), Pyrogenes (Salinem), Icterohaemorrhagiae (RGA), Paidjan (Paidjan), Gurungi (Gurung), Djasiman (Djasiman), Bangkinang (Bangkinang I), Autumnalis (Akiyami A), Samaranga (Veldrat Sem 173), Proechimys (1161 U), Grippotyphosa (Mandemakers), Grippotyphosa (Moskva V), Cynopteri (3522 C), and Celledoni (Celledoni).

Pulsed-field gel electrophoresis. Chromosomal DNA was prepared according to protocol[16] with minor modifications. *Xba*I-digested *Salmonella* Braenderup H9812 was used as the DNA size marker. *Leptospira* DNA plugs were digested with 10 U restriction enzyme *Not*I (Promega, Madison, WI) at 37°C. The restriction fragments obtained were separated by electrophoresis in $0.5 \times 10X$ Tris-borate EDTA (TBE) buffer for 24 h at 14°C in a CHEF Mapper system (Bio-Rad, Hercules, CA) using pulsed times of 2.2–35 s. PFGE data were analyzed using BioNumerics Version 6.0 (Applied Maths, Sint-Martens-Latem, Belgium) software. Clustering was based on the unweighted pair group average method (UPGMA) with the position tolerance of 1.0.

Statistical analysis. All statistical analyses were performed using SPSS software, version 17 (SPSS Inc., Chicago, IL). The data were subjected to an independent-samples t test, and the P values were generated. The confidence level was set at

Location	Rat species		Sex		Age		Lesions		
	R. rattus	*R. norvegicus*	Male	Female	Adult	Juvenile	Liver	Kidney	Skin
Chow Kit	113 (51.6%)	47 (58.0%)	94 (56.3%)	66 (49.6%)	105 (50.5%)	55 (59.8%)	27 (58.7%)	12 (38.7%)	24 (61.5%)
Setapak	68 (31.0%)	22 (27.2%)	45 (26.9%)	45 (33.8%)	69 (33.2%)	21 (22.8%)	14 (30.4%)	15 (48.4%)	8 (20.5%)
Bangsar	38 (17.4%)	12 (14.8%)	28 (16.8%)	22 (16.6%)	34 (16.3%)	16 (17.4%)	5 (10.9%)	4 (12.9%)	7 (18.0%)

95%. A test with a *P* value lower than 0.05 was considered statistically significant.

RESULTS

A total of 300 rodents were captured from all three sites and comprised of two rat species, namely *Rattus rattus* (219, 73%) and *R. norvegicus* (81, 27%). Most rodents were captured from the vicinity of Chow Kit (160 rats, 53.3%) followed by Setapak and Bangsar with 90 and 50 rats, respectively. Based on the age and sex of the rats captured, 208 (69.3%) rats were adults, whereas 92 (30.7%) rates were juveniles; there were more males (167, 55.7%) compared with females (133, 44.3%). The trapping results from the three locations are as indicated in Table 1.

A total of 6.7% (20/300) were positive with cultures that showed the typical morphology and characteristic motility of *Leptospira* genus by dark-field microscopy from 16 urine and 4 kidney samples. *R. rattus* (14/20, 70%) had higher infection rates compared with *R. norvegicus* (6/20, 30%), and statistically significantly more male rats (15/20) were infected compared with females (5/20, *P* = 0.045). According to host age, more adults were infected with *Leptospira* (14/20) than juveniles (6/20, *P* = 0.663); however, the difference was not significant.

According to site, 10 positive samples were obtained from the Chow Kit vicinity followed by Setapak and Bangsar with 6 and 4 positive samples, respectively. Rats with liver lesions recorded higher positives (15.3%) compared with skin (13%) and kidney (10.3%) lesions.

Leptospira genus confirmation by PCR showed that all 20 isolates gave the expected band size of 331 bp, indicating that all the positive isolates were *Leptospira* species (Figure 1). All isolates were pathogenic species as shown by the band size of 285 bp amplified by the primers G1/G2, which targets *secY* genes (Figure 2) (99% similarity; accession numbers EU358040 and DQ882852).

The identification of the serogroup of the isolates was carried out using 25 different hyperimmune antisera. The results showed that 17 isolates had the agglutinating reaction against serovar Javanica antisera (titer > 1:400), whereas 3 isolates showed agglutination to serovar Bataviae (titer > 1:400).

PFGE of *Not*I-digested chromosomal DNA subtyped the 20 isolates into two distinct PFGE profiles: LNot01 and LNot02. The number of DNA fragments generated ranged from 10 to 23, with sizes ranging from approximately 28 to 706 kb (Figure 3). Limited genetic diversity was found among the isolates. The dendrogram generated two major clusters: A and B (Figure 4). Cluster A consisted of three isolates isolated from *R. rattus*; two isolates were from Setapak, and one isolate was from Chow Kit market. Cluster B consisted of 17 isolates; 11 isolates were isolated from *R. rattus*, whereas 6 isolates were isolated from *R. norvegicus*. Of these isolates, nine isolates were from Chow Kit market, four isolates were from Setapak, and four isolates were from Bangsar.

The two PFGE profiles produced were compared with the *Not*I patterns from the database provided by the Centers for Disease Control and Prevention (CDC). The results showed that LNot01 has a similar pattern to *L. interrogans* serovar Bataviae, whereas LNot02 has the same pattern as *L. borgpetersenii* serovar Javanica.

DISCUSSION

In Kuala Lumpur city, *R. rattus* and *R. norvegicus* were found to be the two most dominant commensal rat species living close to human habitation.[26] Urbanization of cities and towns has created favorable conditions for commensal rats to live, because these rats often depend on direct contact with human food. The two species are closely related, and formerly, *R. norvegicus* was classed as a subspecies of *R. rattus*.[27] Similar to the report by Gordon-Smith and others,[28] more than twofold *R. rattus* were captured compared with *R. norvegicus* from the three sites, suggesting the population dominance of *R. rattus* in urban settings. Both species have successfully adapted and established themselves, despite a continuous and extensive
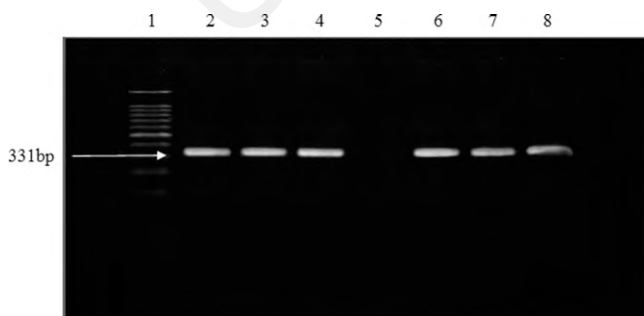
FIGURE 1.   Representative gel of PCR for detection of *Leptospira* genus using LA/LB primers. Lanes 1–8: DNA marker (100 bp); positive control; R2 (urine sample); R18 (urine sample); negative control; R71 (kidney sample); R77 (urine sample); R108 (kidney sample).
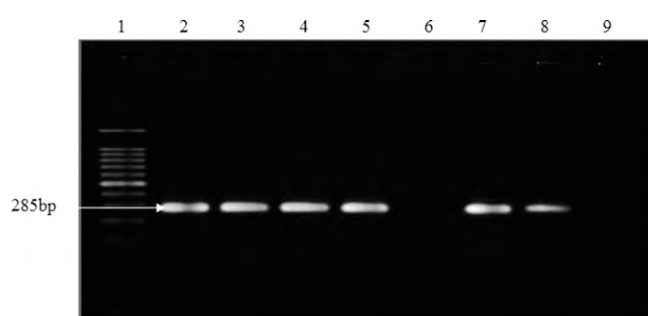

FIGURE 2.   Representative gel of PCR for confirmation of pathogenic *Leptospira* using G1/G2 primers. Lanes 1–8: DNA marker (100 bp); positive control; R77 (urine sample); R78 (urine sample); R84 (urine sample); negative control; R102 (urine sample); R108 (kidney sample).
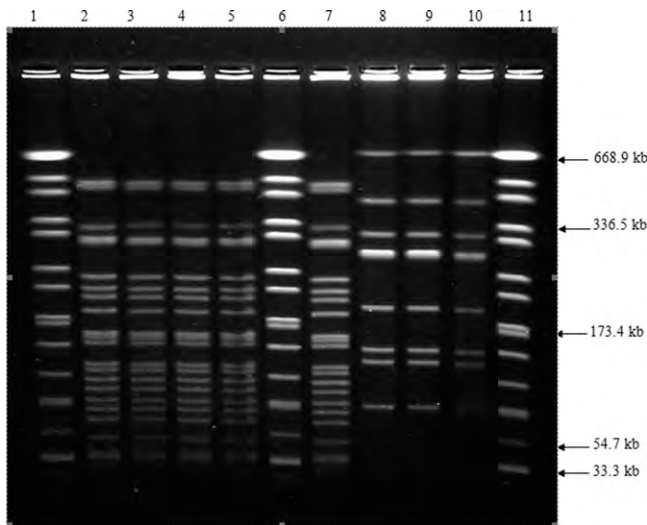
FIGURE 3. PFGE patterns of *Not*I digested chromosomal DNA of *Leptospira* isolates from urban rats of Kuala Lumpur. Lanes 1, 6, 11: *Xba*I digested chromosomal DNA of *Salmonella* H9812 marker strain; lanes: 2–5, 7–10: *L. interrogans* serovar Javanica R2; *L. interrogans* serovar Javanica R18; *L. interrogans* serovar Javanica R7; *L. interrogans* serovar Javanica R78; *L. interrogans* serovar Javanica R84; *L. interrogans* serovar Bataviae R108; *L. interrogans* serovar Bataviae R149; *L. interrogans* serovar Bataviae R183.

rodent control program in the city. Therefore, it is essential that the rodent population be regularly monitored.

Rodents are known as the maintenance hosts of *Leptospira* in Malaysia. To date, 37 serovars of *Leptospira* from 13 serogroups have been identified in Malaysia, and more than one-half of these serovars are carried by rats.[20] However, previous studies focused on rodent populations associated with outbreak areas, such as national training service centers, oil palm estates, and forest habitat.[29–31] The present study serves to highlight this disease in the urban setting, where rodents are known to live closely with humans.

Chow Kit market is the largest market in Malaysia, with plentiful resources for rodents. Rubbish left in open garbage containers attracts high numbers of rats to forage, especially *R. rattus*. Similarly, in Setapak, a smaller market nearby also attracts this pest. In addition, open sewers and poor drainage serve as thriving grounds for rodents to breed. The more affluent residential area, Bangsar, had the smallest number of rats captured. This result could be explained by better sanitary conditions and garbage management practices in this affluent area.

R. rattus was found to be the more prevalent rat species infected with *Leptospira* compared with *R. norvegicus*. Gordon-Smith and others[28] reported that the prevalence of the infected *R. norvegicus* was higher compared with *R. rattus* (2/259). Other rat species have also been reported as carriers of the leptospires, such as *R. tiomanicus*, *R. exulans*, and *R. argentiventer*.[29,30] Faria and others[32] showed that the higher population density of *R. norvegicus* was the main factor facilitating transmission of leptospirosis in Brazil, whereas *R. tiomanicus* was predominant in various habitats in Malaysia.[30]

In this study, the overall prevalence of rats being infected was low (6.7%). This finding is concordant to previous studies.[28,30,33] Studies in Brazil and the Philippines reported a high prevalence of leptospirosis among urban rats.[32,34]

Of 300 captured rats, 38.7% had lesions in liver, kidney, or skin. The *R. rattus* species showed more lesions compared with *R. norvegicus* ($P = 0.029$); however, the frequency of the clinical lesions was not statistically significant ($P > 0.05$) between the rats species and the prevalence of the positive infected rats. A total of 16 urine samples was positive compared with only 4 kidney samples.

Strains	Sample	Location	Sex	Age	Rat species
R108	Kidney	Setapak	Male	Adult	RR
R149	Urine	Setapak	Male	Adult	RR
R183	Kidney	Chow Kit	Female	Adult	RR
R102	Urine	Setapak	Male	Juvenile	RR
R107	Urine	Setapak	Male	Adult	RN
R122	Urine	Setepak	Male	Adult	RR
R139	Urine	Setapak	Male	Adult	RR
R179	Urine	Chow Kit	Male	Adult	RR
R18	Urine	Chow Kit	Female	Juvenile	RR
R2	Urine	Chow Kit	Male	Adult	RR
R255	Urine	Bangsar	Male	Adult	RR
R282	Urine	Chow Kit	Male	Juvenile	RN
R285	Kidney	Chow Kit	Male	Adult	RN
R293	Urine	Chow Kit	Male	Adult	RN
R298	Urine	Chow Kit	Female	Juvenile	RN
R299	Urine	Chow Kit	Male	Adult	RR
R71	Kidney	Chow Kit	Female	Juvenile	RR
R77	Urine	Bangsar	Male	Adult	RR
R78	Urine	Bangsar	Female	Adult	RR
R84	Urine	Bangsar	Male	Juvenile	RN

RR: *Rattus rattus*, RN: *Rattus norvegicus*
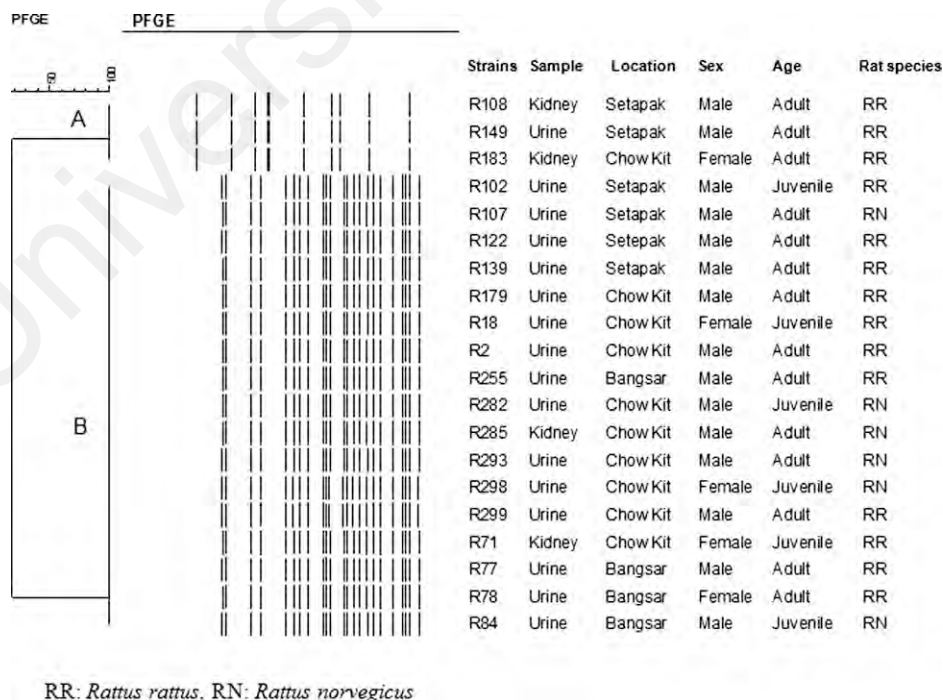
FIGURE 4. Dendrogram based on cluster analysis of the PFGE profiles of *Not*I digested chromosomal *Leptospira* isolated from urban rats generated using BioNumerics Version 6.0 (Applied Maths, Belgium) software and unweighted pair group arithmetic means methods (UPGMA). Cluster A represents *L. interrogans* serovar Bataviae, cluster B represents *L. borgpetersenii* serovar Javanica.

According to host age, adult rats (15/208) were infected more frequently compared with juvenile rats (5/92). This finding is in agreement with other reports that showed that adult rats can carry more *Leptospira* than immature rats.[35,36] Carter and Cordes[37] reported that mature urban *R. rattus* were four times more likely to carry *Leptospira* compared with immature *R. rattus*. Aggression, fighting, and biting among the adult rats can also facilitate the transmission of *Leptospira* between the adults.[30] Other factors include the dynamic movement of adults compared with the young and length of exposure to the environment contaminated with *Leptospira* from rat urine. Transmission by sexual contact in adults can also take place for this age class only. Moreover, young rats are generally confined to the nesting burrows; hence, they experience less exposure to the pathogen.

According to host sex, more male carrier rats were observed compared with females ($P = 0.045$). This result could be explained by the dynamic movement and aggressiveness of male rats, which is a sign of maturity.[30] However, this observation differed from previous studies. Among them, a study on urban rats of Baltimore, Maryland by Easterbrook and others[38] reported more females carrying *Leptospira* compared with male rats. Faria and others,[32] however, found similar carriage between male and female rats. In contrast, Agudelo-Florez and others[39] reported no correlation between sex, age, and health condition of the host and prevalence of the infection.

Many studies reported the low sensitivity of the culture technique in detection of *Leptospira* spp. compared with other techniques, such as PCR, immunofluorescence, and nucleic acid hybridization.[40,41] The success of the isolation step is influenced by various factors, such as the number of organisms per inoculation, the type of media used, and the type of specimen. The difficulty in the isolation of *Leptospira* and the slow growth of this fastidious organism made the culture technique time consuming with low sensitivity. In the present study, PCR was able to successfully determine the genus and pathogenic status of the isolates. With its specificity and rapidity, PCR has become a promising tool for the early detection of *Leptospira* in various specimens.[12,13,33]

However, MAT was able to identify successfully 20 positive *Leptospira* isolates at the serogroup level using 25 different sera. Two serogroups were identified; serogroup Javanica was found to be the predominant serogroup infecting 17 (85%) positive rats, whereas serogroup Bataviae was found only in 3 rats. In a recent study, the prevalence of leptospiral antibody among municipal workers in Kota Bharu, Kelantan, Malaysia showed that antibodies against serogroups of Bataviae and Javanica were predominant.[42]

The rat species *R. rattus* was found to be the main carrier for both serogroups. Interestingly, *R. norvegicus* was the main carrier of serogroup Javanica rather than serogroup Icterohaemorrhagiae, which was recognized as the maintenance host of these leptospires. This finding indicates that carriers of this pathogen are not dependent on host species but the distribution of the respective rat species in the various habitats. Gordon-Smith and others[28] reported that, among the 42 identified strains from Javanica serogroup, 37 (88%) strains were isolated from the rice field rat, *R. argentiventer*. *L. borgpetersenii* serovar Javanica was also one of the predominant strains isolated from rice field *R. norvegicus* trapped in rural areas of Tamil Nadu, India.[43] The existence of this serovar among the urban rats could be explained by the migration of infected rats from the rural area to the urban cities by transportation of goods, such as rice, and animals between the two areas. This reason probably explained the high number of positive rats infected with serogroup Javanica from Chow Kit market, where raw foods were brought from rural areas to be sold in the city.

PFGE results were in concordance with MAT results. Two PFGE profiles were generated using the NotI enzyme. With reference to PFGE profiles available in the leptospiral database at CDC, we were able to identify *L. interrogans* serovar Bataviae in 3 isolates and *L. borgpetersenii* serovar Javanica in 17 isolates.

PFGE showed a limited genetic diversity within the serovars of Javanica and Bataviae. All 17 Javanica isolates had the same PFGE profiles, although these isolates were collected from different locations, different rat species, and different sampling times. Similarly, all the Bataviae that originated from different geographical locations were indistinguishable. This finding was in agreement with the observation of Galloway and Levett[15] that PFGE was unable to discriminate strains that are closely related, such as serovars Icterohaemorragiae and Copenhageni of *L. interrogans* species. However, PFGE showed a high discriminatory power to differentiate different serovars of *L. borgpetersenii* and *L. interrogans*. Hence, PFGE could be applied as a complementary tool to the serological technique to differentiate leptospiral serovars.[15,44]

In conclusion, this study provides important information regarding the infection levels and identification of the pathogenic serovars circulating among the urban rat populations of Kuala Lumpur, namely *Leptospira* serovars Javanica and Bataviae. Despite the low infection rate present in the population, because of its high pathogenicity, long-term monitoring is necessary to keep the safety of the public in check.

Authors' addresses: Douadi Benacer and Kwai Lin Thong, Institute of Biological Sciences, Faculty of Science and Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Kuala Lumpur, Malaysia, E-mails: benacer_dvt@hotmail.com and thongkl@um.edu.my. Siti Nursheena Mohd Zain, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, E-mail: nsheena@um.edu.my. Fairuz Amran, Bacteriology Unit, Institute for Medical Research, Ministry of Health, Kuala Lumpur, Malaysia, E-mail: fairuz@imr.gov.my. Renee L. Galloway, Bacterial Zoonoses Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA, E-mail: zul0@cdc.gov.

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3.5 Publication 5

Determination of *Leptospira borgpetersenii* serovar Javanica and *Leptospira interrogans* serovar Bataviae as the persistent *Leptospira* serovars circulating in the urban rat populations in Peninsular Malaysia.

Benacer D, Mohd Zain SN, Sim SZ, Mohd Khalid NMK, Galloway RL, Souris M, Thong KL.

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3.5.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Mohd Zain SN, Thong KL, Mohd Khalid NMK
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

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Determination of *Leptospira borgpetersenii* serovar Javanica and *Leptospira interrogans* serovar Bataviae as the persistent *Leptospira* serovars circulating in the urban rat populations in Peninsular Malaysia

Douadi Benacer[1], Siti Nursheena Mohd Zain[1], Shin Zhu Sim[2,3], Mohd Khairul Nizam Mohd Khalid[4], Renee L. Galloway[5], Marc Souris[6] and Kwai Lin Thong[1*]

Abstract

Background: Leptospirosis is an emerging infectious disease of global significance, and is endemic in tropical countries, including Malaysia. Over the last decade, a dramatic increase of human cases was reported; however, information on the primary vector, the rat, and the *Leptospira* serovars circulating among the rat population is limited. Therefore, the present study was undertaken to isolate *Leptospira* and characterise the serovars circulating in the urban rat populations from selected main cities in Peninsular Malaysia.

Methods: Rat trappings were carried out between October 2011 to February 2014 in five urban cities which were chosen as study sites to represent different geographical locations in Peninsular Malaysia. Microscopic agglutination test (MAT) and PCR were carried out to identify the Leptospiral serogroup and determine the pathogenic status of the isolates, respectively while pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD)-PCR were used to characterize the isolates.

Results: Three rat species were identified from the three hundred and fifty seven rats captured with *Rattus rattus*, being the dominant rat species (285, 80 %) followed by *Rattus norgevicus* (53, 15 %) and *Rattus exulans* (19, 5 %). Only 39 samples (11.0 %) were positive by culture and further confirmed as pathogenic *Leptospira* by PCR. Significant associations were shown between host infection with locality, season, host-age and species. Based on MAT, two serogroups were identified in the population namely; *L. borgpetersenii* serogroup Javanica (*n* = 16) and *L. interrogans* serogroup Bataviae (*n* = 23). Pulsed-field gel electrophoresis (PFGE) distinguished the two serovars in the urban rat populations: *L. borgpetersenii* serovar Javanica (41 %), and *L. interrogans* serovar Bataviae (59 %). RAPD-PCR yielded 14 distinct patterns and was found to be more discriminative than PFGE.

Conclusions: This study confirms two *Leptospira* serovars circulating among the urban rats population in Peninsular Malaysia namely; *L. borgpetersenii* serovar Javanica and *L. interrogans* serovars Bataviae. Despite the low number of isolates obtained from the rat population, this study suggests that rodent control programs and disease surveillance may help to reduce the possible risk of disease transmission.

Keywords: *Leptospira*, Leptospirosis, Rodents, Outbreaks

* Correspondence: thongkl@um.edu.my
[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
Full list of author information is available at the end of the article

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Background

Leptospirosis is one of the most widespread zoonotic bacterial diseases with a worldwide distribution [1]. It is caused by the pathogenic species from the genus of *Leptospira*. and affects both humans as well as domestic and wild animals. It is maintained and spread throughout the environment through the urine of infected animals. Human infection occurs through exposure to water or soil contaminated with urine of infected animals or through direct contact with contaminated urine [2]. Leptospirosis occurs mainly in tropical and sub-tropical regions where environmental and socioeconomic conditions for its transmission and survival are particularly favourable [3, 4]. Leptospires thrive in warm, moist soil and immersion in water for long periods [5]. At present, leptospirosis is commonly associated with recreational activities in wild environments [6]. Certain occupations and recreational activities are at higher risk of contracting the infection due to the nature of these activities and work environment. In Malaysia, several outbreaks were related to recreational activities [7, 8]. Leptospirosis is also known as an occupational disease, commonly occurring among farmers, veterinarians, fishermen, livestock and abattoir workers [1, 9].

Natural reservoirs of leptospires are rodents and a large variety of other feral and domestic animals. Many serotypes occur predominantly in select mammalian hosts; however, the distribution of a specific serotype in a select host is not exclusive.

