UNIVERSITI PUTRA MALAYSIA

ISOLATION, IDENTIFICATION, CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF LEPTOSPIRA SEROVARS IN RATS TRAPPED IN SELECTED AREAS OF KUALA LUMPUR

GHADA A HASOUN AL KATTAN

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By

GHADA A HASOUN AL KATTAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillments of the Requirements for the Degree of Doctor of Philosophy

August 2017

DEDICATION

Finally, a thesis for;
My ever extremely helpful and understanding husband,
Mr. Aqeel Mohammed Ridha
Who without his help, PhD would never have come to completion.
My late father and mother to them I am much indebted
My loving, helpful sisters who offered an endless stream of love and affection throughout my PhD journey.
My country (Iraq)
Who offered this opportunity to me to do PhD even there was hard situation of war
As well
To Iraqi army and all Iraqi forces who liberate my country from Terrorist invasion.

Abstract of thesis presented to the Senate of the Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Prof. Dato' Dr. Abdul Rani Bahaman, PhD
Faculty : Veterinary Medicine

Leptospirosis is a global zoonotic disease of humans and a wide range of domestic animals caused by pathogenic species of *Leptospira*. The genus *Leptospira* comprises 20 species; nine of them are pathogenic, five species as intermediate and the last six species as saprophytes. DNA-Based methods were used for classification of *Leptospira* besides, the use of Polymerase chain reaction assay (PCR) as an unconventional tool for identification and characterization. Leptospirosis has a significant impact on public health and livestock production. Rodents, particularly rats, have been described as the main source of infection to humans and animals. The importance of leptospirosis is that it is a zoonosis and a public health concern. It is also of great economic impact in Malaysia, since almost all rats are infected with *Leptospira spp*. and they shed the pathogenic leptospires to the environment, subsequently infecting humans and animals within the same zone. Conversely, there is not abundant evidence available about the renal carriage in rats from urban areas in Kuala Lumpur. That influences us towards finding new molecular approaches for early detection and diagnosis of the infection particularly through sensitive, specific and rapid molecular methods.

Prevalence of leptospirosis in rat populations from urban areas has been studied in this project through implementing molecular and classical techniques. The association between rat categories through Pearson Chi square and the agreement between results of tests through Cohen Kappa analysis were done in addition to identifying the predominant rat species which carry and disseminate the infection in the studied locations.

In this study, 112 rats were trapped and sacrificed from four locations (wet markets) in Kuala Lumpur, Malaysia where the poor hygiene renders the probability of infected rats with leptospirosis possible. Sera and both kidneys of each rat were collected and categorized by; species, gender and age which were studied. Serological and molecular detection were performed, using microscopic agglutination test (MAT) for anti-*Leptospira* anti-body detection in sera against 18 standard *Leptospira* strains and multiplex polymerase chain reaction (mPCR) for direct detection of *Leptospira* DNA in rat kidney tissues. Each test was evaluated with respect to its sensitivity and specificity ensuring that the recommendations made are valid to be used in a program for leptospirosis eradication in the country. Isolation of the *Leptospira* isolates from culturing of rat kidney tissues in a selective medium (EMJH) and then characterization and identification by classical and molecular methods were also done. Molecular characterization of recovered *Leptospira* isolates was achieved by PCR-Restriction fragment length polymorphism (PCR-RFLP) through digestion of PCR products amplified with *lipL*32 (819bp) and 16S *rRNA* (541bp) with restriction enzymes *Bam H*I and *Kpn*I respectively. Besides, constructing the tree for phylogenetic analysis of the obtained isolates by sequencing the *lipL*32 (819bp) region and 16S *rRNA* (541bp) region and the genetic relationships were determined.