In Malaysia, the first study on a leptospirosis was recovered from black rats [10] and the rodents are now considered the principal maintenance hosts of *Leptospira* and the carriers for pathogenic *Leptospira* serovars. To date more than 37 *Leptospira* serovars from 13 different serogroups have been identified in Malaysia with more than half found to be carried by rodents [11].

Locally, dramatic increases in human cases were reported over the last decade. In 2013 there were 4,457 cases with 71 deaths marking an increase in disease incidence from 12.5 per 100,000 population in 2012 to 15.0 per 100,000 population the following year (Data extracted from official report from the Ministry of Health Malaysia). Recently an outbreak occurred in Kelantan State where more than 94 human leptospirosis cases were reported after the floods that hit the eastern side of the Peninsular between 1 and 18 of January 2015 [12].

The majority of cases in this country were related to the exposure of humans to environment contaminated by *Leptospira* spp. [13, 14]. However, there is little information regarding the primary carrier vector, the rat and the risk to human transmission in urban settings of Malaysia.

A recent study revealed two predominant pathogenic *Leptospira* serovars from two species; *Leptospira borgpetersenii* serovar Javanica and *Leptospira interrogans* serovars Bataviae circulating in the two dominant rat species, *Rattus rattus* and *Rattus norvegicus* in Kuala Lumpur [11]. The presence of pathogenic *Leptospira* thriving in an urban rat population, facilitated with abundance of food and improper garbage management, in addition to rapid urbanisation and growing of slums with inadequate infrastructures (sewage, water) could ultimately bring rodents in close proximity to humans. Therefore the objective of this study was to determine *Leptospira* serovars circulating in several urban rat populations in Peninsular Malaysia.

Five different States representing North (Penang, Perak), East (Pahang), West (Selangor) and South (Malacca) parts of Peninsular Malaysia were selected as study sites based on the disease incidence over the past few years [15]. The identification of *Leptospira* and determination of its pathogenic status of the isolates were carried using MAT and PCR while PFGE and RAPD-PCR were used to analyze the genetic relatedness of the recovered isolates.
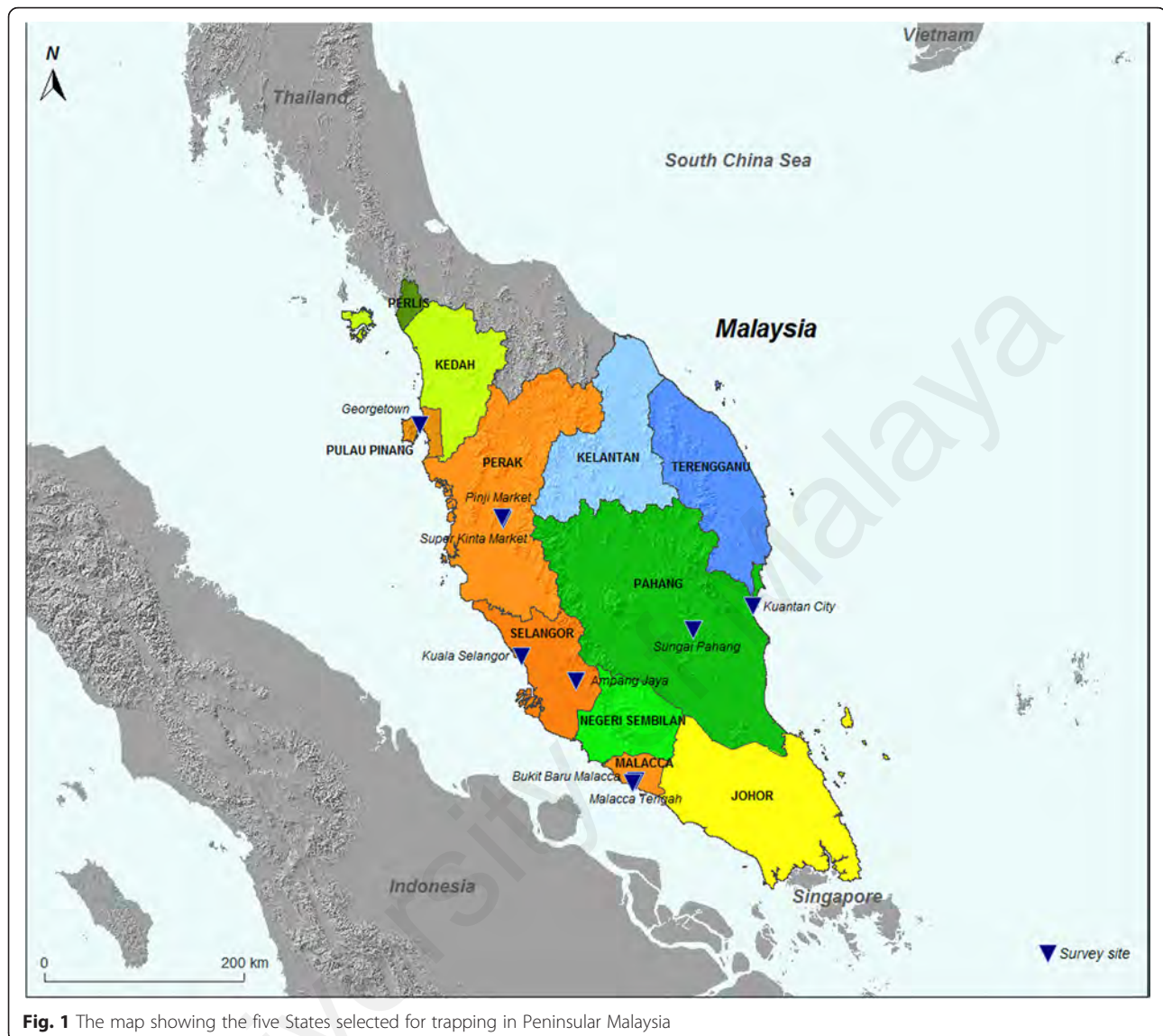
Methods

Ethics statement

This study was approved with the ethics reference no. ISB/31/01/2013/SNMZ (R) by the Institutional Animal Care and Use Committee, University of Malaya, Malaysia (UM IACUC). This study did not involve any endangered or protected species.

Choice of the study sites

Several locations were selected as study sites to represent different geographical locations in Peninsular Malaysia namely; Penang and Perak (North), Selangor (East), Malacca (South) and Pahang (West) (Fig. 1). The trappings were conducted with the assistance of the municipality from each city as part of the vector control programme. The sites chosen were based on the suitability of the habitat for rats to forage, breed and the risk of transmission. This included wet markets, food courts, restaurants and hawker stalls with abundance of leftovers (food remnants). In addition, high density residential areas particularly from the lower income bracket and nearby open wet markets providing fresh produce to the community and poor garbage management, were selected as these sites attracted rodents and stray animals to breed and to source for food. All sites were characterized by tropical climate and high humidity throughout the year with temperatures ranging between 30 °C and 36 °C with heavy rainfall coinciding with the monsoon season. For this purpose, season is divided into wet and dry seasons for each year with dry months falling between March – September and wet season between October – February.

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Fig. 1 The map showing the five States selected for trapping in Peninsular Malaysia

Trapping and host identification

Trapping was conducted over a period of 6 days and 5 nights for each session with a total of 36 trapping sessions conducted between October 2011 and February 2014. The wire traps (18 x 12 x 28 cm) were placed in each site with baits such as dry fish, breads, and peanut butter. The traps were placed in the evening and collected early morning before the market was opened to the public. The captured rats were placed in black plastic bags to reduce the stress and transported to the laboratory for examination. The rats were then euthanized and morphometric measurements recorded [11]. Age and species identification were carried out according to Medway [16] based on the phenotypic characteristics such as fur color (ventral and dorsal), body weight, hind foot and head-body length.

Isolation of *Leptospira* and dark field microscopy examination

Post mortem examination was performed to retrieve selected organs such as blood, livers and kidneys. The urine was collected *via* direct puncture of the bladder and then cultured into the modified semi-solid EMJH medium. Kidney tissue samples were removed using a sterile blade, and a small piece of tissue was placed in a sterile syringe without a needle and squeezed into the EMJH medium supplemented with 5-fluorouracil (Merck, Germany). All the inoculated media were incubated aerobically at 30 °C, examined under a dark field microscope for the presence of *Leptospira* at 10-day intervals for a period of 3 months. Samples that failed to show any evidence of growth after 3 months were considered negative.

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PCR detection and confirmation of leptospiral isolates by PCR

DNA was extracted from 7 day-old culture media using Wizard™ Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The quantity of DNA was measured by Biophotometer (Eppendorf, Germany).

PCR primers, LG1/LG2 [(5′-CGG TGA AAT GCG TAG ATA TC-3′) and (5′- CGG TTT GTC ACC GGC AGT TC-3′)] were designed in-house to target the 16S rDNA gene to confirm the genus of *Leptospira* isolates. The cycling conditions consisted of an initial denaturation at 94 °C for 3 min, 35 cycles each of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and further extension at 72 °C for 10 min. To determine the pathogenic status of the isolates, the published G1/G2 primers were used [(5′-CTG AAT CGC TGT ATA AAA GT-3′) and (5′-GGA AAA CAA ATG GTC GGA AG-3′)], which target the *secY* gene among the pathogenic species except for *L. kirschneri* [17]. The cycling conditions consisted of an initial denaturation at 94 °C for 10 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. In both PCRs, the reactions were done in a final volume of 25 μl containing 1X PCR buffer, 1.5 mM MgCl₂, 200 μM each of dNTPs, 0.3 μM of each primer, 1 U of *Taq* DNA polymerase (Intron, Biotechnology, South Korea) and 100 ng of DNA template. The PCR products were analyzed by electrophoresis through a 1 % agarose gel (Promega, Madison, USA).

DNA sequencing. PCR products from representative isolates were verified by DNA sequencing. The amplicons were purified using DNA purification kit (Qiagen, Germany) and sent to a commercial facility for sequencing (First BASE, Pte. Ltd., Singapore). The resulting DNA sequence data were compared with the GenBank database using the BLAST algorithm available at web site (http://www.ncbi.nlm.nih.gov).

Microscopic agglutination test (MAT)

Live isolates were used for MAT as described by World Health Organization [18]. The isolates were cultured into liquid medium supplemented with 1.0 % rabbit serum to increase bacterial density [11]. The antisera used in this study were raised against serovars Sejroe (M84), Javanica (Veldrat Batavia 46), Canicola (Hond Ultrecht IV), Hebdomadis (Hebdomadis), Pomona (Pomona), Hardjo (Hardjoprajitno), Australis (Ballico), Bataviae (Swart), Pyrogenes (Salinem), Icterohaemorrhagiae (RGA), Autumnalis (Akiyami A), Grippotyphosa (Mandemakers).

Pulsed field gel electrophoresis

PFGE was performed according to protocol [19] with minor modifications. Leptospiral DNA embedded into agarose plugs were digested with 10 U of restriction enzyme *Not*I (Promega, Madison, USA) at 37 °C. The restriction fragments obtained were separated by electrophoresis in 0.5X TBE buffer, for 24 h at 14 °C in a CHEF Mapper system (Bio-Rad, USA) using pulsed times of 2.2 to 35 s. *Xba*I-digested *Salmonella* Braenderup H9812 was used as the DNA size marker. PFGE data were analyzed using BioNumerics Version 6.0 (Applied Maths, Belgium) software. Clustering was based on the unweighted pair group average method (UPGMA) with the position tolerance of 1.0.

Subtyping of *Leptospira* Isolates by using RAPD-PCR

RAPD-PCR fingerprinting was performed according to Ramadass et al. [20] using B11 (5′-CCG GAA GAA GGG GCG CCA T-3′) and B12 (5′-CGA TTT AGA AGG ACT TGC ACA C-3′) primers with some modifications. The reaction was carried out in a final volume of 25 μl containing 1X PCR buffer, 1.5 mM MgCl₂, 200 μM each of dNTPs, 0.3 μM of each primer, 1 U of *Taq* DNA polymerase (Intron, Biotechnology, South Korea) and 100 ng of DNA template. The cycling conditions consisted of two cycles of denaturation at 95 °C for 5 min, annealing of primers for 5 min at 40 °C, and extension for 5 min at 72 °C. The subsequent 35 cycles consisted of denaturation for 1 min at 95 °C, annealing of primers for 1 min at 60 °C, and extension for 3 min at 72 °C, with a final extension step for 10 min during the last cycle. PCR products were analyzed by electrophoresis through a 1 % agarose gel (Promega, Madison, USA).

Statistical analysis

All statistical analyses were performed using the logistic regression model in R software (version 3.1.1) [21]. The infection status of individual rats was investigated based on the effects of host-age, host-sex, host-species, season and location. In our study, the samples that showed the typical morphology and the characteristic motility of *Leptospira* genus under the dark field microscope from urine and kidney samples and confirmed by MAT and PCR were considered as positive samples. Further sequencing of the PCR products were done to confirm the results. Akaike's Information Criterion (AIC) was used for the selection of the best model. It is defined as $AIC = 2k – 2InL$ where k is the number of parameters in the model and InL is the maximum of the log-likelihood function for the data [22]. The model with the lowest AIC value was selected. The significance tests for the individual explanatory variables were performed by computing Wald statistics. A small p-value (<0.05) of the Wald test indicates a detection of a statistical significance variable in our model.

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Results

A total of 357 rodents captured were composed predominantly of *Rattus rattus* 285 (80 %), followed by *Rattus norvegicus* 53 (15 %) and *Rattus exulans* 19 (5 %) (Table 1). The highest capture was from the vicinity of Pasar Super Kinta in Ipoh, Perak (105 rats, 29 %), followed by Taman Bukit Baru residential area in Malacca with 95 (27 %) rats. Based on the host-age and sex, more adults 265 (74 %) were captured than juveniles 92 (26 %) with more females (216, 61 %) compared to males (141, 39 %). Isolation of *Leptospira* was achieved from 39 captured rats (11.0 %) which were recovered from 35 urine and 4 kidney samples. The cultures showed the typical morphology and the characteristic motility of species of *Leptospira* by dark field microscopy.

The statistical analysis revealed that the infection status of individual rats depends on the host-age, host-species, season and location. Note that the variable host-sex was excluded because the Wald test for significance of this variable yielded *p*-value of 0.93. This indicates that the variable host-sex appears to be redundant in the model.

From Table 2, the generalized linear model analysis shows that the odds of positive infection were 93 % less likely among juvenile rats as compared to adult rats (odds ratio = 0.07) after adjusting for host-species, season and location. For host-species, the rat species *R. exulans* had a 5.58 times greater odds of positive infection than the rat species *R. rattus* after adjusting for host-age, season and location. Meanwhile, the variable season was found to be statistically significant (*p*-value = 0.04). The odds of infected rats were about 10 times higher among rats

Table 2 Generalized linear model of urban rats infections from Peninsular Malaysia (October 2011 to February 2014) by *Leptospira* with Binomial distribution and logit link function

Variables	Estimate	(Standard error)	*p*-values
(Intercept)	−5.18	1.23	< 0.001
Host-age: juvenile	−2.66	1.04	0.01
Host-species: *Rattus norvegicus*	0.28	0.62	0.65
Host-species: *Rattus exulans*	1.72	0.76	0.02
Location: Penang	1.97	0.83	0.02
Location: Pahang	1.47	0.75	0.05
Location: Malacca	2.68	1.29	0.04
Location: Perak	1.53	0.77	0.05
Season: wet	2.30	1.09	0.04

Null deviance: 246.28 on 356 degrees of freedom
Residual deviance: 197.83 on 348 degrees of freedom
AIC: 215.83

captured during the wet season as compared to rats captured during the dry season (odds ratio = 9.97) after adjusting for host-age, host-species and location. This indicates that rats captured during the wet season were more likely to be infected than the rats captured during the dry season. Also, the odds of infected rats found in locations Penang, Pahang, Malacca and Perak were higher than the location Selangor after adjusting for host-age, host-species and season.

Genus confirmation by PCR using the designed primers LG1/LG2 showed that all 39 isolates gave the expected band sized 483 bp, indicating that all the positive isolates were *Leptospira* spp. (99 % similarity, accession no. KC662455, JQ988849). In addition, all

Table 1 Summary of the rodent population according to rat species, host-sex and host-age from five states in peninsular Malaysia

States	Location	Host-species			Host-sex		Host-age		Season		Source		Result	
		RR	RN	RE	Male	Female	Adult	Juvenile	Dry	Wet	Urine	Kidney	Negative	Positive
Selangor	Kuala Selangor	10	2	0	4	8	6	6	7	5	8	4	12	0
	Ampang Jaya	22	24	4	17	33	38	12	0	50	14	36	47	3
Penang	Georgetown	4	11	0	10	5	15	0	0	15	8	7	10	5
Pahang	Kuantan city	31	10	12	24	29	48	5	18	35	25	28	41	12
	The Sungai Pahang	18	0	3	9	12	15	6	21	0	5	16	21	0
Malacca	Taman Bukit Baru	90	5	0	31	64	73	22	95	0	82	13	90	5
	Melaka Tengah district	2	1	0	1	2	3	0	3	0	3	0	2	1
Perak	Super Kinta market (Ipoh)	105	0	0	43	62	64	41	14	91	87	18	92	13
	Pinji market (Ipoh)	3	0	0	2	1	3	0	0	3	3	0	3	0
Total (Number of infected rats)		285 (24)	53 (8)	19 (7)	141 (19)	216 (20)	265 (38)	92 (1)	158 (7)	199 (32)	235	122	318	39

RR Rattus rattus
RN Rattus norvegicus
RE Rattus exulans

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isolates were pathogenic species as shown by the band sized 285 bp amplified by the primers G1/G2 which targets *secY* genes (99 % similarity, accession numbers: EU358040, DQ882852).

The identification of the serogroup of the isolates was carried out using the 13 different hyperimmune antisera. Results showed 23/39 isolates reacted by agglutination against the serovar Bataviae antisera (titer > 1:400), while only 16 isolates reacted towards the serovar Javanica (titer > 1:400). All 13 isolates from Ipoh, Perak were identified from the serogroup of Javanica, while 6 isolates from Malacca belong to Bataviae serogroup. The majority of isolates from Kuantan, Pahang State (11 isolates) belong to Bataviae serogroup while only one was from Javanica serogroup. Two isolates from Ampang Jaya in Selangor were from Bataviae serogroup, while only one from Javanica serogroup. Four isolates from Penang were from Bataviae serogroup and only one from Javanica serogroup.
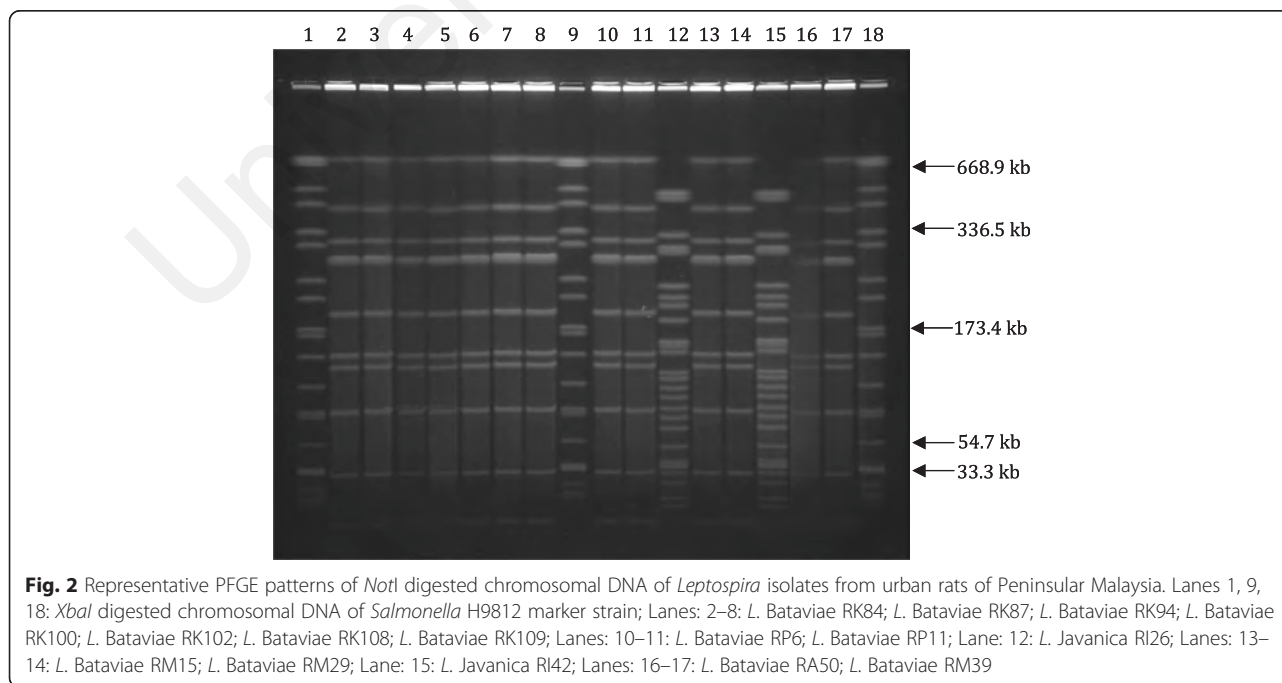
PFGE of *Not*I-digested chromosomal DNA subtyped the 39 isolates into 2 distinct PFGE profiles LNot01, and LNot02. The number of DNA fragments generated ranged from 10 to 23 with sizes ranging approximately from 28 kb to 706 kb (Fig. 2). Limited genetic diversity was found among the isolates.

The 2 PFGE profiles generated were compared to the *Not*I patterns from the database provided by the Centers for Disease Control and Prevention (CDC), USA. The results showed that LR01 were similar to *L. interrogans* serovar Bataviae, whereas LR02 were similar to *L. borgpetersenii* serovar Javanica.
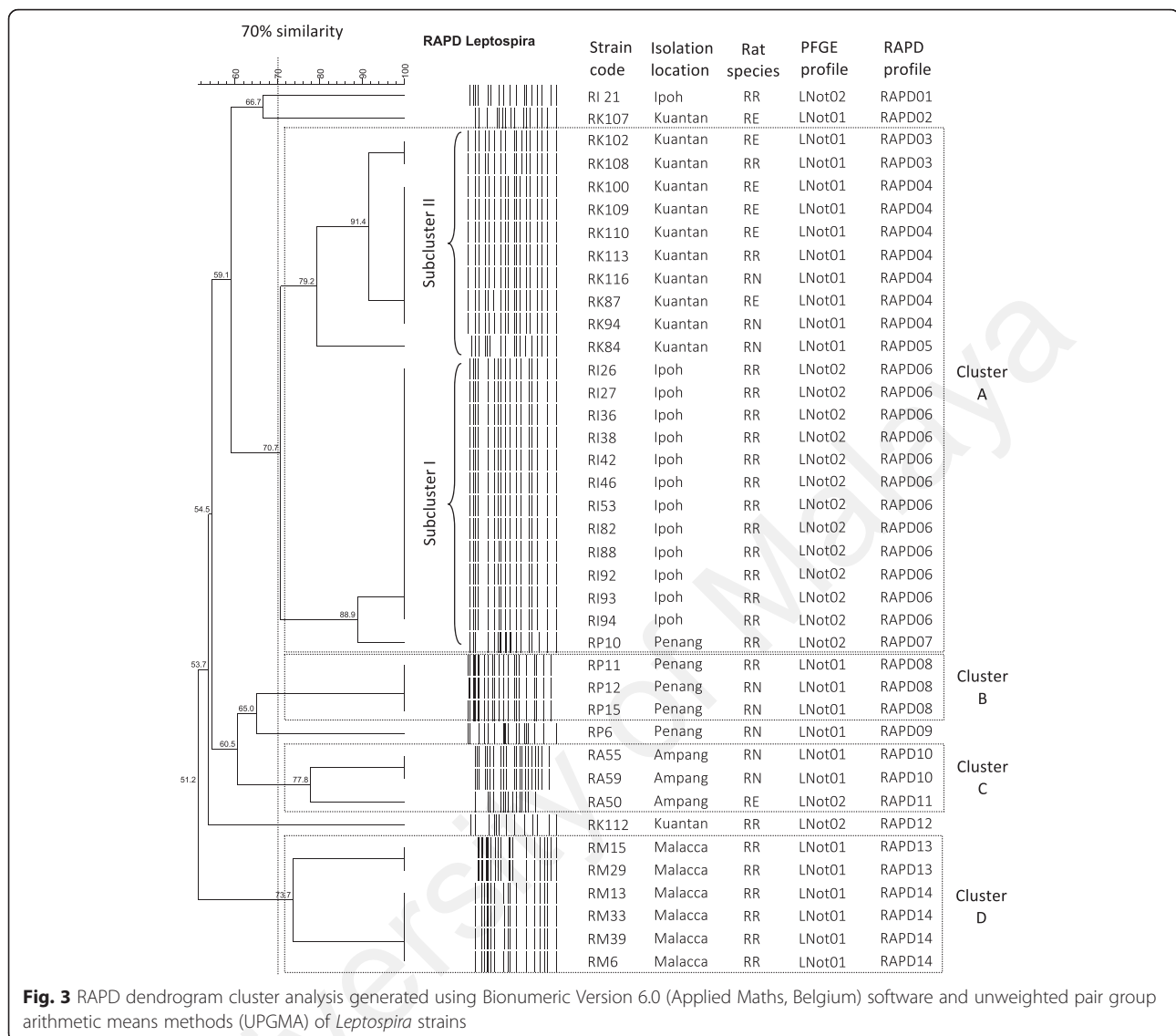
RAPD-PCR using the primers B11 and B12 subtyped the 39 positive isolates into 14 different patterns. All the strains were type-able by using RAPD-PCR and the band patterns were highly variable (polymorphism). Clear genomic patterns were obtained for all strains, even though some variation of band intensity could be observed.

Each strain contained between 15 to 27 bands ranging in size from 200 bp to 2000 bp. Bands below 200 bp and above 2100 bp were not included in the analysis. High genetic diversity was observed among the isolates from Kuantan, Pahang, as the 13 isolates gave six different RAPD patterns. A limited genetic diversity was observed among the isolates isolated from Malacca and Ipoh, Perak. The three isolates from Ampang Jaya in Selangor and the five isolates from Penang gave three different patterns, respectively. The dendrogram generated four major clusters, namely cluster A, B, C and D at 70 % of similarity (Fig. 3). Cluster A consisted of 23 isolates and it is further divided into two subclusters (subcluster I and II). Subcluster I consisted of 13 isolates recovered from *R. rattus* and had the same PFGE profile (LNot02) in both Ipoh (11 isolates) and Penang (2 isolates). Subcluster II consisted of ten isolates recovered from *R. exulans* (5), *R. norvegicus* (3) and *R. rattus* (2). All the ten isolates had the same PFGE profile (LNot01).

Cluster B consisted of three isolates from Penang which had the same PFGE profile (LNot01) and isolated from two *R. norvegicus* and one *R. rattus*. Cluster C consisted also of three isolates from Ampang, Selangor and had two different PFGE profiles (LNot01, LNot02). Two of them were isolated from *R. novegicus* and one

Fig. 2 Representative PFGE patterns of *Not*I digested chromosomal DNA of *Leptospira* isolates from urban rats of Peninsular Malaysia. Lanes 1, 9, 18: *Xba*I digested chromosomal DNA of *Salmonella* H9812 marker strain; Lanes: 2–8: *L.* Bataviae RK84; *L.* Bataviae RK87; *L.* Bataviae RK94; *L.* Bataviae RK100; *L.* Bataviae RK102; *L.* Bataviae RK108; *L.* Bataviae RK109; Lanes: 10–11: *L.* Bataviae RP6; *L.* Bataviae RP11; Lane: 12: *L.* Javanica RI26; Lanes: 13–14: *L.* Bataviae RM15; *L.* Bataviae RM29; Lane: 15: *L.* Javanica RI42; Lanes: 16–17: *L.* Bataviae RA50; *L.* Bataviae RM39

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Fig. 3 RAPD dendrogram cluster analysis generated using Bionumeric Version 6.0 (Applied Maths, Belgium) software and unweighted pair group arithmetic means methods (UPGMA) of *Leptospira* strains

was from *R. exulans*. Cluster D consisted of six isolates which were isolated from *R. rattus* captured in Malacca and had the same PFGE profile (LNot01) (Fig. 3).

Discussion

Rapid urbanization and urban poverty have led to the dramatic growth of slum settlements in many low and middle-income countries [23]. These locations are often characterized by poor infrastructure with an ineffective irrigation system, garbage management system and sanitation facilities that promote proliferation of rodents and poses the risk of rodent-borne transmission. Rats are known as the source of a number of pathogens responsible for significant human morbidity and mortality in many cities around the world [24]. They act as the primary hosts of *Leptospira* spp. and are well recognized as

the most significant mammal species in maintaining and disseminating of leptospires worldwide [25].

In Malaysia, leptospirosis is an endemic disease and recently there has been an increase in the number of reported human cases. Hence it is important to investigate the role of the rats in the dissemination of disease in urban settings. Information with regards to the disease incidence and leptospiral serovars circulating among the urban rat population in the Peninsular Malaysia is essential to have a better understanding of how these reservoirs contribute to disease transmission to humans.

Five major states representing different unique geographical locations in Peninsular Malaysia were selected for rat trapping based on the high incidence of human leptospirosis cases between 2004 and 2012 "unpublished data". Sites were also chosen based on presence of recent rat activity in public spaces. Wet markets were an ideal

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site for both rats and stray animals to forage since food leftovers are aplenty and consequently such places become their breeding grounds. On the other hand, the presence of rats was also observed in slum residential areas due to improper garbage disposal and poor irrigation system with open sewers. These conditions have created favorable habitat for the survival of the host reservoir and subsequently the contamination of environmental waters and soils *via* their excreta and urine.

In this study, *R. rattus* was the dominant rat species captured in the five States ($p < 0.001$) followed by *R. norvegicus*. This result concurred with our previous study [11] indicating that both species are the two dominant rat species in the urban areas of Peninsular Malaysia. Both species are commensal rats and are generally found living closely to human habitation and dependent on human wastes for food, water and space for shelter [26, 27]. Although the Norway rat (*R. norvegicus*) is capable of living in isolation, the convenient human environment is preferable to them and as a result they were found less commonly in the rural environment. A small population of *Rattus exulans* were captured close to the forest fringes of the urban cities. *R. exulans* or known as Polynesian rat is the third most widespread species rat in the world after *R. rattus* and *R. norvegicus.* This species generally inhabits rural scrub, rubber and coconut palm plantations. However, the presence of this species in the cities could be explained by the migrating rats for scavenging as well as the rapid urbanization and the deforestation at the fringes of the cities which have put humans in contact with more animal reservoirs.

The highest number of captured rats was in Ipoh, Perak State. The two trapping locations (Pinji and Super Kinta) were wet markets that sold fresh produce with plentiful resources for the rodents to forage. Both markets lacked proper maintenance with water in containers left unprotected overnight and open sewers and drains that were full of rubbish. These conditions not only attracted rats to forage at night but also pose the risk of possible contamination with their urine, serving as a source of infection for both man and animals.

Taman Bukit Baru in Malacca State recorded the second highest rat capture and this site comprised of residential area with old buildings with poor irrigation and improper rubbish management system. Rats were frequently observed roaming near rubbish dumps close to the housing area. This also poses a high risk of exposure to humans especially children, who play in the surrounding vicinity as living in close proximity to accumulated garbage had been found to significantly increase the risk to leptospirosis [28].

The levels of infection found in the rodent population in Peninsular Malaysia were low (11 %). However these results were comparatively higher to our previous study

(6.7 %) in the capital city of Kuala Lumpur [11]. Despite the low infection rate reported, the high pathogenicity of these serovars raises concern of public health risks caused by the transmission of leptospirosis. Ivanova et al. [29] noted similar infection rates in rodent and shrews from several locations in Cambodia while Cosson et al. [30] noted low *Leptospira* prevalence among trapped rodents from seven localities in Southeast Asia (Thailand, Laos and Cambodia).

For the three species captured, infections were more prevalent in *R. exulans* (38 %) compared to *R. norvegicus* (18 %) and *R. rattus* (7 %). To date, *Leptospira* spp. has been successfully isolated from more than ten rat species including *R. exulans* [31–33] in Malaysia. The finding is also in agreement with studies conducted by Wangroongsarb et al. [34] on 1,164 rodents captured from ten epidemic states in Thailand and where they noted highest infection rate in *R. exulans*. However, their results did not concur with other studies [29, 35].

Host age played an important role in influencing the infection with more adult rats being significantly infected with *Leptospira* than immature rats and this is in agreement with other studies [11, 25, 36]. Physiology, immunology and behavioral characteristics related to age are reported to play an important role in the transmission of the infections [35]. Adult rats forage further and have a more dynamic movement which put them at higher risk of exposure to the infection compared to the juvenile or newborns that are generally confined to the nesting burrows [37]. Aggressive behaviour of the adult rats such as fighting and biting is known to facilitate the transmission of leptospiral infection [32] as reported by Himsworth et al. [38] that the weight, body fat and bite wounds common among the adult rats increases the probability of leptospiral infection.

A similar carriage between males and females rats was recorded in this study with no correlation between infection rates with host-sex. This finding differed from other studies where significantly more males were infected [11, 33]. In contrast, Agudelo-Florez et al. [39] reported no relationship between host-sex, host-age or host health with the disease prevalence.

This investigation also recorded the influence of season on the disease prevalence with significantly more rats infected during the wet season as observed in the States of Pahang, Penang and Perak and this result concurred with other studies [25, 29]. Heavy rainfall during the monsoon months readily facilitates the transmission of waterborne bacteria including *Leptospira* spp. as the moisture and humidity increase and the availability of fresher and cleaner water bodies to thrive in. The combination of all these factors can lead to a more favorable environment for the survival of leptospires [25]. In contrast, initial *Leptospira* survival is likely to be poor in the

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dry season as rodent urine quickly dries up on dry ground. The numbers of leptospirosis-infected rodents and the abundance of leptospires in the environment are both potential indicators of risk of leptospirosis infection to humans [40].

Several studies reported the low sensitivity of the culture technique in the detection of *Leptospira* spp. compared to other techniques such as PCR, immuno-fluorescence and nucleic acid hybridization [41, 42]. Besides this, the success of the isolation step is influenced by various factors such as the number of organisms per inoculation, the type of media used, and also the type of specimen. The difficulty in the isolation of *Leptospira* and the slow growth of this fastidious organism made the culture technique time consuming with low sensitivity.

Presently, PCR analysis is increasingly used as a promising tool in the diagnosis of leptospirosis due to the high rapidity, sensitivity and specificity in the detection of *Leptospira* in different specimens including clinical, animal and environmental samples [14, 43, 44].

In the present study, PCR was able to successfully determine the genus and pathogenic status of the isolates. To determine the *Leptospira* genus of the isolates, the designed primers LG1/LG2 yielded a product band size of 483 bp from cultured positive samples. These primers were designed from *rrs* genes and expected to amplify leptospiral DNA from both saprophytic and pathogenic species. The specificity and the sensitivity of these primers were checked using 12 different *Leptospira* reference strains and 10 non-leptospiral bacteria. All the 39 isolates were confirmed as *Leptospira* genus. The pathogenicity status of the isolates was checked using G1/G2 primers. The result showed that all the confirmed *Leptospira* isolates were pathogenic.

The serogroups of the 39 isolates were determined using 13 different antisera and based on MAT technique. Only 2 serogroups were identified; with serogroup Bataviae (59 %) marginally higher than serogroup Javanica (41 %) with both serogroups previously reported in Kuala Lumpur city [11]. The persistence of these two serogroups circulating in the rat population indicates its role as a maintenance host in the transmission of *Leptospira*. Shafei et al. [45] reported two serogroups, Bataviae and Javanica predominant among town service workers in the north-eastern state of Malaysia.

PFGE of *Not*I-digested *Leptospira* DNA gave two profiles and the results were in concordance with MAT results. With reference to PFGE profiles available in the leptospiral database at CDC, we were able to identify *L. interrogans* serovar Bataviae in 23 isolates and *L. borgpetersenii* serovar Javanica in 16 isolates. Hence, PFGE could be used to complement serogrouping. However, PFGE was not discriminative enough to distinguish

isolates from different sources. Therefore, an apparent lack of genetic diversity was observed within the members of serovars of Javanica and Bataviae. All 23 Bataviae isolates had the same PFGE profiles, despite being collected from different locations in four different States including Malacca, Pahang, Penang and Selangor, different rat species and at different sampling times. Similarly, all the 16 Javanica, which originated from different geographical locations were indistinguishable. This was in agreement with the observation of the previous studies [11, 19] that PFGE was unable to discriminate strains within the same serovars such as Icterohaemorragiae and Copenhageni of *L. interrogans* species. However, PFGE showed a high discriminatory ability to differentiate different serovars of *L. borgpetersenii* and *L. interrogans.*

Hence, a second subtyping method, RAPD-PCR was used to improve the discrimination. In this study a greater genetic variation was observed among the members of the same serovar as the 39 isolates were represented by 14 different patterns. Some of isolates represented the same serovar isolated from different locations gave different RAPD patterns. Our study was in agreement with several previous studies [46, 47]. RAPD-PCR is shown to be a useful technique in epidemiological investigation of leptospirosis. In several studies, RAPD-PCR had the ability to discriminate between the strains at species and even at serovars level [20, 48]. Hence, based on discriminatory power, RAPD is more discriminative compared to PFGE which failed to distinguish strains within the same serovar. However, the reproducibility of the PCR fingerprinting is moderate, which makes pattern analysis more difficult and tedious as compared to PFGE. PFGE examines the genetic variation throughout the genome and is highly reproducible, which makes it the "gold standard" molecular typing of *Leptospira* spp. [19]. RAPD fingerprint could be an alternative tool in subtyping of *Leptospira* isolates, as it is easier and could generate results more rapidly than PFGE.

Conclusions

L. borgpetersenii serovar Javanica and *L. interrogans* serovar Bataviae were predominant among the urban rats in all the States studied which concurred with our previous study of the urban rats in Kuala Lumpur [11]. Despite the low disease prevalence, this finding highlights the risk of close proximity of this maintenance host to humans in urban settings. Therefore, assessment and regular monitoring of infections by wild rodents is necessary to address an important health problem and provide conclusions that can inform the design of effective public health policy.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Author details
[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. [2]Institute of Mathematical Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. [3]Lee Kong Chian Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, Kuala Lumpur, Malaysia. [4]Bacteriology Unit, Institute for Medical Research, Ministry of Health, Kuala Lumpur, Malaysia. [5]Bacterial Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia. [6]UMR_D 190 "Emergence des Pathologies Virales", IRD Aix- Marseille University EHESP, Marseille, France.

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3.6 Publication 6

Serological and Molecular Identification of *Leptospira* spp. in Swine and Stray Dogs from Malaysia.

Benacer D, Thong KL, Ooi PT, Souris M, Lewis JW, Ahmed AA, Mohd Zain SN

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3.6.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Mohd Zain SN, Thong KL, Ooi PT
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Serological and molecular identification of *Leptospira* spp. in swine and stray dogs from Malaysia

Benacer, D.[1], Thong, K.L.[1], Ooi, P.T.[2], Souris, M.[3], Lewis, J.W.[4], Ahmed, A.A[5] and Mohd Zain, S.N.[1*]
[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
[2]Faculty of Veterinary Medicine, University Putra Malaysia, Selangor, Malaysia
[3]UMR_D 190 "Emergence des Pathologies Virales", IRD Aix- Marseille University EHESP, Marseille, France
[4]School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK
[5]Royal Tropical Institute (KIT), Amsterdam, The Netherlands
[*]Corresponding author e-mail: nsheena@um.edu.my
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Abstract. Leptospirosis is endemic in Malaysia with *Leptospira* species extensively isolated from domestic and wild animals. Rats were found to be the principal maintenance hosts followed by cattle, pigs, and dogs. The objectives of this study were to isolate and identify *Leptospira* serovars circulating among swine from three different farms and also from stray dogs and cats from Klang valley, Selangor, Malaysia. Urine and kidney samples collected from 150 stray dogs, 50 cats and 81 swine were inoculated into semi-solid Ellinghausen McCullough Johnson and Harris (EMJH) media supplemented with additional 5-Fluorouracil. Dark field microscopy revealed only one positive culture of *Leptospira* from dog and swine samples, but all cat samples were negative. The PCR technique using published primers detected 11 positives in urine samples of dogs and 5 positives from swine. The microscopic agglutination test (MAT) confirmed the presence of two serovars in both dog and swine populations namely, *L. interrogans* serovar Canicola and *L. interrogans* serovar Pomona (MAT ≥ 100), with *Not* I-PFGE analyses separating these two serovars into distinct profiles. Despite the low prevalence in stray dogs, the latter may play an important role in the contamination of the environment. Swine can also pose a potential risk of infection to humans and other domestic animals, especially those living close to swine farms. Thus improving hygiene and eradication of rodents in swine farms are likely to reduce the risk of infection.

INTRODUCTION

Leptospirosis is one of the most prevalent zoonotic disease found worldwide (Levett, 2001) that is caused by pathogenic species within the genus *Leptospira*. This disease has an major public health impact in view of its occurrence in human as well as in domestic and wild animal hosts. Pathogenic species of *Leptospira* colonize the renal tubules and urine of various mammals including rodents, domestic and wild animals. Humans can be infected indirectly from exposure to water or soil contaminated with urine of infected animals or through direct contact with animal reservoirs through skin or mucous membranes (eyes, nose, or mouth),

especially if the skin is broken from a cut or scratch (Bharti *et al.*, 2003). The clinical manifestation of leptospirosis is biphasic with acute or septicaemic phases lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Levett, 2001). The incidence of leptospirosis is highest in tropical and sub-tropical countries where environmental conditions for transmission and survival are optimal (Pappas *et al.*, 2008). Leptospires can survive for long periods in warm, moist soil and in water (Ullmann & Langoni, 2011). Some occupations especially those with recreational activities, farmers, veterinarians, fishermen, livestock and

abattoir workers constitute high risk populations (Levett, 2001).

Leptospirosis is an endemic disease in Malaysia and in recent years there has been a dramatic increase in the number of reported cases. According to Ministry of Health, Malaysia a marked increase in the disease occurred from 12.5 per 100,000 population in 2012 to 15.0 per 100,000 population in 2013, with 71 of 4,457 cases resulting in mortality (Benacer *et al.*, 2016a).

Within the farming community, leptospirosis can potentially lead to economic losses, causing abortion, stillbirth, infertility, loss of milk production and death (Tilahun *et al.*, 2013; Maleki *et al.*, 2013). In Malaysia, *Leptospira* spp. have been isolated from a wide variety of animals (Bahaman & Ibrahim, 1988) including rodents, cattle, pigs, and dogs, which serve as potential carriers (Thayaparan *et al.*, 2013).

Biodiversity within tropical ecosystems of Malaysia results in the divergence of transmission with isolates of *Leptospira* being detected from a variety of animals living in low land habitats to forests swamps and rivers (Bahaman & Ibrahim, 1988). To date 37 serovars of *Leptospira* from 13 different serogroups have been identified in Malaysia with more than half from rodents (Bahaman & Ibrahim, 1988; Benacer *et al.*, 2013a). Both these studies showed that urban rats in Malaysia harbour two serovars, namely Bataviae and Javanica (Benacer *et al.*, 2013a, Benacer *et al.*, 2016b). The first report in Malaysia occurred in dogs (Fletcher, 1928), although both dogs and cats have been implicated as sources of infection because they mark territory by defecating and urinating indescrimiately and are closely associated with humans (Phumoonna *et al.*, 2009). *L.* Canicola and *L.* Icterohaemorrhagiae are the main serovars infecting dogs although Pomona serotypes have also been reported (Joseph, 1979; Phumoonna *et al.*, 2009). Leptospirosis in cats is rare (Bahaman & Ibrahim, 1988), but the importance of cats shedding *Leptospira* and serving as a source of infection has recently gained attention. An extensive study carried out by Gordon-Smith *et al.* (1961) showed that the serovar Pomona was found in 10.3% of 68 cats examined with non-observable clinical signs.

Leptospirosis in livestock particularly in swine is of great concern to farmers because the disease is usually chronic, occasionally resulting in abortions and still-births with poor survival rates in newborn piglets. An outbreak of *Leptospira* Icterohaemorrhagiae was reported as the cause of abortion in sows from Selangor (Bradenburg & Too, 1981) although serovars Pomona, Icterohaemorrhagiae and Tarassovi are also prominent in swine (Bahaman & Ibrahim, 1987).

The present study was undertaken to identify and assess the prevalence of *Leptospira* in swine and stray dog populations from the Klang Valley, especially as little research had been undertaken in Malaysia since the 1980's except for one study conducted by Phumoonna *et al.* (2009).

MATERIALS AND METHODS

Ethics statement
This study was approved with the ethics reference no. ISB/31/01/2013/SNMZ (R) by the Institutional Animal Care and Use Committee, University of Malaya, Malaysia (UM IACUC).

Study sites
Selected sites occurred in Klang Valley (latitude 3.139003 and longitude 101.686855), where large populations of stray cats and dogs roamed in close contact with human communities. The animals were captured by the workers (dog-catchers) of the Kuala Lumpur City Council from different sites in Klang Valley as part of an animal control program. A total of 3 swine farms located in Selangor State in central of Peninsular Malaysia, during 2012 and 2013. All sites were characterized by a tropical climate and high humidity throughout the year with temperatures ranging from 30°C to 36°C.

Isolation of *Leptospira*
All stray dogs and cats were screened for leptospirosis and in view of unknown

vaccination history, the general physical condition of each animal was noted at the time of sampling and approximate ages were determined by dental examination. Following euthanization, all dogs and cats underwent a post-mortem examination. Urine samples were collected via direct puncture of the bladder and then cultured in a modified semi-solid EMJH medium. Following removal of kidney tissue with sterile blades, small pieces of tissue were each placed in a sterile syringe and placed in EMJH medium (Sigma), supplemented with 5-fluorouracil (Merck, Germany). Cultures were incubated for 30 days at 30°C and examined at intervals of 10 days using a dark field microscope. For swine urine samples up to 2 to 3 drops were inoculated into in 5 ml of EMJH medium, followed by the addition of 10% BSA, incubated at 30°C and then examined every 10 days.

Serology

Serological identification of *Leptospira* isolates was performed using the microscopic agglutination test (MAT) as described by the World Health Organization (WHO, 2003). A set of 10 reference antisera were provided by the Institute of Medical Research (IMR), Malaysia. The antisera raised used in this study were against serovars Javanica (Veldrat Batavia 46), Canicola (Hond Ultrecht IV), Hebdomadis (Hebdomadis), Pomona (Pomona), Hardjo (Hardjoprajitno), Australis (Ballico), Bataviae (Swart), Icterohaemorrhagiae (RGA), Tarassovi (Perepelicin) and Bratislava (Jez Bratislava). Leptospiral isolates were cultured into liquid media with the addition of 1.0% rabbit serum to increase bacterial density. Agglutination of anti-leptospiral antibodies with living leptospires were viewed using dark field microscopy (Benacer *et al.*, 2013a).

PCR analysis

Fresh urine samples collected from dogs, cats and swine were further centrifuged and the sediments were used to detect leptospiral DNA. PCR primers, LA/LB [(5'-GGC GGC GCG TCT TAA ACA TG-3') and (5'-TTC CCC CCA TTG AGC AAG ATT-3')] which target the 16S rDNA gene were used to confirm the genus

Leptospira (Merien *et al.*, 1992). To determine the pathogenic status of isolates, G1/G2 primers [(5'-CTG AAT CGC TGT ATA AAA GT-3') and (5'-GGA AAA CAA ATG GTC GGA AG-3')] were used as these target the secY gene among isolates except for *L. kirschneri* (Gravekamp *el al.*, 1993). Amplified DNA products from representative isolates were verified by DNA sequencing. Amplicons were purified using a DNA purification kit (Qiagen, Germany) and sequenced at a commercial facility (First BASE, Pte. Ltd., Singapore). The resulting DNA sequence data were compared with the GenBank database using a BLAST algorithm available at web site (http://www.ncbi.nlm.nih.gov).

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis was carried out as described by Galloway & Levett (2008), but with minor modifications (Benacer *et al.*, 2013a). The standard size marker strain *S*. Braenderup H9812 was used as the PFGE size marker. Equal volumes of cell suspension were mixed with agarose and dispensed into wells of PFGE plug molds and allowed to solidify at room temperature for 20 min. Plugs were transferred into 50 ml flacon tubes containing 2 ml of cell lysis buffer (CLB) and Proteinase K (20 mg/ml). Plugs were then incubated at 55°C for 2 hours and after lysis, each plug was washed thoroughly in 10-15 ml of sterile deionized water for 2 times and then washed up to 6 times with 1X TE buffer (Benacer *et al.*, 2013a).