The MAT revealed 8 serovars infected 63/112 of the sampled rats; *L. borgpetersenii* serovars Javanica and *L. interrogans* serovars; Bataviae, Icterohaemorrhagiae, Canicola, Pomona, Australis, Andamana and Patoc. While, mPCR showed 56 DNA samples were positive to both genes (16S *rRNA, lipL*32 genes) and only 4 DNA samples positive to 16S *rRNA.* Study of seroprevalence association based on the mentioned categories revealed that; *Rattus rattus* and *Rattus norvegicus* were the predominant rat species with no statistical significance ($\chi 2 = 0.490^{a}$; *P*=0. 484) between both species. Though infected males of rats were more infected than females, ($\chi 2 = 1.765^{a}$; *P*=0. 184), this was not statistically significant. Adult infected rats were more than sub adults and the difference was statistically significant ($\chi 2 = 6.748^{a}$; *P*=0.009).

Cultures of the tissues from rat kidneys were examined by dark field microscopy and found 57/ 112 positive. Identification of the isolates using 8-azaguanine, 1M NaCl and Trypticase soy broth indicated 40 *Leptospira* isolates were pathogenic whereas, 17 *Leptospira* isolates were non-pathogenic. Detection of *Leptospira* DNA in 112 kidney cultures by PCR showed 50 isolates were pathogenic and positive to PCR-*lipL*21 and G1/G2 genes. The sensitivity of isolation was 70.2% in comparison with PCR whilst, the specificity was 81.8%. Typing of isolates by MAT against 16 rabbit hyperimmune anti sera revealed 13/57 isolates belong to *L. interrogans* serovar Bataviae and 17/57 were serovar Javanica belonging to *L. Borgpetersenii*.

Characterization of isolates by Restriction fragment length poly morphism PCR-RFLP of *lipL*32 (819 bp) generated two different patterns on agarose gel that differentiated between *L. interrogans* and *L. borgpetersenii* species. On the other hand, phylogenetic analysis showed horizontal transfer of ribosomal gene through sequencing the 16S *rRNA* gene in serovar Hardjo belonging to both pathogenic *Leptospira* species. Sequencing of *lipL*32 gene showed the genetic nature of isolates that belonged to *L. interrogans* and *L. borgpetersenii spp.* but different in serovars which was revealed by MAT. Combinations of serologic analysis namely MAT and the DNA-based methods particularly PCR and mPCR (16S *rRNA, lipL*32 genes) showed promising results. This combination can be used for rapid, sensitive detection of leptospirosis in humans and animals. Assays of recovered isolates with PCR-RFLP of *lipL*32 is valuable for discriminatory needs. Phylogenetic analysis is decisive in findings of isolate relationships and heterogeneity among serovars circulated in the rat populations. It appears that leptospirosis is endemic in Malaysia and rats are the main source of infection. Although multi-approaches are needed for detection and characterization of *Leptospira spp*., PCR-based methods are rapid, sensitive and accurate. Further studies using advanced molecular methods are required for early detection and diagnosis of leptospirosis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN, PENGENALPASTIAN, PENYIFATAN DAN ANALISIS FILOGENETIK SEROVAR LEPTOSPIRA DALAM TIKUS DIPERANGKAP DI KAWASAN-KAWASAN TERPILIH KUALA LUMPUR