RESULTS

Within the study location, a total of 150 dogs (n=106 males; n=44 females) with an average age of 30 months and 50 cats (n=22 males; n= 28 females) with an average age of 18 months were examined for leptospires (Table 1). Molecular characterization detected a low prevalence (7.3%; 11/150) of *Leptospira* in the urine of adult dogs aged between 2 and 5 years. Only one isolate successfully grew on EMJH medium and this was further identified as *L. interrogans* serogoup Canicola by the microscopic

Table 1. The number of urine and kidney samples examined for *Leptospira* isolates from dogs, cats and swine in Peninsular Malaysia

Number of hosts	Number of samples		Host gender		Positive samples	
	Urine	Kidney	Male	Female	PCR	Culture
Dogs (150)	128	22	106	44	11	1
Cats (50)	36	14	22	28	0	0
Swine (81)	81	0	0	81		

agglutination test (MAT). DNA sequencing of 11 positive samples confirmed that 9 were identified as *L. interrogans* serovars Canicola (98% similarity), with the remaining two samples being Icterohaemorrhagiae (99% similarity). Nine positive dogs (82%) were males while the remaining 2 (18%) were females. Six of the positive dogs (54.5%) presented with skin lessions and the entire cat samples were negative using both culture and PCR.

Of 81 urine samples examined from female swine, only 5 (6%) samples were positive based on the PCR method, and one sample was clearly identified as *L. interrogans* serogroup Pomona using MAT. Using PCR and DNA sequencing resulted in 4 positive samples being identified as *L. interrogans* serovar Pomona and the remaining sample as *L. interrogans* serovar Bratislava, all with up to 99% similarity (Table 1).

PFGE of *Not*I-digested chromosomal DNA subtyped 2 isolates into 2 PFGE profiles (LS01 and LD01) and whereas 10 to 25 DNA fragments ranging from 28 to 706 kb were generated. Both PFGE profiles were compared with the *Not*I patterns from the database of the Center for Disease Control and Prevention (CDC), USA, confirming that LS01 was similar to *L. interrogans* serovar Pomona, and LD01 to *L. interrogans* serovar Canicola (Figure 1).

Figure 1. Representative PFGE patterns of *Not*I digested chromosomal DNA of *Leptospira* isolates from dog and swine hosts in Peninsular Malaysia.

Lanes 1,4: *XbaI* digested chromosomal DNA of *Salmonella* H9812 marker strain; lane 2: *L.* Pomona; lane 3: *L.* Canicola.

DISCUSSION

Human leptospirosis is on the rise in Malaysia and consequently there is an urgent need to update the role of domestic animals in the
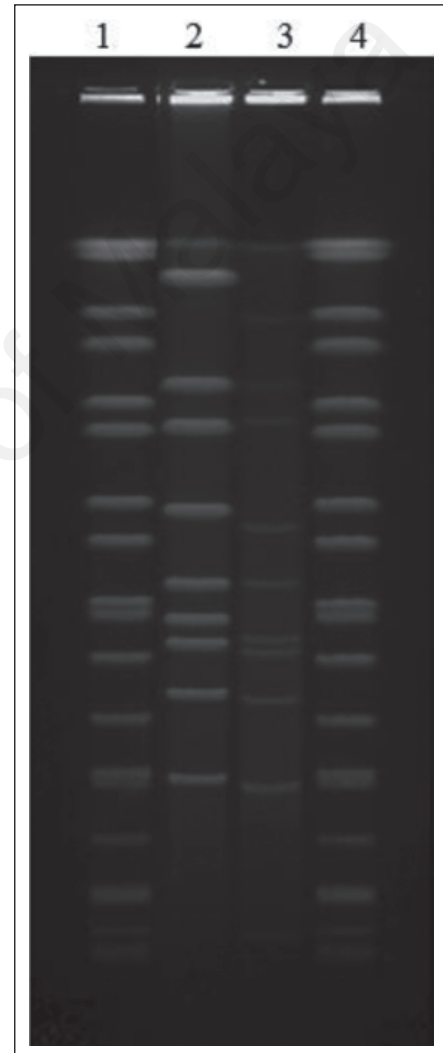
potential spread of the disease locally and nationally. New information on prevalence and serovar types circulating in the population can facilitate better understanding of how these animal reservoirs contribute to disease transmission to humans, thus improving research on existing diagnostic methods i.e MAT and ELISA and contribute to the development of an effective vaccine against the pathogen.

Recently Rafizah *et al*. (2013) determined the seroprevalence of leptospirosis among febrile inpatient cases in north-eastern Malaysia, where 88 of 999 cases admitted to 10 hospitals were positive with over 50% being in direct contact with domesticated animals either through outdoor activity or agriculture. Rodents, cattle, dogs and swine are known to be primary reservoirs of *Leptospira* in Malaysia (Thayaparan *et al*., 2013) and the present study confirmed that infections do occur in stray dogs and swine but not in cats. Therefore the identification of serovars in stray and domesticated animals in Malaysia will help to extrapolate epidemiological patterns of infection of leptospirosis.

In the present study the PCR technique recorded low infections in the stray dog population compared with Phumoonna *et al*. (2009), who found that 17 of 142 IgM seropositive samples were identified as the serovar Pomona. These authors reported that all dogs were in the early stages of the disease with only one being IgG positive, and also confirmed by PCR with low MAT titres (50 to 100). In contrast the present results indicate that infected dogs were in the later stages of the disease or were reservoir hosts for the pathogen. The latter assumption is in accordance with sequencing results of the PCR products which showed 9 of 11 positive samples belong to the serovar Canicola. Dogs with subclinical infections can be reservoir hosts for the serovar Canicola with the ability to shed the bacteria in urine into the environment over long periods (Aedo & Smits, 2014).

In the present study positive samples occurred in dogs older than 1 year, which is similar to the findings of Batista *et al*. (2005)

and Aguiar *et al*. (2007) where dogs in this age category were at higher risk. On the other hand neither age nor gender of dogs influenced infection with leptospires as also shown in studies undertaken by Alton *et al*. (2009) and Kikuti *et al*. (2012). Other risk factors may be involved as 6 of the infected dogs in the present study showed skin lesions, facilitating transmission of the spirochete bacteria through the damaged skin.

Two serovars identified in dogs the present study included Canicola from 9 and Icterohaemorrhagiae from 3 samples and these appeared to be the most prevalent in Malaysia (Gordon-Smith *et al*., 1961; Phumoonna *et al*., 2009) and worldwide (Bolin, 1996; Kikuti *et al*., 2012; Vojinovic *et al*., 2015). Dogs are the main hosts for Canicola, although the occurrence of this serovar has declined in many European countries (Vojinovic *et al*., 2015) mainly attributable to the use of vaccines in recent years. Similarly, from 1970 to the mid 1990's, a decline in the prevalence of both Canicola and Icterohaemorrhagiae serovars in North America was reported following the introduction of vaccination, but infections have since remerged (Bolin, 1996). The serovar Pomona previously reported by Phumoona *et al*. (2009) did not occur in dogs in the present study.

Wild rats are he sources of the infection with Icterohaemorrhagiae in dogs and consequently urban dogs are at a higher risk of infection than rural dogs due to the abundance of rodents in the urban environment (Ampily, 2013; Alton *et al*. 2009). Peripheral urban and slum areas with inadequate sanitation, open sewers and poor garbage management will attract large numbers of rodents and consequently expose dogs to infection (Lelu *et al*., 2015).

Unlike dogs, cats in this study were free from infection although, the serovar Pomona was previously identified in Malaysia but with no observable clinical signs of leptospirosis (Gordon-Smith *et al.*, 1961). In a more recent serological survey, Mosallanezhad *et al.*, (2011) found only 5 of 105 cats infected with the serovars Ballum and Australis. Cats can be incidental hosts to a variety of *Leptospira*

serovars prevalent in wildlife or domestic hosts. In Southern India, Natarajaseenivasan *et al.* (2002) showed that up to 33% of cats were infected with theserovar Autumnalis and 16.6% both with Canicola and Icterohaemorrhagiae. The serovar Autumnalis was also detected in rats from rice fields in the same region. In the metropolitan area of Goiania, Brazil, Parreira *et al.* (2010) found that 23 of 330 cats were infected with up to five serovars including Cynopteri, Djasiman, Butembo, Castellonis and Patoc.

Wisseman *et al.* (1995) conducted the earliest study on swine leptospirosis and found 3 of 5 pigs positive for Autumnalis, Pomona and Sentot. In the present study, low infections (6.2%) of the serovar Pomona and Bratislava occurred in female pigs over 2 years of age. The presence of Pomona in swine is in agreement with previous studies (Bahaman *et al.*, 1987; Tan, 1981) although Icterohaemorrhagiae, Cynopteri, Pomona and Pyrogenes were also previously reported by Joseph (1979).

The decline in infections may be due to improved husbandry practices and the extensive use of antibiotics in feeding regimes in swine farms (Bahaman *et al.*, 1987). Icterohaemorrhagiae, which primarily occurs in rats acting as reservoir hosts, was previously found in swine in Selangor resulting in an abortion epidemic (Brandenburg & Too, 1981).

Culturing *Leptospira* is tedious and time consuming as this slow growing bacterium is normally displaced by other contaminating microorganisms in enrichment media (Benacer *et al.*, 2013b). The PCR technique accompanied with sequencing proved to be the more sensitive method for serovar identification compared with culture techniques. This method was also used in the early detection of serovars of *Leptospira* especially in clinical, animal and environment samples (Fonseca *et al.*, 2006; Bomfim *et al.*, 2008; Benacer *et al.*, 2013b). However, one limitation of the present study is the use of urine as the only source available for PCR screening. The occasional negative result can occur mainly because infections in susceptable hosts are influenced by several factors such as the type of infection and the timing of sampling or if reservoir or accidental hosts are involved. PCR screening of urine samples from reservoir hosts can occasionally be negative and not always accurate due to the intermittent shedding of leptospires, whereas in accidental hosts, leptospires can only be detected in urine at the later stages of the disease. The use of kidney tissues for conventional PCR screening may also be limiting when material with a high concentration of host's DNA is used. Such a limitation could be overcome by using real time PCR to investigate kidney tissue for the colonization of leptospires.

In the present study, PFGE of *Not*I-digested *Leptospira* DNA showed two profiles which were in accordance with MAT when referencing PFGE profiles in the leptospiral database at CDC. Consequently the serovars *L. interrogans* Canicola in dogs and *L. borgpetersenii* Pomona in swine were identified, thus confirming PFGE as a promising and useful tool in the identification of leptospire isolates.

The present findings indicate that stray dogs rather than cats are potential carriers of this zoonotic disease and contribute to the spread and maintenance of *Leptospira* spp. in Malaysia. Further studies using larger samples of hosts from a variety of sites and habitats will contribute further to our understanding of the epidemiology of leptospirosis infections in stray canine and feline hosts, but combined with methods of disease prevention and control including vaccination.

Swine can also be potential carriers of *Leptospira* serovars, resulting in the spread of disease to farmers, livestock handlers and other animals. Therefore, apart from vaccination, preventive measures should be used in situations where spirochete bacteria are likely to thrive and these include the exclusion of rodents from swine farms and avoiding where possible habitats such as stagnant water, ponds, marshes and muddy areas.

Conflict of interest:
The authors declare that they have no competing interests.

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3.7 Publication 7

Predominance of the ST143 and ST50 *Leptospira* Clones in the Urban Rat Populations of Peninsular Malaysia.

Benacer D, Mohd Zain SN, Ahmed AA, Mohd Khalid NMK, Hartskeerl RA, Kwai Thong KL.

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3.7.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Mohd Zain SN, Thong KL, Mohd Khalid NMK
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Predominance of the ST143 and ST50 *Leptospira* clones in the urban rat populations of Peninsular Malaysia

Leptospirosis is a zoonotic and an emerging infectious disease caused by the pathogenic *Leptospira* species (Levett, 2001). Leptospirosis is transmitted directly or indirectly through urine of infected animals or environments contaminated with urine of reservoir animals (Bharti *et al.*, 2003; Ullmann & Langoi, 2011). Serovar identification of *Leptospira* isolates constitutes an important component in understanding leptospiral epidemiology and establishment of appropriate control and preventive measures, especially in areas where the disease is prevalent (Bourhy *et al.*, 2013). To date, more than 300 serovars that belong to 29 serogroups have been identified and described (Saito *et al.*, 2013; Bourhy *et al.*, 2014).

In addition to serological methods, molecular techniques such as fluorescent amplified fragment length polymorphism (FAFLP) (Vijayachari *et al.*, 2004), pulsed-field gel electrophoresis (PFGE) (Galloway & Levett, 2008, Galloway & Levett, 2010) and multiple locus variable number tandem repeat analysis (MLVA) (Majed *et al.*, 2005; Slack *et al.*, 2005) have been applied for the characterization of leptospiral isolates. Recently, multilocus sequence typing (MLST) schemes were developed for pathogenic *Leptospira* species (Ahmed *et al.*, 2006; Boonsilp *et al.*, 2013). MLST has become the gold standard in the molecular epidemiological studies of various bacterial pathogens (Maiden, 2006) and this is attributed to its portability and standardized approach to data collection in addition to being cost-effective due to the dwindling cost in DNA sequencing. The usefulness of *Leptospira* MLST was exemplified by the successful identification of a pathogenic clone, ST34 that was responsible for outbreak of human leptospirosis in Thailand (Thaipadungpanit *et al.*, 2007).

Leptospirosis is an endemic disease in Malaysia. The number of reported cases has increased significantly since the Ministry of Health Malaysia gazetted leptospirosis as a notifiable disease in 2010. Consequently, various publications have appeared addressing several epidemiological questions, such as the prevalent *Leptospira* species and serovars in rodent hosts and the environment (Benacer *et al.*, 2013a; , b; Mohamed-Hassan *et al.*, 2010; , Mohamed-Hassan *et al.*, 2012; Sapian *et al.*, 2012; Benacer *et al.*, 2013b). However, there is no study available to date from Malaysia that employs MLST to explore the genetic diversity of pathogenic *Leptospira* species. Therefore, we initiated this research by analyzing local pathogenic *Leptospira* isolates by MLST to examine their genetic relationships.

Of the 63 *Leptospira* isolates analyzed, 60 isolates were recovered from rats and we included one isolate each from dog, swine and human (Table 1). The dog isolate (D7) was isolated from urine sample of a male stray dog, aged more than 2 years and that showed some skins lesions, while the swine isolate (LS5) was from a healthy domestic female swine from a farm in Selangor state. Isolates in this study were identified sand characterized as *Leptospira* using culture, microscopic agglutination test (MAT) and PFGE techniques; in addition, the pathogenic status of the strains was determined using PCR targeting the 16S rDNA and *secY* genes followed by sequencing (Benacer *et al.*, 2016; Benacer *et al.*, 2013a). The human isolate was provided by the Royal Tropical Institute (KIT), Amsterdam, Netherlands, and was originally isolated from a patient in Malaysia (Alexander *et al.*, 1957). All isolates were maintained in culture in Ellinghausen McCullough Johnson Harris (EMJH) medium.

Genomic DNA was extracted from seven days-old culture using Wizard™Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The quantity of DNA was measured by Biophotometer (Eppendorf). MLST was performed according to the procedures described by Boonsilp *et al.*, (2013). Briefly, a 25 µl PCR reaction consisted of 1×X PCR buffer, 1.5 mM MgCl$_2$, 200 µM each of dNTPs, 0.3 µM of each primer, 1 U of *Taq* DNA polymerase (Intron Biotechnology) and 100 ng of DNA template. The cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for *pntA, sucA, pfkB, tpiA, mreA, caiB* and 50 °C for *glmU* for 30 s, and extension at 72 °C for 2 min, and lastly a final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis, purified using QIAquick PCR purification kit (Qiagen) and sent to a commercial sequencing facility (First BASE Laboratories Sdn Bhd).

Sequencing data were analyzed using Seq Scanner 2 (Applied Biosystems) and BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Both the allelic numbers and sequence types (STs) were obtained from the *Leptospira* MLST database (http://leptospira.mlst.net/). In one case (isolate ID R183) raw sequencing data for both *tpiA* and *caiB* were submitted to the database curator for assignment of new allelic numbers and ST. The eBURST algorithm (http://leptospira.mlst.net/eburst/) was used to assign STs into clonal complexes and clones were considered genetically related if they are either single-locus variants (SLVs) or double-locus variants (DLVs) to each other. Genetic diversity was measured by using the Simpson's index of diversity as described previously (Hunter & Gaston, 1988).

MLST analysis identified seven unique STs comprising of six known clones and one new clone (ST205) (Table 1). The genetic diversity of the Malaysian pathogenic *Leptospira* population was low with Simpson's index of diversity of 0.579. The most prevalent clone was ST143 (32 isolates, 50.8 %), followed by ST50 (26 isolates, 41.3 %). Two clonal complexes were identified by eBURST, one consisted of ST143–ST144 and the other one, ST37–ST140 with predicted primary founder

Table 1. Properties of *Leptospira* isolates included in this study

Isolate ID	Source	Sample	Location	Species and serovar when available	ST
LD7	Dog	Urine	Setepak (KL)	*L. interrogans* serovar Canicola	37
R108	*Rattus rattus*	Kidney	Setepak (KL)	*L. interrogans* serovar Bataviae	50
R149	*Rattus rattus*	Urine	Setepak (KL)	*L. interrogans* serovar Bataviae	50
R286	*Rattus norvegicus*	Kidney	Chow Kit (KL)	*L. interrogans* serovar Bataviae	50
RP6	*Rattus norvegicus*	Urine	Penang	*L. interrogans* serovar Bataviae	50
RP11	*Rattus rattus*	Urine	Penang	*L. interrogans* serovar Bataviae	50
RP12	*Rattus norvegicus*	Kidney	Penang	*L. interrogans* serovar Bataviae	50
RP15	*Rattus norvegicus*	Urine	Penang	*L. interrogans* serovar Bataviae	50
RM6	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RM13	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RM15	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RM29	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RM33	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RM39	*Rattus rattus*	Urine	Malacca	*L. interrogans* serovar Bataviae	50
RK84	*Rattus norvegicus*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK87	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK94	*Rattus norvegicus*	Kidney	Kuantan	*L. interrogans* serovar Bataviae	50
RK100	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK102	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK107	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK108	*Rattus rattus*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK109	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK110	*Rattus exulans*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK113	*Rattus rattus*	Urine	Kuantan	*L. interrogans* serovar Bataviae	50
RK116	*Rattus norvegicus*	Kidney	Kuantan	*L. interrogans* serovar Bataviae	50
RA55	*Rattus norvegicus*	Urine	Selangor	*L. interrogans* serovar Bataviae	50
RA59	*Rattus norvegicus*	Urine	Selangor	*L. interrogans* serovar Bataviae	50
LH52*	Human	Blood	–	*L. interrogans* serovar Ricardi	56
LS5	Swine	Urine	Selangor	*L. interrogans* serovar Pomona	140
R2	*Rattus rattus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R18	*Rattus rattus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R71	*Rattus ratus*	Kidney	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R77	*Rattus rattus*	Urine	Bangsar (KL)	*L. borgpetersenii* serovar Javanica	143
R78	*Rattus rattus*	Urine	Bangsar (KL)	*L. borgpetersenii* serovar Javanica	143
R84	*Rattus norvegicus*	Urine	Bangsar (KL)	*L. borgpetersenii* serovar Javanica	143
R112	*Rattus rattus*	Urine	Setapak (KL)	*L. borgpetersenii* serovar Javanica	143
R107	*Rattus norvegicus*	Urine	Setapak (KL)	*L. borgpetersenii* serovar Javanica	143
R122	*Rattus rattus*	Urine	Setapak (KL)	*L. borgpetersenii* serovar Javanica	143
R139	*Rattus rattus*	Urine	Setapak (KL)	*L. borgpetersenii* serovar Javanica	143
R179	*Rattus rattus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R225	*Rattus rattus*	Kidney	Bangsar (KL)	*L. borgpetersenii* serovar Javanica	143
R282	*Rattus norvegicus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R293	*Rattus norvegicus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R294	*Rattus rattus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R300	*Rattus norvegicus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
R301	*Rattus rattus*	Urine	Chow Kit (KL)	*L. borgpetersenii* serovar Javanica	143
RP10	*Rattus rattus*	Urine	Penang	*L. borgpetersenii* serovar Javanica	143
RI21	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI26	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI27	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI36	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI38	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143

Table 1. cont.

Isolate ID	Source	Sample	Location	Species and serovar when available	ST
RI42	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI46	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI53	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI82	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI88	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI92	*Rattus rattus*	Kidney	Ipoh	*L. borgpetersenii* serovar Javanica	143
RI93	*Rattus rattus*	Urine	Ipoh	*L. borgpetersenii* serovar Javanica	143
RK112	*Rattus rattus*	Urine	Kuantan	*L. borgpetersenii* serovar Javanica	143
RA50	*Rattus exulans*	Urine	Selangor	*L. borgpetersenii* serovar Javanica	143
RI94	*Rattus rattus*	Kidney	Ipoh	*L. borgpetersenii* serovar Javanica	144
R183	*Rattus norvegicus*	Kidney	Chow Kit (KL)	*L. interrogans*	205†*

KL, Kuala Lumpur.
*Location information for LH52 isolate recovered from human is not available.
†*denotes new ST identified in this study. Location information for LH52 isolate recovered from human is not available. Abbreviation used: KL, Kuala Lumpur.

ST143 and ST37, respectively. The remaining three clones appeared as singletons. The predicted primary founder of each clonal complex differed with single-locus variants (SLVs) from its subgroup clone at the *caiB* locus by a single base pair substitution.

A unidirectional correlation in which the isolates with the same ST belonging to the same serovar was apparent in our study. However, in the MLST database, there are at least 18 STs that include two or three different serovars (Boonsilp *et al.*, 2013). The genotypes were also host-specific and therefore no evidence of clonal transmission from maintenance hosts such as rodent, swine and dog to human was observed. Nevertheless, more data from non-rodent hosts and humans are required especially to track the source of infection in epidemic areas and during leptospirosis outbreaks.

Our findings suggested that despite the wide geographical distribution of our isolates, the genetic diversity for the pathogenic *Leptospira* population was surprisingly low. The success of two major clones, ST143 and ST50 was responsible for the low genetic diversity because both clones constitute about 92.0 % of the genotypes in our study. These clones could have adapted selective advantages in the environment or in maintenance hosts that allowed them to thrive and spread ubiquitously (Kanagavel *et al.*, 2016). Kanagavel *et al.* (2016) also highlighted

the existence of specific dominant clones in different countries and our findings supported his observation.

This work also confirmed the results of a recent publication which proposed two *Leptospira* species, *Leptospira interrogans* and *Leptospira borgpetersenii* as the most abundant species circulating amongst rodents in Southeast Asia (Cosson *et al.*, 2014). However, further comparison at strain level was not possible due to the different molecular typing methods used to determine the *Leptospira* genotype.

Despite successfully identifying two dominant clones in the urban rat population, their significance especially in causing human infections remained unknown. Future studies involving pathogenic *Leptospira* isolates recovered from human cases are needed to complement the present study.

Douadi Benacer[1], Siti Nursheena Mohd Zain[1], Ahmed A Ahmed[2], Mohd Khairul Nizam Mohd Khalid[3], Rudy A Hartskeerl[2] and Kwai Lin Thong[1]

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia

[2]Royal Tropical Institute (KIT), Amsterdam, The Netherlands

[3]Bacteriology Unit, Institute for Medical Research, Ministry of Health, Malaysia

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3.8 Publication 8

Antimicrobial Susceptibility of *Leptospira* spp. Isolated from Environmental, Human

and Animal Sources in Malaysia

Benacer D, Mohd Zain SN, Ooi PT, Thong KL

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3.8.1 Contribution of Co-Authors.

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Mohd Zain SN, Thong KL
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

Antimicrobial Susceptibility of *Leptospira* spp. Isolated from Environmental, Human and Animal Sources in Malaysia

Douadi Benacer, Siti Nursheena Mohd Zain, Peck Toung Ooi[1], Kwai Lin Thong

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, [1]Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia

Abstract

Leptospirosis is a zoonosis with worldwide distribution caused by pathogenic spirochetes of the genus *Leptospira*. The aim of this study was to evaluate the susceptibility of isolates obtained from different hosts. A total of 65 *Leptospira* isolates from humans ($n = 1$), zoonoses (rat, $n = 60$; dog, $n = 1$; swine, $n = 1$) and environment ($n = 2$) were tested against six antibiotics. All the isolates were resistant to trimethoprim and sulphamethoxazole and had high MIC toward chloramphenicol (MIC_{90}: 6.25 μg/ml). All except one environment isolate were sensitive to ampicillin, doxycycline and penicillin G.