Oleh

GHADA A HASOUN AL KATTAN

Ogos 2017

Pengerusi : Prof. Dato 'Dr. Abdul Rani Bahaman, PhD
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Leptospirosis adalah penyakit zoonosis global manusia dan pelbagai jenis haiwan domestic yang disebabkan oleh spesies patogenik *Leptospira.* Genus *Leptospira* terdiri daripada 20 spesies; sembilan daripada mereka patogenik, lima spesies sebagai perantaraan dan enam spesies terakhir sebagai saprofit. Kaedah berasaskan-DNA telah digunakan untuk pengelasan *Leptospira* selain penggunaan cerakin tindak balas rantai Polimeras (PCR) sebagai alat tidak konvensional untuk pengenalpastian dan penyifatan. Leptospirosis mempunyai kesan yang besar terhadap kesihatan awam dan pengeluaran ternakan. Rodensia, terutamanya tikus, telah digambarkan sebagai sumber utama jangkitan kepada manusia dan haiwan. Kepentingan leptospirosis adalah bahawa ianya merupakan zoonosis dan merupakan kebimbangan kesihatan awam. Ia juga mempunyai kesan ekonomi yang besar di Malaysia, kerana hampir semua tikus dijangkiti *Leptospira spp.* dan mereka menggugurkan leptospires patogenik kepada alam sekitar, seterusnya menjangkiti manusia dan haiwan di dalam zon yang sama. Sebaliknya, tidak banyak bukti yang boleh didapati mengenai pengangkutan buah pinggang pada tikus dari kawasan bandar di Kuala Lumpur. Ini mempengaruhi kami ke arah mencari pendekatan molekul baru untuk pengesanan dan diagnosis awal jangkitan terutamanya melalui kaedah molekul yang sensitif, spesifik dan pantas.
Kekerapan leptospirosis di kalangan populasi tikus dari kawasan bandar telah dikaji di dalam projek ini melalui pelaksanaan teknik molekul dan klasik. Hubungan antara kategori tikus melalui Khi Kuasa Dua Pearson dan persetujuan antara keputusan ujian-ujian melalui analisis Cohen Kappa dilakukan selain daripada mengenal pasti spesies tikus utama yang membawa dan menyebarkan jangkitan tersebut di lokasi yang dikaji.

Dalam kajian ini, 112 tikus telah terperangkap dan dikorbankan dari empat lokasi (pasar basah) di Kuala Lumpur, Malaysia di mana kebersihan yang tidak baik menyebabkan kebarangkalian tikus dijangkiti leptospirosis menjadi mungkin. Serum-serum dan kedua-dua ginjal setiap tikus dikumpulkan dan dikategorikan menurut spesies, jantina dan umur yang telah dikaji. Pengesanan serologi dan molekul telah dilakukan, dengan menggunakan ujian pengaglutinatan mikroskop (MAT) untuk pengesanan antibodi anti-*Leptospira* di dalam serum-serum 18 jenis *Leptospira* standard dan tindak balas rantai polymerase multipleks (mPCR) untuk pengesanan langsung DNA *Leptospira* di dalam tisu ginjal tikus. Setiap ujian dinilai dari segi sensitiviti dan spesifiknya untuk memastikan bahawa syor-syor yang dibuat adalah sah untuk digunakan di dalam sesuatu program untuk pembasmian leptospirosis di negara ini. Pemencilan hasil dari pencilan *Leptospira* dari pengkulturan tisu ginjal tikus di dalam medium terpilih (EMJH) dan kemudiannya penyifatan dan pengenalpastian dengan kaedah-kaedah klasik dan molekul juga telah dilakukan. Penyifatan molekul pencilan *Leptospira* yang didapati telah dicapai dengan PCR-polimorfisme batasan kepanjangan serpihan (PCR-RFLP) melalui penghadaman produk-produk PCR dikuatkan dengan *lipL*32 (819bp) dan 16S *rRNA* (541bp) dengan enzim batasan *Bam H*I dan *Kpn*I masing-masing. Selain itu, membina pokok untuk analisis filogenetik pencilan yang diperolehi oleh penjujukan kawasan *lipL*32 (819bp) dan kawasan 16S *rRNA* (541bp) serta hubungan-hubungan genetik ditentukan.