Keywords: Antimicrobial susceptibility, *Leptospira*, leptospirosis, minimal inhibitory concentration

INTRODUCTION

Leptospirosis is an emerging zoonotic disease with worldwide distribution.[1] It is caused by pathogenic spirochetes of the genus *Leptospira.* Approximately, one-half of the pathogenic serovars belong to *Leptospira interrogans* or *Leptospira borgpetersenii*.[2] Rodents are the principal known maintenance hosts, besides domestic animals, livestock and wild animals.[2] Humans become accidental hosts by acquiring the infection through direct contact with urine, blood or infected animal tissue or indirect contact with water or soil contaminated with the urine from reservoir animals.[1] Clinically, symptoms of infection may range in severity from mild to fatal, depending on the infection stage. However, the clinical presentation of leptospirosis is unspecific, which frequently leads to misdiagnosis.[3] In Malaysia, misdiagnosis of this infection has become a critical issue, where dengue, malaria and other infectious diseases with overlapping clinical presentations are endemic. Treatment normally follows an empirical chemotherapy route, which requires information regarding the susceptibilities of *Leptospira* isolates to various antimicrobial agents. Effective and appropriate antibiotic selection for treatment is essential to prevent complications. Several studies have been carried out on *Leptospira* isolates' susceptibilities. However, these studies have a number of limitations, such as using laboratory-passaged strains or a small number of

Leptospira strains.[4] Due to the endemicity of leptospirosis in Malaysia[5] as well as the dramatic increase in reported cases over the last decade, there is a critical need to determine the effectiveness of common antibiotics in controlling this organism. However, no information is available on the antimicrobial susceptibilities of Malaysian *Leptospira* isolates. Therefore, the aim of this study is to monitor and evaluate the susceptibility of recent local isolates obtained from different hosts and sites to traditional antimicrobial compounds used for leptospirosis treatment.

MATERIALS AND METHODS

Bacterial strains

Sixty-five *Leptospira* isolates representing four different species and at least five serovars were included in the testing. These isolates from environmental (water, $n = 2$) and animal sources (rat, $n = 60$; dog, $n = 1$; swine, $n = 1$) were isolated between 2011 and 2014 from different sites in six states in Peninsular Malaysia.[2,5] One clinical isolate was provided by

Address for correspondence: Dr. Kwai Lin Thong,
Faculty of Science, Institute of Biological Sciences, University of Malaya,
50603 Kuala Lumpur, Malaysia.
E-mail: thongkl@um.edu.my

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the Royal Tropical Institute (KIT), Amsterdam, Netherlands. The strain was originally isolated from a human in Malaysia by Alexander *et al.*[6] The isolates were maintained in culture in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. Information on the isolates is summarised in Table 1.

Antimicrobial agents

The antimicrobial solutions employed in this study were prepared from reagent grade powders to produce 1 mg/ml solutions using solvents and diluents suggested in the Clinical and Laboratory Standards Institute document M100-S22[7] or according to the manufacturer's suggestions if available. Six antimicrobial agents, including doxycycline, penicillin G, trimethoprim, ampicillin, chloramphenicol (MP Biomedicals, France) and sulphamethaxazole (Sigma-Aldrich; St. Louis, MO, USA) were used to test the *Leptospira* isolates' antimicrobial susceptibility. The stock antimicrobial solutions were stored at −80°C in divided one-time use aliquots.

Susceptibility testing

Broth microdilution testing was performed as reported by Murray and Hospenthal[4] and Chakraborty *et al.*[8] The antibiotic concentrations ranged from 25.0 to 0.01 µg/ml (units/ml for penicillin), with the exception of sulphamethaxazole and trimethoprim, which were tested at 3200 to 12.5 µg/ml. The strain *L. interrogans* serovar Icterohaemorrhagiae was used for internal validation with minimal inhibitory concentration (MIC) parameters and served as the control strain.[4] All tests were performed in triplicate and included positive (bacteria only) and negative controls (medium only). The *Leptospira* inoculum used for testing was prepared from 7-day-old cultures grown in liquid EMJH medium at 30°C. Then, 100 µl *Leptospira* inoculum of 2×10^6 leptospiral organisms/ml was added, and the 96 well plates were incubated at 30°C, with each well having a final volume of 200 µl. Following 3 days of incubation, 20 µl of 10X alamarBlue (Invitrogen, Cleveland, OH, USA) was added to each well. AlamarBlue is an oxidation-reduction indicator that changes colour from dark blue to bright pink in response to the chemical reduction of the growth medium resulting from cell development. The colour of each well was documented on the 5th day of incubation, and the MICs were recorded as the concentration in the well containing the lowest concentration without blue-to-pink colour change.

Results

The MIC_{90} values are reported in Table 1. All isolates were resistant to trimethoprim and sulphamethoxazole (MIC_{90}: 1600 µg/ml). All isolates had an MIC_{90} range of 3.13–6.25 µg/ml to chloramphenicol, except that from swine (LS01/11) and one isolate from rat (LR31/13) which showed higher MICs of 12.5 and 25 µg/ml, respectively. Overall, penicillin G and ampicillin appeared to be effective for all clinical and zoonotic isolates with MIC_{90} between 0.1 and 0.2 µg/ml. Only the dog isolate showed a slightly higher MIC toward penicillin G (0.39 µg/ml) compare to the rest

while the lowest was toward the swine isolate (<0.01 µg/ml). However, the environmental isolate *L. kirschneri* (LE02/11) was resistant to penicillin G and ampicillin as the MIC reported was 25 µg/ml. The MICs of doxycycline ranged from 0.2 to 0.78 µg/ml, but one zoonotic (LS01/11) and 2 environmental isolates (LE01/11, LE02/11) displayed a higher MIC toward doxycycline (3.13 µg/ml) compared to the other isolates although still remained susceptible. Overall, doxycycline was more effective on all isolates tested compared to ampicillin and penicillin G.

Discussion

Leptospirosis is an endemic disease in Malaysia, occurring in both urban and rural locations. The local absence of sensitive, specific and rapid methods of diagnosing leptospirosis makes it difficult to distinguish it from other febrile illnesses. The misdiagnosis of leptospirosis often leads to treatment with a broad range of antimicrobials that cover the febrile syndromes of various local illnesses. Therefore, this study was carried out to monitor and determine the susceptibility patterns in the different isolates of leptospirosis refl cting on their effectiveness in the treatment of leptospirosis.

In our study, both trimethoprim and sulphamethoxazole were found ineffective against all isolates tested. This findin is in agreement with previous studies.[8,9] Trimethoprim and sulphamethoxazole have sometimes been applied in combination for their synergistic action in providing a broad-spectrum bactericidal antimicrobial coverage before definitive diagnosis.[10] However, the resistance of *Leptospira* strains to these antibiotics may compel health workers to consider other antileptospiral drugs in cases where the diagnosis of leptospirosis is inconclusive.

The local isolates demonstrated a higher MIC toward chloramphenicol, which is in agreement with the finding of Murray and Hospenthal.[4] Previously, chloramphenicol appeared to be effective against *Leptospira* in experimental mice.[11] However, a higher concentration was required to produce an inhibition or a bactericidal effect on *Leptospira* strains.[4] Unlike chloramphenicol, ampicillin displayed a lower MIC to the isolates tested, except for one environmental isolate that was resistant (MIC: 25 µg/ml). Administration of ampicillin was a potential option in the treatment of this illness both *in vivo* and *in vitro*.[8,12,13] However, the action of ampicillin against leptospirosis is restricted and cannot be distributed to all organ tissues, such as the kidneys and the heart, rendering it ineffective in clearing leptospires located in protected sites.[13] Currently, penicillin G and doxycycline are recognised as ideal drugs for the treatment of leptospirosis.[9] The MIC results of penicillin G were similar to those produced by ampicillin. However, penicillin G is generally recommended for treating severe leptospirosis. The advantages of using penicillin G include low toxicity and the potential to administer the drug intramuscularly or intravenously at high doses in the early stages of infection.[14]

Table 1: Strains of *Leptospira* tested and their susceptibility to six antimicrobial agents

Strains number	Species/serovar	Isolation site	Isolation source	MIC (µg/ml)[a]					
				PenG	AMP	CAM	DOXY	SMX	TMP
LR01/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.05	3.13	0.39	800	800
LR02/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR03/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR04/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR05/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	1.56	1600	1600
LR06/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR07/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR08/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR09/11	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR10/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR11/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.02	0.05	6.25	0.39	800	1600
LR12/11	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR13/11	*L. interrogans*/Bataviae	Penang	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR14/11	*L. borgpetersenii*/Javanica	Penang	Rat	0.2	0.05	6.25	0.39	1600	1600
LR15/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR16/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR17/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR18/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR19/12	*L. interrogans*/unknown	Kuala Lumpur	Rat	0.2	0.05	3.13	0.39	800	800
LR20/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR21/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR22/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR23/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR24/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR25/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR26/12	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	800
LR27/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	1600	1600
LR28/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR29/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.2	25	0.78	>3200	3200
LR30/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	>1600	1600
LR31/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	>1600	1600
LR32/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	1600	1600
LR33/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	1600	1600
LR34/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	3200	1600
LR35/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	>1600	1600
LR36/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600

Contd...

Table 1: Contd...

Strains number	Species/serovar	Isolation site	Isolation source	MIC (µg/ml)[a]					
				PenG	AMP	CAM	DOXY	SMX	TMP
LR37/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR38/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.78	1600	1600
LR39/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR40/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR41/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR42/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR43/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR44/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR45/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR46/13	*L. borgpetersenii*/Javanica	Ampang	Rat	0.2	0.1	6.25	0.39	1600	1600
LR47/13	*L. interrogans*/Bataviae	Ampang	Rat	0.1	0.05	6.25	0.39	3200	1600
LR48/13	*L. interrogans*/Bataviae	Ampang	Rat	0.1	0.05	6.25	0.39	3200	1600
LR49/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.1	0.05	6.25	0.39	3200	1600
LR50/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.1	0.05	6.25	0.39	3200	1600
LR51/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR52/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR53/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR54/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR55/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR56/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR57/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR58/14	*L. borgpetersenii*/Javanica	Kuantan	Rat	0.2	0.1	6.25	0.39	1600	1600
LR59/14	*L. interrogans*/Bataviae	Kuantan	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR60/14	*L. interrogans*/Bataviae	Kuantan	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LD01/11	*L. interrogans*/Canicola	Kuala Lumpur	Dog	0.39	0.2	6.25	0.39	1600	1600
LS01/11	*L. interrogans*/Pomona	Selangor	Swine	≤0.01	0.02	12.5	3.13	1600	800
LH01/57	*L. interrogans*/Ricardi	Pahang	Human	0.2	0.1	3.13	0.78	800	800
LE01/11	*L. kmetyi*	Ipoh	Water	0.2	0.1	6.25	3.13	800	800
LE02/11	*L. kirschneri*	Ipoh	Water	25	25	6.25	3.13	400	800
MIC$_{90}$				>0.2	0.1	6.25	0.78	1600	1600

[a]Values for penicillin are in units/ml; all others are in µg/ml. MIC$_{90}$: The concentration at which 90% of the *Leptospira* isolates are inhibited.
PenG: Penicillin G, AMP: Ampicillin, CAM: Chloramphenicol, DOXY: Doxycycline, SMX: Sulphamethoxazole, TMP: Trimethoprim,
L. borgpetersenii: *Leptospira borgpetersenii*, *L. interrogans*: *Leptospira interrogans*, *L. kmetyi*: *Leptospira kmetyi*, *L. kirschneri*: *Leptospira kirschneri*,
MIC: Minimal inhibitory concentration

The efficacy of penicillin was compared with ceftriaxone in a trial study in treatment of severe leptospirosis, where one group (*n* = 86) was given intravenous penicillin G 1.5 million unit/6 h and second group (*n* = 87) was given intravenous ceftriaxone 1 g daily for 7 days. After 7 days follow-up, no significant difference was observed for median duration of fever, mortality and complications such as renal failure, jaundice and thrombocytopenia.[15]

In this study, one environmental and one zoonotic isolate showed a slightly higher MIC with doxycycline than the rest of the isolates. However, this antibiotic was still effective on all isolates – a finding which correlated with previous studies.[8,16] Doxycycline has been widely recommended and utilised for the prophylaxis and treatment of mild leptospirosis.[17] In the leptospirosis outbreak that occurred in an eco-challenge multisport competition in Sabah, Malaysia, athletes who had taken doxycycline before the challenge were spared from

infection.[18] Truccolo *et al*.[13] used quantitative PCR assay to evaluate ampicillin, ofloxacin and doxycycline for treatment of experimental leptospirosis. The results showed the ability of ampicillin at a high dose (100 mg/kg of body weight) to clear leptospires from the host, except from kidneys and heart, where 10^2 leptospires/g remained at day 6. Ofloxacin (30 mg/kg) was unable to clear bacteria from blood or kidneys. With doxycycline (10 mg/kg), the clearance of leptospires occurred in 2 days in all the target organs studied, with the exception of liver, which required 3 days. They concluded that doxycycline had the potential for the treatment of leptospirosis cases compared to other two antibiotics used.

The environmental isolate *L. kirschneri* had higher MIC with the antibiotics tested compared to clinical and zoonotic isolates. In a study performed by Murray and Hospenthal,[7] the results indicated that *L. kirschneri* had a higher MIC toward some of the antibiotics tested compared to other species.

CONCLUSION

Doxycycline, ampicillin and penicillin G are still effective against all clinical and zoonotic isolates. However, further testing on larger numbers of environmental isolates is required to determine the most suitable antibiotic treatment for leptospirosis.

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Conflicts of interest

There are no conflicts of interest

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3.9 Publication 9

A duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains.

Benacer D, Mohd Zain SN, Lewis JW, Mohd Khalid NMK, Thong KL.

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3.9.1 Contribution of Co-Authors

Design and concept of study	Benacer D
Acquisition of data	Benacer D, Mohd Zain SN, Thong KL
Analyses of data	Benacer D, Mohd Zain SN, Thong KL, Mohd Khalid NMK
Drafting of manuscript	Benacer D
Revising manuscript for intellectual content	All authors

A duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains

Douadi Benacer[1], Siti Nursheena Mohd Zain[1], John W. Lewis[2], Mohd Khairul Nizam Mohd Khalid[3] and Kwai Lin Thong[1]

[1]. Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.
[2]. School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, United Kingdom.
[3]. Bacteriology Unit, Institute for Medical Research, Ministry of Health, Kuala Lumpur, Malaysia.

Abstract

Introduction: This study aimed to develop a duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains. **Methods:** Primers were designed to target the *rrs* (LG1/LG2) and *ligB* (LP1/LP2) genes to confirm the presence of the *Leptospira* genus and the pathogenic species, respectively. **Results:** The assay showed 100% specificity against 17 *Leptospira* strains with a limit of detection of 23.1pg/µl of leptospiral DNA and sensitivity of 10^3 leptospires/ml in both spiked urine and water. **Conclusions:** Our duplex endpoint PCR assay is suitable for rapid early detection of *Leptospira* with high sensitivity and specificit .

Keywords: *Leptospira*. Leptospirosis. Duplex endpoint. PCR.

Leptospirosis is a zoonotic disease of global importance[1]. It is caused by pathogenic species of the genus *Leptospira* and affects humans as well as domestic and wild animals. Leptospirosis has a much higher incidence in subtropical and tropical climates, where environmental conditions are ideal for the growth and transmission of the pathogens[2]. Rodents are the main reservoirs of this disease, as they continuously shed leptospires in urine without exhibiting clinical manifestations[3]. Human infections usually occur through direct contact with animal reservoirs or indirect exposure with contaminated freshwater or soil[2]. Symptoms range from flu-l ke illness to organ failure, and the disease is often misdiagnosed as dengue fever or malaria.

The characterization of *Leptospira* strains is essential for better epidemiological understanding of the disease. To date, at least 22 *Leptospira* species have been classified according to analysis of deoxyribonucleic acid (DNA)-DNA hybridization, nucleotide identity, genome-to-genome distances, and over 300 serovars based on agglutinating lipopolysaccharide (LPS) antigens[4]. Species within the *Leptospira* genus can be further divided into pathogenic, non-pathogenic, and opportunistic/ possibly pathogenic groups. Classically, diagnosis is based on serological tests, such as the microscopic agglutination test (MAT), but the sensitivity of this method is low. It is also highly laborious and requires extensive collection of reference strains[2,3] and therefore is more suitable for reference laboratories.

Recently, polymerase chain reaction (PCR)-based methods are increasingly used in the diagnosis of leptospirosis owing to their rapidity, high sensitivity, and specificity as well as robust detection of *Leptospira* in a wide range of specimens, including environmental, clinical, and animal samples[2,5,6]. PCR assays developed by Merien et al.[5] and Gravekamp et al.[7] have been extensively used for diagnosis over the past two decades, although these assays do have several limitations. The PCR assay described by Merien et al.[5] is a genus-specific assay that amplifies both pathogenic and non-pathogenic serovars, whereas the assay described by Gravekamp et al.[7] and evaluated by Brown et al.[8] requires the amplification of two distinct targets in order to detect all pathogenic species.

In this study, we report the development of a rapid and simple assay, known as the duplex endpoint PCR assay, for specific detection and differentiation of *Leptospira* species. To achieve this, we combined both genus-specific primers that target the *rrs* gene and pathogenic species-specifi primers that target the *ligB* gene. The latter codes for a high-molecular-weight leptospiral immunoglobulin-like repeat (Lig) protein that was previously identifi d as a putative virulence factor in pathogenic *Leptospira*[9]. Furthermore, the *ligB* gene has been found in all pathogenic *Leptospira* species identified to date[10]. Our results show that the duplex endpoint PCR assay is rapid, as well as highly specifi and sensitive for the simultaneous detection and differentiation of *Leptospira* strains.

A total of 17 leptospiral strains were included in this study (**Table 1**). Leptospires were maintained in semi-solid or liquid Ellinghausen-McCulloughJohnson-Harris (EMJH) medium supplemented with 5-fluorouracil (Merck, Germany). All inoculated media were incubated aerobically at 30ºC and were examined under dark-field microscope for the presence of *Leptospira* at 10-day intervals for a period of three months. Genomic DNA was extracted from fresh culture media using Wizard™ Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The quantity and quality of extracted DNA was measured using a Biophotometer (Eppendorf, Germany).

Primer sequences used for the duplex endpoint PCR assay were LG1 (5′- CGGTGAAATGCGTAGATATC-3′), LG2 (5′-CGGTTTGTCACCGGCAGTTC-3′), LP1 (5′-TCGTTTTAGAATCGATAG-3′), and LP2 (5′-ATACTTCCATTATGTA-3′). A *Leptospira* genus-specific primer set (LG1/LG2) published previously[3] was obtained from a multiple sequence alignment of *rrs* gene sequences of all known *Leptospira* species using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Primers were selected within conserved regions, and amplicon sizes predicted using The National Center for Biotechnology Information (NCBI) Primer-*Basic Local Alignment Search Tool* (BLAST) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) ranged from 479 to 483bp.

The pathogenic *Leptospira*-specific primer set was obtained from a multiple sequence alignment of full-length *ligB* gene sequences of pathogenic *Leptospira* species using Clustal Omega. The accession numbers for sequences used were: *Leptospira interrogans* serovar Copenhageni (AE016823.1), *Leptospira borgpetersenii* serovar Hardjo-bovis (CP000348.1), *Leptospira kirschneri* serovar Grippotyphosa (AY190126.2), *Leptospira noguchii* strain Cascata (EU700273.1), and *Leptospira weilii* strain Ecochallenge (EU700274.1). Primers were selected based on two criteria: 1) annealing to regions conserved across all aligned sequences and 2) covering regions with large structural differences to allow for direct *Leptospira* speciation based on amplicon sizes. The amplicon sizes predicted using NCBI Primer-BLAST were 192bp for *L. interrogans* and *L. kirschneri,* 252bp for *L. weilii* and *L. borgpetersenii,* and 282bp for *L. noguchii.*

PCR cycling conditions consisted of initial denaturation at 95ºC for 2 min; 30 cycles of 95ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min; and a final extension at 72ºC for 5 min. The reaction mix consisted of $1\times$ PCR buffer, 1.5mM $MgCl_2$, 200μM each deoxyribonucleotide triphosphate (dNTP), 60pmol each primer (LG1, LG2, LP1, and LP2), 1U *Taq* DNA polymerase (Intron Biotechnology, South Korea), and 5μl DNA template in a final volume of 25μl. PCR products were analyzed by electrophoresis of a 1% Tris-borate-EDTA (TBE) agarose gel (Promega, USA).

DNA sequencing was performed on PCR products obtained from *Leptospira alexanderi, Leptospira santarosai,* and *Leptospira alstonii,* since their *ligB* sequences were not available in GenBank. The amplicons were purified and sent to a commercial sequencing facility (First BASE Laboratories,

TABLE 1
List of *Leptospira* strains used in this study.

Leptospira reference strains	ligB	Rrs
L. interrogans serovar Canicola	+	+
L. interrogans serovar Bataviae	+	+
L. kirschneri serovar Grippotyphosa	+	+
L. kirschneri serovar Cynopteri	+	+
L. noguchii serovar Panama	+	+
L. weilii serovar Celledoni	+	+
L. borgpetersenii serovar Hardjobovis	+	+
L. borgpetersenii serovar Javanica	+	+
L. alstonii serovar Sichuan	+	+
L. alstonii serovar Pingchang	+	+
L. alexanderi serovar Manhao	+	+
L. santarosai serovar Canalzonae	+	+
L. santarosai serovar Shermani	+	+
L. inadai serovar Lyme	-	+
L. fainei serovar Hurstbridge	-	+
L. biflexa serovar Patoc	-	+
L. biflexa serovar Andaman	-	+

L.: *Leptospira;* **ligB:** leptospiral immunoglobulin-like gene; **rrs:** 16 ribosomal RNA gene; **-:** absent; **+:** present.

Malaysia). Sequencing data were analyzed using Seq Scanner 2 (Applied Biosystems, USA) and BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). All sequences were deposited in GenBank.

The limit of detection of the duplex endpoint PCR was determined using serially diluted leptospiral DNA from *L. interrogans* serovar Bataviae and *Leptospira borgpetersenii* serovar Javanica. A tenfold dilution of leptospiral DNA was prepared from the starting concentration of 231ng/μl to produce 23.1pg/μl. PCR was performed using the diluted DNA as a template. Next, *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica were spiked into both urine from a healthy individual and sterile water. Leptospiral cell concentrations were measured by spectrophotometry and were adjusted to reach an optical density (OD_{420}) of 0.14

(approximately 1×10^8 cells/ml). A tenfold dilution of the spiked urine and water were prepared to reduce the concentration to 1×10^3 cells/ml using urine and water.

The specificity of the duplex endpoint PCR was evaluated using 17 *Leptospira* strains and 10 strains of commensal and pathogenic but non-*Leptospira* bacteria commonly encountered in clinical specimens, including *Escherichia coli*, *Shigella* spp., *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Typhi, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Listeria monocytogenes,* and *Yersinia enterocolitus*. These strains were previously identified and confirmed by the Laboratory of Biomedical Science and Molecular Microbiology, University of Malaya, Kuala Lumpur.

All pathogenic *Leptospira* samples resulted in amplificatio of both target genes (*rrs* and *ligB*) while non-pathogenic *Leptospira* resulted in amplification of only the *rrs* gene (**Table 1**). The limit of detection was 23.1pg/µl of leptospiral DNA (**Figure 1**). The sensitivity of our duplex assay was 1×10^3 cells/ml in both urine and water. The assay was 100% specific with no detectable amplification of 10 non-*Leptospira* bacteria commonly found in clinical specimens.

Based on the gel image, the amplicon sizes for the LP1/LP2 primers predicted by Primer-BLAST were confirmed (**Figure 2**). Even though the *ligB* sequences for *L. alstonii*, *L. santarosai,* and *L. alexanderi* were not available from GenBank, PCR amplification was successful for all three species. Amplicon sizes for *L. alstonii*, *L. santarosai,* and *L. alexanderi* were 324bp, 252bp, and 252bp, respectively. Partial *ligB* sequences for the three species were deposited in GenBank under the accession numbers KX538904, KX538905, KX538906, KX538907, and KX538908.

Four unique amplicon sizes representing different pathogenic *Leptospira* species were obtained by LP1/LP2 primers: 192 bp for *L. interrogans* and *L. kirschneri*; 252bp for *L. borgpetersenii*, *L. weilii*, *L. alexanderi,* and *L. santarosai*; 282 bp for *L. noguchii;* and 324bp for *L. alstonii*. *Leptospira kmetyi* was the only remaining pathogenic *Leptospira* species that was not tested using the LP1/LP2 primers, and its *ligB* sequence was not available from GenBank.

The similarity between the clinical symptoms of leptospirosis and those of dengue and malaria may potentially lead to misdiagnosis and underestimation of the prevalence of leptospirosis worldwide[3]. Leptospires are fastidious organisms that take a long time to grow, thus making culturing difficult for diagnosis. In addition, direct observation of blood samples using dark-field microscopy is notoriously unreliable and not recommended as the sole diagnostic test[1]. Serological tests, such as MAT, have become the gold standard technique; however, MAT has several drawbacks in that the method is laborious, time-consuming, and requires extensive collection of reference strains.

The widespread application of PCR-based techniques has improved the diagnosis of leptospirosis because of its advantages in speed, sensitivity, and specificit . Several PCR-based methods have been developed recently for the detection of *Leptospira* in different specimens[7,11]. In this study, we developed a duplex
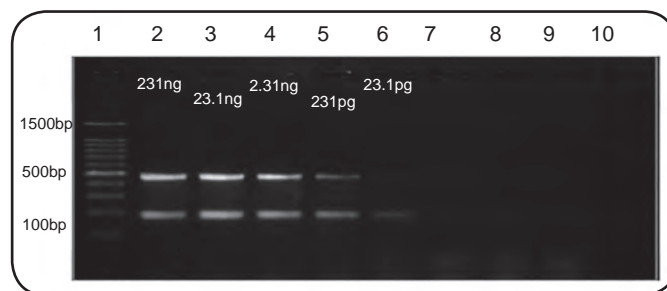
FIGURE 1 - Sensitivity testing of duplex PCR assay relative to the quantity (ng/µl) of genomic DNA. Lane 1: DNA ladder, 100bp; **lane 2:** 231ng/µl; **lane 3:** 23.1ng/µl; **lane 4:** 2.31ng/µl; **lane 5:** 231pg/µl; **lane 6:** 23.1pg/µl. **ng:** nanogram; **bp:** basepair; **PCR:** polymerase chain reaction; **DNA:** deoxyribonucleic acid.
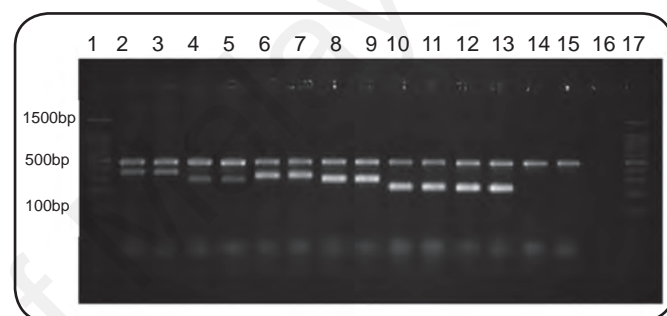
FIGURE 2 - Representative gels of the duplex endpoint PCR for seven reference *Leptospira* strains. Lanes 1 & 17: DNA ladder, 100bp; **lane 2:** *L. alstonii* serovar Sichuan; **lane 3:** *L. alstonii* serovar Pingchang; **lane 4:** *L. borgpetersenii* serovar Hardjobovis; **lane 5:** *L. borgpetersenii* serovar Javanica; **lanes 6 & 7:** *L. noguchii* serovar Panama; **lane 8:** *L. weilii* serovar Celledoni; **lane 9:** *L. borgpetersenii* serovar Javanica; **lane 10:** *L. interrogans* serovar Canicola; **lane 11:** *L. interrogans* serovar Bataviae; **lane 12:** *L. kirschneri* serovar Grippotyphosa; **lane 13:** *L. kirschneri* serovar Cynopteri; **lane 14:** *L. biflexa* serovar Patoc; **lane 15:** *L. biflexa* serovar Andaman; **lane 16:** negative control (sterile water);. **PCR:** polymerase chain reaction; **DNA:** deoxyribonucleic acid; **bp:** basepair..

endpoint PCR assay using primer pairs designed to target the *rrs* and *ligB* genes for the simultaneous detection and differentiation of *Leptospira* species based on their pathogenic status.

The choice of appropriate target genes and optimization of primer designs are critical for ensuring PCR sensitivity and specificit . Our duplex endpoint PCR assay showed a low limit of detection of 23.1pg/µl of genomic DNA and a high sensitivity of 1×10^3 leptospires/ml in spiked urine and water. The limit of detection for this assay is comparable with those of previous studies[6,12].

Our assay was also specific, amplifying only *Leptospira* species and not other commensal and pathogenic non-*Leptospira* bacteria. *rrs* gene sequencing is rapidly becoming a common technique for the identification of unknown bacterial isolates, especially those fastidious organisms such as *Leptospira*[13]. This gene has also been used in previous studies on the genus *Leptospira*[3,13]. However, to determine the pathogenicity of *Leptospira* strains, we designed primers that targeted the *ligB* gene, a decision that was based on previous typing of pathogenic

Leptospira strains at the species level[9,10], though only a few studies have used this gene as a marker for differentiating pathogenic and non-pathogenic *Leptospira* species[14,15]. Lig proteins, including LigA, LigB, and LigC, belong to a superfamily of bacterial immunoglobulin-like proteins[9]. They are present only in pathogenic *Leptospira* spp. and are highly conserved[13]. The *ligB* gene has been found in every pathogenic *Leptospira* spp. studied[9] to date. Sera from patients with leptospirosis were found to contain antibodies to Lig proteins[9]. Thus, Lig proteins appear to be closely associated with infection of the mammalian host, suggesting that they may be protective immunogens.

The primer pair LP1/LP2 was successfully used to differentiate between pathogenic and non-pathogenic *Leptospira*. Even though a few different pathogenic *Leptospira* species produced amplicons with identical sizes, candidate species could be directly inferred based on the amplicon size. The detection of pathogenic *Leptospira* combined with candidate species identification offered by LP1/LP2 primers is useful, especially given that most methods for species identification rely upon DNA sequencing, which is not available to many laboratories.

The present assay is a convenient, single-tube PCR that allows for the simultaneous detection and species classification of pathogenic *Leptospira*.. This rapid assay is therefore suitable for screening, especially during leptospiral outbreaks and in settings where access to sequencing facilities is not possible. However, validation studies using clinical samples are required to establish the clinical utility of this assay.

The duplex endpoint PCR assay is a promising tool for the rapid screening and diagnosis of leptospirosis owing to its high sensitivity and specifi ity. The assay is simple and provides useful information, such as the pathogenicity and possible species of the detected *Leptospira*.

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CHAPTER 4: CONCLUSIONS

Leptospirosis is reemerging in Malaysia. This disease is still underestimated because of misdiagnosis and underreporting, and the epidemiology of this infection remains unclear. Therefore, the present study provides more information for better understanding of the epidemiological features of leptospirosis in this country.

Data for all reported human cases with leptospirosis between 2004 and 2012 provided by The Ministry of Health, Malaysia was thoroughly analysed statistically and disease incidence was found influenced by intrinsic (sex, age, and ethnicity) and extrinsic (location, rainfall, and temperature) factors with positive relationships between the number of reported cases with the number of raining days per month and monthly average temperature. However, no significant associations were noted between rainfall volume and number of reported leptospirosis cases. This findings calls for implementation of a robust surveillance system to monitor temporal and spatial changes in incidence and prevalence in order to identify risk areas and risk behavior and, in turn, design adequate control and prevention measures.

In order to investigate the type of the serovars from various sources including environment (water and soil) and host reservoir (rats, pigs, stray cats and dogs) in different sites in Peninsular Malaysia, isolation, detection and identification of the *Leptospira* spp. was carried out. Isolation step was a challenge as the contamination was high among the samples especially in enviromental samples. Despite this, we were able to provide evidence of the presence of pathogenic *Leptospira* in water and soil unfortunately, we were unable to identify the serogroup types for those positive samples

because of the limited number of the antisera available. PFGE and RAPD showed a high genetic diversity among these eight positive isolates.

The incidence in animal hosts particularly pigs, stray dogs and cats was low compared in the urban rat population. Based on MAT, four serogroups were identified including Bataviae, Javanica, Pomona and Canicola. PFGE results were in concordance with MAT results and four serovars were determined. However, PFGE showed a limited genetic diversity among the zoonotic isolates compared to RAPD. Identification of the four serovars gave the priority of PFGE as a complementary tool to the serological technique to differentiate leptospiral serovars. *L. interrogans* serovar Bataviae and *L. borgpetersenii* serovar Javanica are the two major serovars circulating in urban rat population in Peninsular Malaysia.

This study was also the first to apply MLST scheme on Malaysian *Leptospira* isolates and also contributed to the limited genotypic data of leptospires using PFGE. MLST analysis identified seven unique STs comprising of six known clones and one new clone (ST205) with ST143 being the most prevalent clone followed by ST50. Despite successfully identifying two dominant clones in the urban rat population, their significance especially in causing human infections remained unknown. Future studies involving pathogenic *Leptospira* isolates recovered from human cases are necessary to complete the transmission cycle of the present study.

Six antimicrobial agents used for leptospirosis treatment, doxycycline, ampicillin and penicillin G showed to be effective against all clinical and zoonotic isolates. However, further testing on larger numbers of environmental isolates is required to determine the most suitable antibiotic treatment for leptospirosis.

Of all the techniques, PCR proved to be a promising tool for future diagnostic of leptospirosis, particularly when only method adopted for screening is primarily dependent on MAT. The latter encounters several drawbacks as this method is laborious and time-consuming, and it requires extensive collection of reference strains. The end point PCR showed a higher sensitivity and specificity, as it can be used to detect and determine the pathogenic status of *Leptospira* spp. in many different samples.

In summary, leptospirosis is an endemic disease in Malaysia with a clear dramatic increase of the reported cases since a decade. This study proved the presence of the pathogenic *Leptospira* spp. in enviroment and animals hosts in Malaysia and two major pathogenic *Leptospira* serovars were found dominant in urban rat populations of five states in Peninsular Malaysia. However, the present study was unable to link the type of serovars infecting the human population to the reservoir host and the environment. Therefore, further investigation is required to complete full understanding of transmission of leptospires between the environment and potential hosts reservoir to humans and to gain a better insight understanding of the epidemiology of the disease. Future work that could be beneficial includes; whole genome sequencing (WGS) and Next Generation Technology (NGS) of *Leptospira* spp. isolated from humans, animal host and the environment. The knowledge gain from these studies could potentially gain better insight into the genetic potential of these strains and identify genes that contribute in the virulence and the pathogenicity in the different hosts. In addition, comparative genomic sequences studies of Malaysian *Leptospira* strains with other reference genomes would also help to reveal survival mechanism in the bacteria to adapt in their preferred hosts and habitats.

This study is evidence of a successful collaboration between the medical, academic and governmental institutions that has enabled the construction of this comprehensive database that is essential to understand the disease trends in Malaysia and gain insights into the prevention and control of this disease.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **Benacer, D.**, Thong, K. L., Verasahib, K. B., Galloway, R. L., Hartskeerl, R. A., Lewis, J. W., & Mohd Zain, S. N. (2016). Human Leptospirosis in Malaysia: Reviewing the Challenges After 8 Decades (1925-2012). *Asia Pacific Journal of Public Health*, *28*, 290–302.

2. **Benacer, D.**, Thong, K. L., Min, N. C., Verasahib, K. B., Galloway, R. L., Hartskeerl, R. A., Souris, M., & Mohd Zain, S. N. (2016). Epidemiology of human leptospirosis in Malaysia, 2004–2012. *Acta Tropica Journal*, *157*, 162–168.

3. **Benacer D**, Who, P. Y., Mohd Zain., S. N., Amran., F., & Thong., K. L. (2013). Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in Peninsular Malaysia. *Microbes and Environments Journal*, *28*, 135-140.

4. **Benacer, D.**, Mohd Zain, S. N., Amran, F., Galloway, R. L., & Thong, K. L. (2013). Molecular Characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* from urban rat populations of Kuala Lumpur, Malaysia. *American Journal of Tropical Medicine and Hygiene*, *88*, 704–709.

5. **Benacer, D.**, Mohd Zain, S. N., Sim, S. Z., Mohd Khalid, N. M. K., Galloway, R. L., Souris, M., & Thong, K. L. (2016). Determination of *Leptospira borgpetersenii* serovar Javanica and *Leptospira interrogans* serovar Bataviae as the persistent *Leptospira* serovars circulating in the urban rat populations in Peninsular Malaysia. *Parasites and Vectors Journal*, *9*, 117.

6. **Benacer, D.**, Thong, K. L., Ooi, P. T., Souris, M., Lewis, J. W., Ahmed, A. A., & Mohd Zain, S. N. (2017). Serological and Molecular Identification of *Leptospira* spp. in Swine and Stray Dogs from Malaysia. *Journal of Tropical Biomedicine*, *34*, 1–9.

7. **Benacer, D.**, Mohd Zain, S. N., Ahmed, A. A., Mohd Khalid, N. M. K., Hartskeerl, R. A., & Thong, K. L. (2016). Predominance of the ST143 and ST50 *Leptospira* Clones in the Urban Rat Populations of Peninsular Malaysia. *Journal of Medical Microbiology*, *65*, 574-577.

8. **Benacer, D.**, Mohd Zain, S. N., Ooi, P. T., & Thong, K. L. (2017). Antimicrobial Susceptibility of *Leptospira* spp. Isolated from Environmental, Human and Animal Sources in Malaysia. *Journal of Indian Medical Microbiology*, *35*, 124-128.

9. **Benacer, D.**, Mohd Zain, S. N., Lewis, J. W., Mohd Khalid, N. M. K., & Thong, K. L. (2017). A duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains. *Journal of the Brazilian Society of Tropical Medicine*, *50*, 1-4.

PAPERS PRESENTED

NATIONAL

1. **Benacer Douadi**, Siti Nursheena Mohd Zain, Fairuz Amran, Thong Kwai Lin. Prevalence of *Leptospira* spp. in Urban Rat Population of Kuala Lumpur, Malaysia. National Postgraduate Seminar 2012. Research Management & Innovation Complex. University of Malaya. 11 July, 2012.

2. **Benacer Douadi**, Siti Nursheena Mohd Zain, Fairuz Amran, Thong Kwai Lin. Molecular Characterization of *Leptospira* Species Isolated from Animal and Environment Hosts. 31st Symposium of the Malaysian Society for Microbiology. Kota Kinabalu, Malaysia. 13-15 December, 2012.

3. **Benacer Douadi**, Siti Nursheena Mohd Zain, Mohamed Khalid Nizam, Fairuz Amran, Thong Kwai Lin.
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APPENDIX I

Background Data for *Leptospira* Strains

Table A1: *Leptospira* spp. strains isolated from rats.

Strains No.	Species/Serovar	Isolation site	Isolation source	Rat Species
LR01/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR02/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR03/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR04/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR05/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR06/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR07/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR08/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR09/11	*L.interrogans*/Bataviae	Kuala Lumpur	Rat	*Rattus rattus*
LR10/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR11/11	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR12/11	*L.interrogans*/Bataviae	Kuala Lumpur	Rat	*Rattus rattus*
LR13/11	*L.interrogans*/Bataviae	Penang	Rat	*Rattus norvegicus*
LR14/11	*L.borgpetersenii*/Javanica	Penang	Rat	*Rattus rattus*
LR15/11	*L.interrogans*/Bataviae	Penang	Rat	*Rattus rattus*
LR16/11	*L.interrogans*/Bataviae	Penang	Rat	*Rattus norvegicus*
LR17/11	*L.interrogans*/Bataviae	Penang	Rat	*Rattus norvegicus*
LR18/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR19/12	*L.interrogans/* Unknown	Kuala Lumpur	Rat	*Rattus norvegicus*
LR20/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR21/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR22/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR23/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR24/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus rattus*
LR25/12	*L.borgpetersenii*/Javanica	Kuala Lumpur	Rat	*Rattus norvegicus*
LR26/12	*L.interrogans*/Bataviae	Kuala Lumpur	Rat	*Rattus rattus*
LR27/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR28/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR29/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR30/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR31/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR32/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR33/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR34/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR35/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR36/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR37/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR38/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR39/13	*L.borgpetersenii*/Javanica	Ipoh	Rat	*Rattus rattus*
LR40/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*
LR41/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*
LR42/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*
LR43/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*

Table A1 continued.

LR44/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*
LR45/13	*L.interrogans*/Bataviae	Malacca	Rat	*Rattus rattus*
LR46/13	*L.borgpetersenii*/Javanica	Ampang	Rat	*Rattus annandalei*
LR47/13	*L.interrogans*/Bataviae	Ampang	Rat	*Rattus norvegicus*
LR48/13	*L.interrogans*/Bataviae	Ampang	Rat	*Rattus norvegicus*
LR49/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus norvegicus*
LR50/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus annandalei*
LR51/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus norvegicus*
LR52/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus annandalei*
LR53/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus annandalei*
LR54/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus annandalei*
LR55/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus rattus*
LR56/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus annandalei*
LR57/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus norvegicus*
LR58/14	*L.borgpetersenii*/Javanica	Kuantan	Rat	*Rattus rattus*
LR59/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus rattus*
LR60/14	*L.interrogans*/Bataviae	Kuantan	Rat	*Rattus norvegicus*

Table A2: *Leptospira* spp. strains isolated from swine and dogs.

Strains	Age	Sex	Year	Source
LD01/11	1 year	Female	19/10/2011	Dog (urine)
LS01/11	2 years	Female	2011	Swine (urine)

Table A3: *Leptospira* spp. strains isolated from water and soil.

Sampling sites	Coordinates	Water			Soil		
		No. of samples	Average temperature	Average pH	No. of samples	Average temperature	Average pH
Kuala Lumpur							
1. Cheras	3°11'53"N 101°40'27"E	10	28°C	7.27	-	-	-
2. Pantai Dalam	3°6'49"N 101°39'45"E	10	28°C	6.47	10	28°C	6.54
3. Setapak	3°11'32"N 101°43'1"E	11	28°C	7.49	-	-	-
4. Taman Tasik Titiwangsa	3°10'42"N 101°42'25"E	10	30°C	7.55	-	-	-
5. Taman Setapak Jaya	3°11'27"N 101°43'41"E	10	29°C	7.01	-	-	-
6. Taman Tasik Perdana	3°8'30"N 101°41'4"E	10	30°C	7.54	-	-	-
7. UM Lake	3°7'9"N 101°39'26"E	10	29°C	7.76	-	-	-
Selangor							
1. SS2	3°7'6"N 101°37'17"E	10	28°C	7.57	10	28°C	6.56
2. Section 17	3°7'10"N 101°38'14"E	10	28°C	7.45	-	-	-
3. Taman Jaya	3°6'18"N 101°38'54"E	10	29°C	7.91	-	-	-
4. Taman Paramount	3°6'10"N 101°37'30"E	10	30°C	5.77	-	-	-
Johor							
1. Gemas Baru	2°34'46"N 102°36'43"E	10	28°C	6.63	10	28°C	6.38
Total of sample	-	121	-	-	30	-	-

Preparation of EMJH Media

I

Chemical Reagents	Chemical Formula	g/100 ml distilled water
Ammonium chloride	NH_4Cl	25.0
Calcium chloride dihydrate	$CaCl_2.2H_2O$	1.5
Magnesium chloride hexahydrate	$MgCl_2.6H_2O$	1.5
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	0.4
Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.3
Iron (II) sulphate heptahydrate	$FeSO_4.7H_2O$	0.5
Vitamin B1	-	0.5
Vitamin B12	-	0.02
Sodium Pyruvate	-	10.0
5-Flurouracil	-	2.0
Glycerol	-	10.0 ml
Tween 80	-	10.0 ml

II

Chemicals	Chemical Formula	g/900 ml
Disodium hydrogen phosphate (anhydrous)	Na_2HPO_4	1.0
Potassium dihydrogen phosphate (anhydrous)	KH_2HPO4	0.3
Sodium chloride	NaCl	1.0
Stock Solutions	**Chemical Formula**	**ml**
Ammonium chloride	NH_4Cl	1.0
Vitamin B1	-	1.0
Sodium Pyruvate	-	1.0
Glycerol	-	1.0
Double distilled deionized water	-	900

III

Chemicals	Chemical Formula	g/100 ml
Bovine Serum Albumin Fraction V	-	20
Stock Solutions	**Chemical Formula**	**ml**
Calcium chloride dihydrate	$CaCl_2.2H_2O$	2.0
Magnesium chloride hexahydrate	$MgCl_2.6H_2O$	2.0
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	2.0
Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.2
Iron (II) sulphate heptahydrate	$FeSO_4.7H_2O$	20.0
Vitamin B12	-	2.0
Tween 80	-	25.0

APPENDIX III

Rats Species Identification Guide as According to Medway (1983)

Species	*Rattus rattus*	*R. norvegicus*	*R. argentiventus*	*R. tiomanicus*	*R. annandalei*	*R. exulans*
Dorsal fur colouring	Olive brown, fur rough without prominent spines	Brown, hair course without prominent spines	Fur rough without prominent spines, olive brown with yellow and black hairs intermixed with brown	Olive brown, slightly darker in midline, hair relatively smooth without prominent spines	Soft fur without prominent spines, grayish brown	Dark-grey drown intermixed with spines
Ventral fur colouring	Olive brown or grayish brown	Grey, grayish brown	Silvergrey, darker longitudinal streak in midline	Poor white or dull white	White, sometimes tinged with yellow	Dull grey
Body weight (g)	85-240	150-400	85-180	55-152	145-250	30-65
Body length (mm)	125-205	163-265	140-210	125-190	145-220	100-135
Hind foot (mm)	31-39	40-45	32-35	28-32	35-40	20-25
Tail length (mm)	90-120 Uniform dark brown	170-230 Brown on the upper side, unpigmented underside	130-192 Uniform dark	125-198 Uniform dark	158-270 Uniform dark	108-147 Uniform dark
Ear length (mm)	18-23	18-25	19-23	16-22	18-23	

Multilocus Sequence Type Results

ST 143

glmU (444 bp)

CTACTTCTCCATGTACTCGATCATCTGAAAGGCTCCGGCATAGAACGAATCG

TAGTCGTAGTAGGTTACAAAAAAGAATTGGTCCAAGCACTTTGCTCTGAAAT

ATCCGGAGTCTCCTTCGTTGAACAAAAAGAACAACTGGGAACCGCCCACGC

ACTACTTTGTGCAGAACCCGAACTCAAAAACTTTCAAGGTTCCGTAATCGTC

GCCTGTGGCGACGTTCCTATGATCACTTCCAAAACGTTTGCCGATATCGTAA

AAGAACATAGAGAGAATGAATTCTCCGCTACGATTCTTTCCGCGGTCGTAG

AAAAACCGACAGGTTATGGAAGAATCATTCGCAACGCATCGGGTGATGTTA

CCGCTATCGTCGAAGAAAAGGATTCTTCTTCCGAAGAAAAATTGATCAACG

AAATCAATACCGGAACCTACGTCTTCGAAGGA

pntA (525 bp)

GCCATAAATGCATCAACCATCCAAAAACTCGCGGCTAAAAAAGTGGAAGTT

TTAAGTTTGGATGCGATCGCAAGAATTACAAGAGCTCAGTCGATGGATGTTC

TTTCTTCCCAAGCGACCGTTGCTGGCTACAAAGCAGTGTTACTTGCGGCTTC

TCATTTGGCTAGATTTTTCCCAATGCTTACGACTGCAGCTGGAACGATCACA

CCTGCTTCTGTGTTGATTATTGGAGCCGGAGTCGCAGGTTTACAGGCGATCG

CTTCTAGTAGAAGGCTGGGTGCGGTAGTGGATGTATTTGATACAAGACCGG

AAGTAAAAGAGCAAGTTCATTCTCTTGGTGCAAAGTTCGTGGAAGTGGAAG

GCGCCTCGCATTCCGCTGCGGCCGGTGGTTACGCAGTAGAACAGACTGAAG

AATACAAAAAGCGTCAGCAAGAAGCCATCGATAGATACGCTCAGAAGGCG

GACGTAATCATAACGACTGCATTGATTCCGGGAAAAAAAGCCCCTTTACTA

ATTACGAAGAAG

sucA (447 bp)

GGAATGGCGCACAGAGGGAGACTGAACGTTCTCGTTAACATCATCGAAAAA

CCCGCGTCACTCATCTTTGCCGAGTTCGAGGAAAAGACGGATAGGGATAAC

TTAAGTTATGCGGACGTTAAGTATCATCTCGGGTATTCCAACAGCAGAATGA

CAACCGCGGGAAAAGAAGTGAAACTTTCCCTCATGTTCAATCCGAGTCATTT

GGAATGTGTGGGTCCTGTCGTAACAGGATCTGTTCGCGCTCGTCAGGAACTT

ATCGGAAATAAGGATCGTACGAAATATATGCCGATTCTGATTCACGGCGAC

GCCGCGTTTGCGGGTCAGGGTGTAGTAGCGGAAACCCTCAATCTGATGAAT

CTTGAAGGTTATACTACGGGGGGGAACCTTTCACATCGTGGTCAATAATCAGA

TCGGATTTACTACTCTTCCGGACGAATCCAGATCC

tpiA (426 bp)

ACATTGCATCTTGCGGGTGTTGCGAAAATTTTACAAGGAACAGAAATTCTAG

TCGGAGCGCAGAATGTGTATCCTTCCGGGCTTGCGGCCTTTACCGGTGAAAC

CTCACCGGAACAACTAAAAGAACTCGGCGTGAAGGTCGTTATGATCGGACA

TTCGGAAAGAAGACAATTTCTAGGCGAGACGAATTCCTTTTGTAACGAAAA

GATTCATTTCTTATTAAAGAATGATTTTATCGTGCTCTATTGTGTAGGTGAAA

CTCTTATGGAAAGAGAATCCGGAAAAACCTTCGAAGTAATTTCTTCCCAAAT

TCGAGAAGGCTTGAAAGGAATCCACAGTCATTCTTTTTCCAATCTGATCCTA

GCTTATGAGCCGGTCTGGGCGATCGGAACCGGAAAAGTGGCAACACCGGCA

CAAGCACAAGAG

pfkB (432 bp)

ACTCCCGATGCTGAAAGAACGATGCTCACCCACTTGGGGATTTCCATCACAT
TACAAAAATCGGATGTGGATTTGGACAAACTCAAGTCCTCCGGCATTTCCTA
TATCGAAGGATATCTCTGGGATGGTCAGGGAACCAAAGAAGCTTCTCTTTTG
ACGATGGAAGAATCCAAAAGAAACGGGGTAAAAGTCGCTTACACATACAG
CGATCCGTTTTGTGTGAATCGTTCGAGAGAAGACTTCGTTCGTTTAACAAAA
GATTACTTCGATATCGTTTTCTGCAACGCGGAAGAAGCCAAAGCCCTTTCTC
AAAGGGAAGATAAACTCGAAGCTTTGAAATTTATCGCGAATCTTTCTCCTCT
CGTTTTTATGACCGATTCCGCAAATGGGGCCTATTTTGCGGAGAACGGAGTG
GTTGCTCACGTTGGTGGA

mreA (435 bp)

GAGGGAGGATTTTTCTTTAAGATTTTCTCCAATCAAACTCTGCTTTTGACTTT
TGGAGCGATCTTTTTGATCGCTTGTCTTGTTATGGTGGGAATCCGAATTGCC
AGAGGAAGCAGAGCATTAAGACGATATTATATACCTTTGGGGATTTTGGGA
ATCAGTTTGATCTCCGCGGTTGTAGTGATGAAAATCGTGCCCTTTAGAGAGA
ACCAGGTGATTCGATTAACCGCGTTTTTAAATCCGGAAGAATTCAAACAAG
GAGCCGGGTATCAACTCAGGGCTTCGAAACCCGCGGTCGGATCCGGTAGAT
TTTTCGGAAAAGGTTTGATGAATGCAGAGATGACGGAAGGAAGAATCCCGC
ACGTCCCCGAATCGAGCACCGATTTTATTTTCGCTTCTTGGGCCGAGCAGAC
CGGGTTTTTAGGGTCCGTATTT

caiB (402 bp)

GCTCTGTATGCGAGGGAAAAGACGGGGAAAGGACAGAAGATCGCGATTTCT
ATGATGGATTCTTCCCTTCCGTTTTTATCTTTGTATGCGGGAATATTCGCGGC
TACCGGGAAAAATCCGGAAGGGGGAAACGAACTTCTATCTGGTAAATTACC
GAATTATAATATATCAAACTAAGGAAGGGCGTTGGGTTGCATTAGGCGC
GCTTGAGGATATGTTTTTTAAGACCTTTTTACGTCAGTCGGGATTAGATGGA
CATTTGGGGGAATTACCGACGGAAGAGAAAAACTTTTCCAAGTGGAAAGAA
ATTCTTACCGCTTACTTTGCATCCAAGACGTTTGAAGATTTGAACTTTTTATT
TGAGAATGAGGATTCTTGTCTGACCCCCGTTAAGACGATG

ST 50

glmU (444 bp)

CTGCTTTTACATGTACTCGATCATCTAAAAGGTTCTGGTGTAGAACGGATCG
TAGTAGTAGTAGGTTACAAAAAAGAATTGGTTCAGTCTCTTTGCTCCAAAAT
TCCAGGTGTTACTTTTGTCGAACAAAAGAACAACTTGGAACTGCTCATGCT
CTTCTTTGCGCCGAAACGGAACTGAAAAATTTCCAAGGTTCTGTGATCGTTG
CCTGTGGAGACGTTCCAATGATTACTTCCGAAACGTTTTCTAATATAGTAAA
ACAACACAAAGAGAATGAATTTTCTGCAACGATCCTTTCTGCAGTCGTAGA
AAAACCTACAGGATATGGAAGAATTATCCGCAACTCCTCCGGTGAAGTAAC
AGCTATCGTAGAAGAAAAGATTCTTCTACCGAAGAAAAATTGATCAACGA
AATTAACACTGGAACCTATGTATTCGACGGA

pntA (525 bp)

GCGATGAATGCACAAACAGTTCAAAAACTTGCTTCTAAAAAGGTAGAAGTT
TTAAGTTTAGATGCAATCGCAAGGATTACGAGAGCCCAGTCTATGGATGTA
CTTTCTTCTCAGGCAACGGTTGCCGGTTACAAAGCGGTATTGCTTGCGGCTT
CTCATTTAGCCAGATTTTTTCCAATGCTTACTACGGCGGCTGGAACCATCAC
ACCTGCTTCCGTGTTGATTATTGGAGCCGGAGTTGCTGGATTACAAGCGATC
GCTTCCAGTAGAAGATTGGGCGCGGTAGTAGACGTATTCGATACAAGACCA
GAAGTAAAAGAGCAGGTTCATTCTCTTGGAGCGAAGTTTGTAGAAGTAGAA
GGCGCTTCCCATTCTGCTGCTGCTGGCGGTTATGCGGTTGAACAAACCGAAG
AATATAAAAAACGTCAACAAGAAGCGATCGATAAATACGCTCAAAAGGCG
GATGTAATCATAACAACTGCGCTCATTCCTGGAAAAAAGCCCCATTATTGA
TCACTAAAAAG

sucA (447 bp)

GGAATGGCACATAGGGGAAGACTGAATGTTCTCGTGAACATTATCGAAAAA
CCTGCGTCACTCATCTTTGCGGAGTTTGAAGAAAAAACGGACAAAGATAAC
TTAAGTTATGCGGATGTAAAATACCATTTGGGGTATTCTAACAGTAGAATGA
CTACCTCTGGTAAAGAAGTGAAACTTTCACTCGCGTTCAACCCAAGTCATTT
AGAATGTGTGGATCCAGTGGTAACCGGATCGGTTCGTGCCCGTCAGACTTTG
ATCGGAGATAAGGATCGTTCCAAATATATGCCGATTTTGATTCATGGTGACG
CTGCGTTTGCGGGTCAGGGAGTTGTTGCGGAAACTCTCAACTTGATGAATTT
AGAAGGTTATACTACCGGTGGAACGTTTCATATTGTGGTCAATAACCAGATT
GGTTTTACCACTCTTCCCGATGAATCTAGATCT

tpiA (426 bp)

ACTCTTCATCTTGAAAACGTTTCCAAAATATTAGAAGGTAGCAGTGTAATTG

TTGGAGCCCAAAACTGTTATCACTCCGGACTTGCAGCTTTTACCGGCGAAAC

CTCTCCCGATCAACTGAAAGAAATTGGTGTTAAAGTTGTAATGGTCGGTCAT

TCGGAACGTCGTCAATTTTTAGGAGAGTCTAACTTTTTTTGTAACGATAAAA

TCCGTTTTCTTTTAAAGAACGAATTTACAGTCCTTTATTGTGTTGGAGAAACT

CTCTCCGAAAGAGAATCCGGTAAAACATTGGAAGTACTGTCTTCTCAAATTA

GAGAAGGTTTAAAAGGAATTGATAGCGTATTTTTTTCCAATTTAATTCTGGC

TTATGAACCAGTTTGGGCCATTGGAACCGGAAAAGTTGCCACCCCCTCTCAA

GCCCAAGAA

pfkB (432bp)

ACACCGGACGCAGAAAGAACGATGCTTACTCATTTAGGAATTTCAATTACA

TTACAAAAATCAGATGTAGATTTAGAAAAACTAAAATCTTCTAGTATTTCTT

ATATCGAAGGTTATCTTTGGGATGGTCAAGGAACGAAAGAGGCTTCACTTTT

AACCATGGAGGAATCCAAGAAAAATGGTGTAAAAGTAGCTTATACTTATAG

TGATCCGTTTTGTGTAAATCGTTCCAGAGAGGACTTTATTCGTTTAACAAAA

GAATATTTTGACATCGTTTTTTGTAATACGGAAGAGGCGAAGGCTCTTTCTC

AAAGAGAAGATAAGTTGGAAGCTCTTAAGTTTATTTCTGGCCTTTCTGCTCT

TGTGTTTATGACTGATTCTGCAAACGGAGCTTACTTTGCAGAGGATGGAAAA

ATTTCTCACGTAGATGGG

mreA (435 bp)

GAAGGAGGATTTTTCTTTAAGATTTTTTCTAATCAAAAATTACTTTTGACCTT
TGGGGCTTTATTTTTAATTGCGAGTATAGTGATGGTGGGAATTCGAATTGCA
AGAGGAAGTAAAACATTAAGACAATATTATATACCTCTTGGAATTTTGGGA
ATCAGTTTGATTTCTGCGGTTGTAGTAATGAAAACCGTTCCGTTTCGAGAAA
ACCAAGTCATTCGATTGACTGCATTTTTAAATCCGGAGGAATTTAAACAAGG
TGCCGGTTATCATCTGAGAGCTTCTAAACCTGCGGTTGGATCGGGAAGATTT
TTCGGAAAAGGGCTGATGAATGCAGAAATGACCGAAGGAAGAATTCCACAC
GTACCAGAATCCAGCACTGATTTTATTTTTGCTTCCTGGGCGGAACAAACCG
GGTTTTTAGGATCTGTATTT

caiB (402 bp)

GCTTTGTATGCGAGGGAAAAAACGGGAAAAGGACAAAAAATTTCCGTTTCT
ATGATGGATTCGTCATTGCCGTTTTTATCCTTGTATGGAGGGATTTACGGAG
CCACCGGAAAAAAATCCAGAAGGAGGAAATGAACTACTTTCTGGTAAATTAC
CTAATTATAATGTTTATCAAACGAAGGAGGGTCGTTGGGTAGCGTTAGGTGC
TCTTGAAGATATGTTTTTTAAAACTTTTTTACGACAGACTGGGTTGGATAAA
CATTTGGAAGAATTACCTGCAGAAGAAAAAAACTTTTCTAAATGGAAAGAA
ATACTTACTACCTATTTTTCTACAAAAACATTCGAAGATTTGAATGTTCTTTT
TGAAAACCAAGATTCCTGTCTAACTCCTGTAAAAACAAT

ST 144

glmU (444 bp)

CTACTTCTCCATGTACTCGATCATCTGAAAGGCTCCGGCATAGAACGAATCG
TAGTCGTAGTAGGTTACAAAAAAGAATTGGTCCAAGCACTTTGCTCTGAAAT
ATCCGGAGTCTCCTTCGTTGAACAAAAAGAACAACTGGGAACCGCCCACGC
ACTACTTTGTGCAGAACCCGAACTCAAAAACTTTCAAGGTTCCGTAATCGTC
GCCTGTGGCGACGTTCCTATGATCACTTCCAAAACGTTTGCCGATATCGTAA
AAGAACATAGAGAGAATGAATTCTCCGCTACGATTCTTTCCGCGGTCGTAG
AAAAACCGACAGGTTATGGAAGAATCATTCGCAACGCATCGGGTGATGTTA
CCGCTATCGTCGAAGAAAAGGATTCTTCTTCCGAAGAAAAATTGATCAACG
AAATCAATACCGGAACCTACGTCTTCGAAGGA

pntA (525 bp)

GCCATAAATGCATCAACCATCCAAAAACTCGCGGCTAAAAAAGTGGAAGTT
TTAAGTTTGGATGCGATCGCAAGAATTACAAGAGCTCAGTCGATGGATGTTC
TTTCTTCCCAAGCGACCGTTGCTGGCTACAAAGCAGTGTTACTTGCGGCTTC
TCATTTGGCTAGATTTTTCCCAATGCTTACGACTGCAGCTGGAACGATCACA
CCTGCTTCTGTGTTGATTATTGGAGCCGGAGTCGCAGGTTTACAGGCGATCG
CTTCTAGTAGAAGGCTGGGTGCGGTAGTGGATGTATTTGATACAAGACCGG
AAGTAAAAGAGCAAGTTCATTCTCTTGGTGCAAAGTTCGTGGAAGTGGAAG
GCGCCTCGCATTCCGCTGCGGCCGGTGGTTACGCAGTAGAACAGACTGAAG
AATACAAAAAGCGTCAGCAAGAAGCCATCGATAGATACGCTCAGAAGGCG
GACGTAATCATAACGACTGCATTGATTCCGGGAAAAAAAGCCCCTTTACTA
ATTACGAAGAAG

sucA (447 bp)

GGAATGGCGCACAGAGGGAGACTGAACGTTCTCGTTAACATCATCGAAAAA
CCCGCGTCACTCATCTTTGCCGAGTTCGAGGAAAAGACGGATAGGGATAAC
TTAAGTTATGCGGACGTTAAGTATCATCTCGGGTATTCCAACAGCAGAATGA
CAACCGCGGGAAAAGAAGTGAAACTTTCCCTCATGTTCAATCCGAGTCATTT
GGAATGTGTGGGTCCTGTCGTAACAGGATCTGTTCGCGCTCGTCAGGAACTT
ATCGGAAATAAGGATCGTACGAAATATATGCCGATTCTGATTCACGGCGAC
GCCGCGTTTGCGGGTCAGGGTGTAGTAGCGGAAACCCTCAATCTGATGAAT
CTTGAAGGTTATACTACGGGGGGGAACCTTTCACATCGTGGTCAATAATCAGA
TCGGATTTACTACTCTTCCGGACGAATCCAGATCC

tpiA (426 bp)

ACATTGCATCTTGCGGGTGTTGCGAAAATTTTACAAGGAACAGAAATTCTAG
TCGGAGCGCAGAATGTGTATCCTTCCGGGCTTGCGGCCTTTACCGGTGAAAC
CTCACCGGAACAACTAAAAGAACTCGGCGTGAAGGTCGTTATGATCGGACA
TTCGGAAAGAAGACAATTTCTAGGCGAGACGAATTCCTTTTGTAACGAAAA
GATTCATTTCTTATTAAAGAATGATTTTATCGTGCTCTATTGTGTAGGTGAAA
CTCTTATGGAAAGAGAATCCGGAAAAACCTTCGAAGTAATTTCTTCCCAAAT
TCGAGAAGGCTTGAAAGGAATCCACAGTCATTCTTTTTCCAATCTGATCCTA
GCTTATGAGCCGGTCTGGGCGATCGGAACCGGAAAAGTGGCAACACCGGCA
CAAGCACAAGAG

pfkB (432 bp)

ACTCCCGATGCTGAAAGAACGATGCTCACCCACTTGGGGATTTCCATCACAT
TACAAAAATCGGATGTGGATTTGGACAAACTCAAGTCCTCCGGCATTTCCTA
TATCGAAGGATATCTCTGGGATGGTCAGGGAACCAAAGAAGCTTCTCTTTTG
ACGATGGAAGAATCCAAAAGAAACGGGGTAAAAGTCGCTTACACATACAG
CGATCCGTTTTGTGTGAATCGTTCGAGAGAAGACTTCGTTCGTTAACAAAA
GATTACTTCGATATCGTTTTCTGCAACGCGGAAGAAGCCAAAGCCCTTTCTC
AAAGGGAAGATAAACTCGAAGCTTTGAAATTTATCGCGAATCTTTCTCCTCT
CGTTTTTATGACCGATTCCGCAAATGGGGCCTATTTTGCGGAGAACGGAGTG
GTTGCTCACGTTGGTGGA

mreA (435 bp)

GAGGGAGGATTTTTCTTTAAGATTTTCTCCAATCAAACTCTGCTTTTGACTTT
TGGAGCGATCTTTTTGATCGCTTGTCTTGTTATGGTGGGAATCCGAATTGCC
AGAGGAAGCAGAGCATTAAGACGATATTATATACCTTTGGGGATTTTGGGA
ATCAGTTTGATCTCCGCGGTTGTAGTGATGAAAATCGTGCCCTTTAGAGAGA
ACCAGGTGATTCGATTAACCGCGTTTTTAAATCCGGAAGAATTCAAACAAG
GAGCCGGGTATCAACTCAGGGCTTCGAAACCCGCGGTCGGATCCGGTAGAT
TTTTCGGAAAAGGTTTGATGAATGCAGAGATGACGGAAGGAAGAATCCCGC
ACGTCCCCGAATCGAGCACCGATTTTATTTTCGCTTCTTGGGCCGAGCAGAC
CGGGTTTTTAGGGTCCGTATTT

caiB (402 bp)

GCTCTGTATGCGAGGGAAAAGACGGGGAAAGGACAGAAGATCGCGATTTCT
ATGATGGATTCTTCCCTTCCGTTTTTATCTTTGTATGCGGGAATATTCGCGGC
TACCGGGAAAAATCCGGAAGGGGGAAACGAACTTCTATCTGGTAAATTACC
GAATTATAATATATCAAACTAAGGAAGGGCGTTGGGTTGCGTTAGGCGC
GCTTGAGGATATGTTTTTTAAGACCTTTTTACGTCAGTCGGGATTAGATGGA
CATTTGGGGGAATTACCGACGGAAGAGAAAAACTTTTCCAAGTGGAAAGAA
ATTCTTACCGCTTACTTTGCATCCAAGACGTTTGAAGATTTGAACTTTTTATT
TGAGAATGAGGATTCTTGTCTGACCCCCGTTAAGACGATG

ST 205 (New sequence type)

glmU (444 bp)

CTGCTTTTACATGTACTCGATCATCTAAAAGGTTCTGGTGTAGAACGGATCG
TAGTAGTAGTAGGTTACAAAAAAGAATTGGTTCAGTCTCTTTGCTCCAAAAT
TCCAGGTGTTACTTTTGCCGAACAAAAAGAACAACTTGGAACTGCTCATGCT
CTTCTTTGCGCCGAAACGGAACTGAAAAATTTCCAAGGTTCTGTGATCGTTG
CCTGTGGAGACGTTCCAATGATTACTTCCGAAACGTTTTCTAATATAGTAAA
ACAACACAAAGAGAATGAATTTTCTGCAACGATCCTTTCTGCAGTCGTAGA
AAAACCTACAGGATATGGAAGAATTATCCGCAACTCCTCCGGTGAAGTAAC
AGCTATCGTAGAAGAAAAGATTCTTCTACCGAAGAAAAATTGATCAACGA
AATTAACACTGGAACCTATGTATTCGACGGA

pntA (525 bp)

GCGATGAATGCACAAATAGTTCAAAAACTTGCTTCTAAAAAGGTAGAAGTT
TTAAGTTTAGATGCAATCGCAAGGATTACGAGAGCCCAGTCTATGGACGTA
CTTTCTTCTCAGGCAACAGTTGCCGGTTACAAAGCGGTATTGCTCGCGGCTT
CTCATTTAGCCAGATTTTTTCCAATGCTTACTACGGCGGCTGGAACCATCAC
ACCTGCTTCCGTGTTGATTATCGGAGCCGGAGTTGCTGGATTACAAGCGATC
GCTTCCAGTAGAAGATTGGGCGCGGTAGTAGACGTATTCGATACAAGACCA
GAAGTAAAAGAGCAGGTTCATTCTCTTGGAGCGAAGTTTGTAGAAGTAGAA
GGTGCTTCCCATTCTGCTGCTGCTGGCGGTTATGCGGTTGAACAAACCGAAG
AATATAAAAAACGTCAACAAGAAGCGATCGATAAATACGCTCAAAAGGCG
GATGTAATCATAACAACTGCGCTCATTCCTGGAAAAAAAGCCCCATTATTGA
TCACTAAAAAG

sucA (447 bp)

GGAATGGCGCATAGGGGAAGACTGAATGTTCTCGTGAACATTATCGAAAAA
CCTGCGTCACTCATCTTTGCGGAATTTGAAGAAAAAACGGACAAAGATAAC
TTAAGTTATGCGGATGTAAAATACCATTTGGGGTATTCTAACAGTAGAATGA
CTACCTCTGGTAAAGAAGTGAAACTTTCACTCGCGTTCAACCCAAGTCATTT
AGAATGTGTGGATCCAGTGGTAACCGGATCGGTTCGTGCCCGTCAGACTTTG
ATCGGAGATAAGGATCGTTCCAAATATATGCCTATTTTGATTCATGGTGACG
CTGCGTTTGCGGGTCAGGGAGTTGTTGCGGAAACTCTCAACTTGATGAATTT
AGAAGGTTATACTACCGGTGGAACGTTTCATATTGTGGTCAATAACCAGATT
GGTTTTACCACTCTTCCCGATGAATCTAGATCT

tpiA (426 bp)

ACTCTTCATCTTGAAAACGTTTCCAAAATATTAGAAGGTAGCAGTGTAATTA

TTGGAGCCCAAAACTGTTATCAGTCCGGACTTGCAGCTTTTACCGGCGAAAC

CTCTCCCGATCAACTGAAAGAAATTGGTGTTAAAGTTGTGATGGTCGGTCAT

TCGGAACGTCGTCAATTTTTAGGAGAGTCTAACTTTTTTTGTAACGATAAAA

TCCGTTTTCTTTTAAAGAACGAATTTACAGTCCTTTATTGTATTGGAGAAACT

CTCTCCGAAAGAGAATCCGGTAAAACATTGGAAGTACTGTCTTCTCAAATTA

GAGAAGGTTTAAAAGGAATTGATAGCGTACTTTTTTCCAATTTAATTCTGGC

TTATGAACCAGTTTGGGCCATCGGAACCGGAAAAGTTGCCACCCCCTCTCAA

GCCCAAGAA

pfkB (432 bp)

ACACCGGACGCAGAAAGAACGATGCTTACTCATTTAGGAATTTCAATTACA

TTACAAAAATCAGATGTAGATTTAGAAAAACTAAAATCTTCTAGTATTTCTT

ATATCGAAGGTTATCTTTGGGATGGTCAAGGAACGAAAGAGGCTTCACTTTT

AACCATGGAGGAATCCAAGAAAAATGGTGTAAAAGTAGCTTATACTTATAG

TGATCCGTTTTGTGTAAATCGTTCCAGAGAGGACTTTATTCGTTTAACAAAA

GAATATTTTGATATCGTTTTTTGTAATACGGAAGAGGCGAAGGCTCTTTCTC

AAAGAGAAGATAAGTTGGAAGCTCTTAAGTTTATTTCTGGCCTTTCTGCTCT

TGTATTTATGACTGATTCCGCAAACGGAGCTTACTTTGCAGAGAATGGAAAA

ATTTCTCACGTAGATGGG

143

mreA (435 bp)

GAAGGAGGATTTTTCTTTAAGATTTTTTCTAATCAAAAATTACTTTTGACCTT
TGGGGCTTTATTTTTAATTGCGAGTATAGTGATGGTGGGAATTCGAATTGCA
AGAGGAAGTAAAACATTAAGACAATATTATATACCTCTTGGAATTTTGGGA
ATCAGTTTGATTTCTGCTGTTGTAGTAATGAAAACCGTTCCGTTTCGAGAAA
ACCAAGTCATTCGATTGACCGCATTTTTAAATCCGGAGGAATTTAAACAAGG
TGCCGGTTATCATCTGAGAGCTTCTAAACCTGCGGTTGGATCGGGAAGATTT
TTCGGAAAAGGGCTGATGAATGCAGAAATGACCGAAGGAAGAATTCCACAC
GTACCAGAATCCAGCACTGATTTTATTTTTGCTTCCTGGGCGGAACAAACCG
GGTTTTTAGGATCTGTATTT

caiB (402 bp)

GCTTTGTATGCGAGGGAAAAAACGGGAAAAGGACAAAAAATTTCCGTTTCT
ATGATGGATTCGTCATTGCCGTTTTTATCCTTGTATGGAGGGATTTACGGAG
CCACCGGAAAAAAATCCAGAAGGAGGAAATGAACTACTTTCTGGTAAATTAC
CTAATTATAATGTTTATCAAACGAAGGAGGGTCGTTGGGTCGCGTTAGGTGC
TCTTGAAGATATGTTTTTTAACACTTTTTTACGACAGACTGGGTTGGATAAA
CATTTGGAAGAATTACCTGCAGAAGAAAAAAAACTTTTCTAAATGGAAAGA
AATACTTACTACCTATTTTTCTACAAAAACATTCGAAGATTTGAATGTTCTTT
TTGAAAACCAAGATTCCTATCTAACTCCTGTAAAAACAATA

144

ST 140

glmU (444 bp)

CTGCTTTTACATGTACTCGATCATCTAAAAGGTTCTGGTGTAGAACGGATCG
TAGTAGTAGTAGGTTACAAAAAAGAATTGGTTCAGTCTCTTTGTTCCAAAAT
TCCAGGTGTTACTTTTGCCGAACAAAAAGAACAACTTGGAACTGCTCATGCT
CTTCTTTGCGCCGAAACGGAACTGAAAAATTTCCAAGGTTCTGTGATCGTTG
CCTGTGGAGACGTTCCAATGATTACTTCCGAAACGTTTTCTAATATAGTAAA
ACAACACAAAGAGAATGAATTTTCTGCAACGATCCTTTCTGCAGTCGTAGA
AAAACCTACAGGATATGGAAGAATTATCCGCAACTCCTCCGGTGAAGTAAC
AGCTATCGTAGAAGAAAAGATTCTTCTACCGAAGAAAAATTGATCAACGA
AATTAACACTGGAACCTATGTATTCGACGGA

pntA (525 bp)

GCGATGAATGCACAAACAGTTCAAAAACTTGCTTCTAAAAAGGTAGAAGTT
TTAAGTTTAGATGCAATCGCAAGGATTACGAGAGCCCAGTCTATGGACGTA
CTTTCTTCTCAGGCAACAGTTGCCGGTTACAAAGCGGTATTGCTCGCGGCTT
CTCATTTAGCCAGATTTTTTCCAATGCTTACTACGGCGGCTGGAACCATCAC
ACCTGCTTCCGTGTTGATTATCGGAGCCGGAGTTGCTGGATTACAAGCGATC
GCTTCCAGTAGAAGATTGGGCGCGGTAGTAGACGTATTCGATACAAGACCA
GAAGTAAAAGAGCAGGTTCATTCTCTTGGAGCGAAGTTTGTAGAAGTAGAA
GGTGCTTCCCATTCTGCTGCTGCTGGCGGTTATGCGGTTGAACAAACCGAAG
AATATAAAAAACGTCAACAAGAAGCGATCGATAAATACGCTCAAAAGGCG
GATGTAATCATAACAACTGCGCTCATTCCTGGAAAAAAAGCCCCATTATTGA
TCACTAAAAAG

sucA (447 bp)

GGAATGGCGCATAGGGGAAGACTGAATGTTCTCGTGAACATTATCGAAAAA
CCTGCGTCACTCATCTTTGCGGAGTTTGAAGAAAAACGGACAAAGATAAC
TTAAGTTATGCGGATGTAAAATACCATTTGGGGTATTCTAACAGTAGAATGA
CTACCTCTGGTAAAGAAGTGAAACTTTCACTCGCGTTCAACCCAAGTCATTT
AGAATGTGTGGATCCAGTGGTAACCGGATCGGTTCGTGCCCGTCAGACTTTG
ATCGGAGATAAGGATCGTTCCAAATATATGCCGATTTTGATTCATGGTGACG
CTGCGTTTGCGGGTCAGGGAGTTGTTGCAGAAACTCTCAACTTGATGAATTT
AGAAGGTTATACTACCGGTGGAACGTTTCATATTGTGGTCAATAATCAGATT
GGTTTTACCACTCTTCCCGATGAATCTAGATCT

tpiA (426 bp)

ACTCTTCATCTTGAAAACGTTTCCAAAATATTAGAAGGTAGCAGTGTAATTG
TTGGAGCCCAAAACTGTTATCACTCCGGACTTGCAGCTTTTACCGGCGAAAC
CTCTCCCGATCAACTGAAAGAAATTGGTGTTAAAGTTGTGATGGTCGGTCAT
TCGGAACGTCGTCAATTTTTAGGAGAGTCTAACTTTTTTTGTAACGATAAAA
TCCGTTTTCTTTTAAAGAACGAATTTACAGTCCTTTATTGTGTTGGAGAAACT
CTCTCCGAAAGAGAATCCGGTAAAACATTGGAAGTACTGTCTTCTCAAATTA
GAGAAGGTTTAAAAGGAATTGATAGCGTACTTTTTTCCAATTTAATTCTGGC
TTATGAACCAGTTTGGGCCATCGGAACCGGAAAAGTTGCCACCCCCTCTCAA
GCCCAAGAA

pfkB (432 bp)

ACACCGGATGCAGAAAGAACGATGCTTACTCATTTAGGAATTTCAATTACAT
TACAAAAATCAGATGTAGATTTGGAAAAACTAAAATCTTCTAGTATTTCTTA
TATCGAAGGTTATCTTTGGGATGGTCAAGGAACGAAAGAGGCTTCACTTTTA
ACCATGGAGGAATCCAAGAAAAATGGTGTAAAGTAGCTTATACTTATAGT
GATCCGTTTTGTGTAAATCGTTCCAGAGAGGACTTTATTCGTTTAACAAAAG
AATATTTTGATATCGTTTTTTGTAATACGGAAGAGGCGAAGGCTCTTTCTCA
AAGAGAAGATAAGTTGGAAGCTCTTAAGTTTATTTCTGGCCTTTCTGCTCTT
GTGTTTATGACTGATTCCGCAAACGGAGCTTACTTTGCAGAGAATGGAAAA
ATTTCTCACGTAGATGGG

mreA (435 bp)

GAAGGAGGATTTTTCTTTAAGATTTTTTCTAATCAAAAATTACTTTTGACCTT
TGGGGCTTTATTTTTAATTGCGAGTATAGTGATGGTGGGAATTCGAATTGCA
AGAGGAAGTAAAACATTAAGACAATATTATATACCTCTTGGAATTTTGGGA
ATCAGTTTGATTTCTGCTGTTGTAGTAATGAAAACCGTTCCGTTTCGAGAAA
ACCAAGTCATTCGATTGACCGCATTTTTAAATCCGGAGGAATTTAAACAAGG
TGCCGGTTATCATCTGAGAGCTTCTAAACCTGCGGTTGGATCGGGAAGATTT
TTCGGAAAAGGGCTGATGAATGCAGAAATGACCGAAGGAAGAATTCCACAC
GTACCAGAATCCAGCACTGATTTTATTTTTGCTTCCTGGGCGGAACAAACCG
GGTTTTTAGGATCTGTATTT

caiB (402 bp)

GCTTTGTATGCGAGGGAAAAAACGGGAAAAGGACAAAAAATTTCCGTTTCT
ATGATGGATTCGTCATTGCCGTTTTTATCCTTGTATGGAGGGATTTACGGAG
CCACCGGAAAAAATCCAGAAGGAGGAAATGAACTACTTTCTGGTAAATTAC
CTAATTATAATGTTTATCAAACGAAGGAGGGTCGTTGGGTAGCATTAGGTGC
TCTTGAAGATATGTTTTTTAAAACTTTTTTACGACAGACTGGGTTGGATAAA
CATTTGGAAGAATTACCTGCAGAAGAAAAAAACTTTTCTAAATGGAAAGAA
ATACTTACTACCTATTTTTCTACAAAAACATTCGAAGATTTGAATGTTCTTTT
TGAAAACCAAGATTCCTGTCTAACTCCTGTAAAAACAATA

ST 56

glmU (444 bp)

CTGCTTTTACATGTACTCGATCATCTAAAAGGTTCTGGTGTAGAACGGATCG
TAGTAGTAGTAGGTTACAAAAAAGAATTGGTTCAATCTCTTTGCTCCAAAAT
TCCAGGTGTTACTTTTGCCGAACAAAAAGAACAACTTGGAACTGCTCATGCT
CTTCTTTGCGCCGAAACGGAACTGAAAAATTTCCAAGGTTCTGTGATCGTTG
CCTGTGGAGACGTTCCAATGATTACTTCCGAAACGTTTTCTAATATAGTAAA
ACAACACAAAGAGAATGAATTTTCTGCAACGATCCTTTCTGCAGTCGTAGA
AAAACCTACAGGATATGGAAGAATTATCCGCAACTCCTCCGGTGAAGTAAC
AGCTATCGTAGAAGAAAAGATTCTTCTACCGAAGAAAAATTGATCAACGA
AATTAACACTGGAACCTATGTATTCGACGGA

pntA (525 bp)

GCGATGAATGCACAAACAGTTCAAAAACTTGCTTCTAAAAAGGTAGAAGTT
TTAAGTTTAGATGCAATCGCAAGGATTACGAGAGCCCAGTCTATGGACGTA
CTTTCTTCTCAGGCAACGGTTGCCGGTTACAAAGCGGTATTGCTCGCGGCTT
CTCATTTAGCCAGATTTTTTCCAATGCTTACTACGGCGGCTGGAACCATCAC
ACCTGCTTCCGTGTTGATTATTGGAGCCGGAGTTGCTGGATTACAAGCGATC
GCTTCCAGTAGAAGATTGGGCGCGGTAGTAGACGTATTCGATACAAGACCA
GAAGTAAAAGAGCAGGTTCATTCTCTTGGAGCGAAGTTTGTAGAAGTAGAA
GGCGCTTCCCATTCTGCTGCTGCTGGCGGTTATGCGGTTGAACAAACCGAAG
AATATAAAAAACGTCAACAAGAAGCGATCGATAAATACGCTCAAAAGGCG
GATGTAATCATAACAACTGCGCTCATTCCTGGAAAAAAAGCCCCATTATTGA
TCACTAAAAAG

sucA (447 bp)

GGAATGGCACATAGGGGAAGACTGAATGTTCTCGTGAACATTATCGAAAAA
CCTGCGTCACTCATCTTTGCGGAGTTTGAAGAAAAAACGGACAAAGATAAC
TTAAGTTATGCGGATGTAAAATACCATTTGGGGTATTCTAACAGTAGAATGA
CTACCTCTGGTAAAGAAGTGAAACTTTCACTCGCGTTCAATCCAAGTCATTT
AGAATGTGTGGATCCAGTGGTAACCGGATCGGTTCGTGCCCGTCAGACTTTG
ATCGGAGATAAGGATCGTTCCAAATATATGCCGATTTTGATTCATGGTGACG
CTGCGTTTGCGGGTCAGGGAGTTGTTGCGGAAACTCTCAACTTGATGAATTT
AGAAGGTTATACTACCGGTGGAACGTTTCATATTGTGGTCAATAACCAAATT
GGTTTTACCACTCTTCCCGATGAATCTAGATCT

tpiA (426 bp)

ACTCTTCATCTTGAAAACGTTTCCAAAATATTAGAAGGTAGCAGTGTAATTG

TTGGAGCCCAAAACTGTTATCACTCCGGACTTGCAGCTTTTACCGGCGAAAC

CTCTCCCGATCAACTGAAAGAAATTGGTGTTAAAGTTGTAATGGTCGGTCAT

TCGGAACGTCGTCAATTTTTAGGAGAGTCTAACTTTTTTTGTAACGATAAAA

TCCGTTTTCTTTTAAAGAACGAATTTACAGTCCTTTATTGTGTTGGAGAAACT

CTCTCCGAAAGAGAATCCGGTAAAACATTGGAAGTACTGTCTTCTCAAATTA

GAGAAGGTTTAAAAGGAATTGATAGCGTATTTTTTTCCAATTTAATTCTGGC

TTATGAACCAGTTTGGGCCATTGGAACCGGAAAAGTTGCCACCCCCTCTCAA

GCCCAAGAA

pfkB (432 bp)

ACACCGGACGCAGAAAGAACGATGCTTACTCATTAGGAATTTCAATTACA

TTACAAAAATCAGATGTAGATTTAGAAAAACTAAAATCTTCTAGTATTTCTT

ATATCGAAGGTTATCTTTGGGATGGTCAAGGAACGAAAGAGGCTTCACTTTT

AACCATGGAGGAATCCAAGAAAAATGGTGTAAAAGTAGCTTATACTTATAG

TGATCCGTTTTGTGTAAATCGTTCCAGAGAGGACTTTATTCGTTTAACAAAA

GAATATTTTGATATCGTTTTTTGTAATACGGAAGAGGCGAAGGCTCTTTCTC

AAAGAGAAGATAAGTTGGAAGCTCTTAAGTTTATTTCTGGCCTTTCTGCTCT

TGTGTTTATGACTGATTCTGCAAACGGAGCTTACTTTGCAGAGAATGGAAAA

ATTTCTCACGTAGATGGG

mreA (435 bp)

GAAGGAGGATTTTTCTTTAAGATTTTTTCTAATCAAAAATTACTTTTGACCTT
TGGGGCTTTATTTTTAATTGCGAGTATAGTGATGGTGGGAATTCGAATTGCA
AGAGGAAGTAAAACATTAAGACAATATTATATACCTCTTGGAATTTTGGGA
ATCAGTTTAATTTCTGCGGTTGTAGTAATGAAAACCGTTCCGTTTCGAGAAA
ACCAAGTCATTCGATTGACCGCATTTTTAAATCCGGAGGAATTTAAACAAGG
TGCCGGTTATCATCTGAGAGCTTCTAAACCTGCGGTTGGATCGGGAAGATTT
TTCGGAAAAGGGCTGATGAATGCAGAAATGACCGAAGGAAGAATTCCACAC
GTGCCAGAATCCAGCACTGATTTTATTTTTGCTTCCTGGGCGGAACAAACCG
GGTTTTTAGGATCTGTATTT

caiB (402 bp)

GCTTTGTATGCGAGGGAAAAAACGGGAAAAGGACAAAAAATTTCCGTTTCT
ATGATGGATTCGTCATTGCCGTTTTTATCCTTGTATGGAGGGATTTACGGAG
CCACCGGAAAAAAATCCAGAAGGAGGAAATGAACTACTTTCTGGTAAATTAC
CTAATTATAATGTTTATCAAACGAAGGAGGGTCGTTGGGTAGCGTTAGGTGC
TCTTGAAGATATGTTTTTTAAAACTTTTTTACGACAGACTGGGTTGGATAAA
CATTTGGAAGAATTACCTGCAGAAGAAAAAAACTTCTCTAAATGGAAAGAA
ATACTTACTACCTATTTTTCTACAAAAACATTCGAAGATTTGAATGTTCTTTT
TGAAAACCAAGATTCTTGTCTAACTCCTGTAAAAACAATA

ST 37

glmU (444 bp)

CTGCTTTTACATGTACTCGATCATCTAAAAGGTTCTGGTGTAGAACGGATCG
TAGTAGTAGTAGGTTACAAAAAAGAATTGGTTCAGTCTCTTTGTTCCAAAAT
TCCAGGTGTTACTTTTGCCGAACAAAAAGAACAACTTGGAACTGCTCATGCT
CTTCTTTGCGCCGAAACGGAACTGAAAAATTTCCAAGGTTCTGTGATCGTTG
CCTGTGGAGACGTTCCAATGATTACTTCCGAAACGTTTTCTAATATAGTAAA
ACAACACAAAGAGAATGAATTTTCTGCAACGATCCTTTCTGCAGTCGTAGA
AAAACCTACAGGATATGGAAGAATTATCCGCAACTCCTCCGGTGAAGTAAC
AGCTATCGTAGAAGAAAAGATTCTTCTACCGAAGAAAAATTGATCAACGA
AATTAACACTGGAACCTATGTATTCGACGGA

pntA (525 bp)

GCGATGAATGCACAAACAGTTCAAAAACTTGCTTCTAAAAAGGTAGAAGTT
TTAAGTTTAGATGCAATCGCAAGGATTACGAGAGCCCAGTCTATGGACGTA
CTTTCTTCTCAGGCAACAGTTGCCGGTTACAAAGCGGTATTGCTCGCGGCTT
CTCATTTAGCCAGATTTTTTCCAATGCTTACTACGGCGGCTGGAACCATCAC
ACCTGCTTCCGTGTTGATTATCGGAGCCGGAGTTGCTGGATTACAAGCGATC
GCTTCCAGTAGAAGATTGGGCGCGGTAGTAGACGTATTCGATACAAGACCA
GAAGTAAAAGAGCAGGTTCATTCTCTTGGAGCGAAGTTTGTAGAAGTAGAA
GGTGCTTCCCATTCTGCTGCTGCTGGCGGTTATGCGGTTGAACAAACCGAAG
AATATAAAAAACGTCAACAAGAAGCGATCGATAAATACGCTCAAAAGGCG
GATGTAATCATAACAACTGCGCTCATTCCTGGAAAAAAAGCCCCATTATTGA
TCACTAAAAAG

sucA (447 bp)

GGAATGGCGCATAGGGGAAGACTGAATGTTCTCGTGAACATTATCGAAAAA

CCTGCGTCACTCATCTTTGCGGAGTTTGAAGAAAAACGGACAAAGATAAC

TTAAGTTATGCGGATGTAAAATACCATTTGGGGTATTCTAACAGTAGAATGA

CTACCTCTGGTAAAGAAGTGAAACTTTCACTCGCGTTCAACCCAAGTCATTT

AGAATGTGTGGATCCAGTGGTAACCGGATCGGTTCGTGCCCGTCAGACTTTG

ATCGGAGATAAGGATCGTTCCAAATATATGCCGATTTTGATTCATGGTGACG

CTGCGTTTGCGGGTCAGGGAGTTGTTGCAGAAACTCTCAACTTGATGAATTT

AGAAGGTTATACTACCGGTGGAACGTTTCATATTGTGGTCAATAATCAGATT

GGTTTTACCACTCTTCCCGATGAATCTAGATCT

tpiA (426 bp)

ACTCTTCATCTTGAAAACGTTTCCAAAATATTAGAAGGTAGCAGTGTAATTG

TTGGAGCCCAAAACTGTTATCACTCCGGACTTGCAGCTTTTACCGGCGAAAC

CTCTCCCGATCAACTGAAAGAAATTGGTGTTAAAGTTGTGATGGTCGGTCAT

TCGGAACGTCGTCAATTTTTAGGAGAGTCTAACTTTTTTTGTAACGATAAAA

TCCGTTTTCTTTTAAAGAACGAATTTACAGTCCTTTATTGTGTTGGAGAAACT

CTCTCCGAAAGAGAATCCGGTAAAACATTGGAAGTACTGTCTTCTCAAATTA

GAGAAGGTTTAAAAGGAATTGATAGCGTACTTTTTTCCAATTTAATTCTGGC

TTATGAACCAGTTTGGGCCATCGGAACCGGAAAAGTTGCCACCCCCTCTCAA

GCCCAAGAA

pfkB (432 bp)

ACACCGGATGCAGAAAGAACGATGCTTACTCATTTAGGAATTTCAATTACAT
TACAAAAATCAGATGTAGATTTGGAAAAACTAAAATCTTCTAGTATTTCTTA
TATCGAAGGTTATCTTTGGGATGGTCAAGGAACGAAAGAGGCTTCACTTTTA
ACCATGGAGGAATCCAAGAAAAATGGTGTAAAAGTAGCTTATACTTATAGT
GATCCGTTTTGTGTAAATCGTTCCAGAGAGGACTTTATTCGTTTAACAAAAG
AATATTTTGATATCGTTTTTTGTAATACGGAAGAGGCGAAGGCTCTTTCTCA
AAGAGAAGATAAGTTGGAAGCTCTTAAGTTTATTTCTGGCCTTTCTGCTCTT
GTGTTTATGACTGATTCCGCAAACGGAGCTTACTTTGCAGAGAATGGAAAA
ATTTCTCACGTAGATGGG

mreA (435 bp)

GAAGGAGGATTTTTCTTTAAGATTTTTTCTAATCAAAAATTACTTTTGACCTT
TGGGGCTTTATTTTTAATTGCGAGTATAGTGATGGTGGGAATTCGAATTGCA
AGAGGAAGTAAAACATTAAGACAATATTATATACCTCTTGGAATTTTGGGA
ATCAGTTTGATTTCTGCTGTTGTAGTAATGAAAACCGTTCCGTTTCGAGAAA
ACCAAGTCATTCGATTGACCGCATTTTTAAATCCGGAGGAATTTAAACAAGG
TGCCGGTTATCATCTGAGAGCTTCTAAACCTGCGGTTGGATCGGGAAGATTT
TTCGGAAAAGGGCTGATGAATGCAGAAATGACCGAAGGAAGAATTCCACAC
GTACCAGAATCCAGCACTGATTTTATTTTTGCTTCCTGGGCGGAACAAACCG
GGTTTTTAGGATCTGTATTT

caiB (402 bp)

GCTTTGTATGCGAGGGAAAAAACGGGAAAAGGACAAAAAATTTCCGTTTCT

ATGATGGATTCGTCATTGCCGTTTTTATCCTTGTATGGAGGGATTTACGGAG

CCACCGGAAAAAATCCAGAAGGAGGAAATGAACTACTTTCTGGTAAATTAC

CTAATTATAATGTTTATCAAACGAAGGAGGGTCGTTGGGTAGCGTTAGGTGC

TCTTGAAGATATGTTTTTTAAAACTTTTTTACGACAGACTGGGTTGGATAAA

CATTTGGAAGAATTACCTGCAGAAGAAAAAAACTTTTCTAAATGGAAAGAA

ATACTTACTACCTATTTTTCTACAAAAACATTCGAAGATTTGAATGTTCTTTT

TGAAAACCAAGATTCCTGTCTAACTCCTGTAAAAACAATA

APPENDIX V

Antibiotic Agents for Antimicrobial Susceptibility Test

Antibiotic discs
Ampicillin (AMP)
Chloramphenicol (CAM)
Sulfonamides (SMX)
Trimethoprim (TMP)
Doxycline (DOXY)
Penicilin G(PenG)

The Partial *ligB* Sequences Deposited in GenBank

LOCUS   Seq1   252 bp

DEFINITION   *Leptospira alexanderi* serovar Manhao *LigB* gene partial CDS.

ACCESSION:

   1   tcgttttaga atcgatagaa tgccttactt cggtggggag agtttggatg acgaatataa

  61   gggtcgcgta ggttcgacgg ctcgttttat tcgaactgtc gcggctagaa ataacggtaa

121   caaattggat cagcttggcg gttcgacagt caattggggc tattatgtgg gaatagattc

181   tcttttgtt tttaaggaaa aactttatgc cgcaaacggg ggatttccga attccttaca

241   taatggaagt at

LOCUS   Seq2   252 bp

DEFINITION   *Leptospira santarosai* serovar Canalzonae *LigB* gene partial CDS.

ACCESSION:

   1   tcgttttaga atcgatagaa tgccttactt cggcggggag agtttggacg aggaatataa

  61   aggtcgagta ggttctacgg ctcgttttat tcggacccctt gcggctaaaa ataacggtaa

121   taaaccgaat caacccggcg actcgacgat caattggggc tattatgtgg gaatcgattc

181   tctgtttgtt tttaagggaa aactttatgc cgcaaacggg ggatttccga attccttaca

241   taatggaagt

LOCUS   Seq3   252 bp

DEFINITION *Leptospira santarosai* serovar Shermani *LigB* gene partial CDS.

ACCESSION:

     1 tcgtttttaga atcgatagaa tgccttactt cggcggggag agtttggacg aggaatataa

    61 aggtcgagta ggttctacgg ctcgtttttat tcggacccctt gcggctaaaa ataacggtaa

   121 taaaccgaat caacccggcg actcgacgat caattggggc tattatgtgg gaatcgattc

   181 tctgtttgtt tttaagggaa aactttatgc cgcaaacggg ggatttccga attccttaca

   241 taatggaagt

LOCUS   Seq4   324 bp

DEFINITION *Leptospira alstonii* serovar Pingchang *LigB* gene partial CDS.

ACCESSION:

     1 tcgtttttaga atcgatagaa tgccttatttt cggcggaggg agcctggact tcactcctaa

    61 taatgcgaat ataaaacctc tccctcgaat gagcttggac ttcactccta ataatgcgaa

   121 tataaaacgt ctccctcgaa tgagtctggg cttagctccc gtcggcaaga ctcaagttga

   181 gaaaaaagtt aatagactga ataaactcaa caactcgtcc atcaactggg gttttttacgt

   241 gggagtggac tctctcttcg tgtttaacga aaaactttac gccgcaaacg gagggtttcc

   301 aaattcatta cataatggaa gt

LOCUS   Seq5   324 bp

DEFINITION *Leptospira alstonii* serovar Sichuan *LigB* gene partial CDS.

ACCESSION:

    1 tcgttttaga atcgatagaa tgccttattt cggcggaggg agcctggact tcactcctaa

   61 taatgcgaat ataaaacctc tccctcgaat gagcttggac ttcactccta ataatgcgaa

  121 tataaaacgt ctccctcgaa tgagtctggg cttagctccc gtcggcaaga ctcaagttga

  181 gaaaaaagtt aatagactga ataaactcaa caactcgtcc atcaactggg gtttttacgt

  241 gggagtggac tctctcttcg tgtttaacga aaaactttac gccgcaaacg gagggtttcc

  301 aaattcatta cataatggaa gtat