MAT menunjukkan 8 serovar menjangkiti 63/112 daripada tikus yang disampel; serovar *L. borgpetersenii* Javanica dan serovar *L. interrogans*; Bataviae, Icterohaemorrhagiae, Canicola, Pomona, Australis, Andamana dan Patoc. Manakala, mPCR menunjukkan 56 sampel DNA adalah positif kepada kedua-dua gen (gen-gen 16S *rRNA, lipL*32) dan hanya 4 sampel DNA positif kepada 16S *rRNA.* Kajian hubungan seroprevalens berdasarkan kategori yang disebutkan menunjukkan bahawa; *Rattus rattus* dan *Rattus norvegicus* adalah spesies tikus utama tanpa kepentingan statistik ($\chi 2 = 0.490^{a}$; $P = 0.484$) antara kedua-dua spesies. Walaupun tikus jantan yang dijangkiti lebih dijangkiti berbanding yang betina, ($\chi 2 = 1.765^{a}$; $P = 0.184$), ini tidaklah penting dari segi statistik. Tikus dewasa yang dijangkiti lebih daripada sub dewasa dan perbezaan secara statistik adalah signifikan ($\chi 2 = 6.748^{a}$; $P = 0.009$).

Kultur tisu dari ginjal tikus diperiksa oleh mikroskopi medan gelap dan mendapati 57/112 positif. Pengenalpastian pencilan menggunakan 8-azaguanine, 1M NaCl dan Trypticase air rebusan soya menunjukkan 40 pencilan *Leptospira* adalah patogenik manakala, 17 pencilan *Leptospira* adalah bukan-patogenik. Pengesanan DNA *Leptospira* di 112 kultur ginjal dengan PCR menunjukkan 50 pencilan adalah patogenik dan positif kepada PCR-*lipL*21 dan gen-gen G1/G2. Kepekaan pemencilan adalah 70.2% berbanding dengan PCR manakala, spesifiknya adalah 81.8%. Penjenisan pencilan oleh MAT terhadap 16 anti-serum hiperimun arnab menunjukkan 13/57 pencilan adalah milik

serovar Bataviae *L. interrogans* dan 17/57 adalah serovar Javanica milik *L. Borgpetersenii*. Penyifatan pencilan oleh poli morfisme batasan kepanjangan serpihan PCR-RFLP daripada *lipL*32 (819 bp) menjana dua corak yang berbeza pada gel agarose yang membezakan antara spesies *L. interrogans* dan *L. borgpetersenii* . Sebaliknya, analisis filogenetik menunjukkan pemindahan mendatar gen ribosom melalui penjujukan gen 16S *rRNA* dalam serovar Hardjo yang dimiliki kedua-dua spesies *Leptospira* patogenik. Penjujukan gen *lipL*32 menunjukkan sifat genetik pencilan kepunyaan *L. interrogans* dan *L. borgpetersenii spp.* tetapi berbeza dalam serovar-serovar yang ditunjukkan oleh MAT. Gabungan analisis serologi iaitu MAT dan kaedah-kaedah berasaskan-DNA khususnya PCR dan mPCR (gen-gen 16S *rRNA, lipL*32) menunjukkan hasil yang menggalakkan. Gabungan ini boleh digunakan untuk pengesanan leptospirosis yang pantas dan sensitif bagi manusia dan haiwan. Cerakin untuk pencilan yang didapati dengan PCR-RFLP daripada *lipL*32 adalah tinggi nilainya bagi keperluan diskriminasi. Analisis filogenetik adalah tegas dalam dapatan mengenai hubungan pencilan dan keheterogenan di kalangan serovar yang beredar dalam populasi tikus. Nampaknya leptospirosis adalah endemik di Malaysia dan tikus merupakan punca utama jangkitan. Walaupun pendekatan-pelbagai diperlukan untuk pengesanan dan penyifatan *Leptospira spp.,* kaedah-kaedah berasaskan-PCR adalah pantas, sensitif dan tepat. Kajian-kajian lanjut menggunakan kaedah molekul yang lebih maju diperlukan untuk pengesanan awal dan diagnosis leptospirosis.

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I certify that a Thesis Examination Committee has met on 21/2/2014 to conduct the final examination of Heshu Sulaiman Rahman on her thesis entitled "**ANTI-LEUKEMIC EFFECTS OF ZERUMBONE NANOPARTICLE ON HUMAN JURKAT T LYMPHOBLASTOID CELL LINES IN VITRO AND MURINE LEUKEMIC WEHI-3B MODEL IN VIVO**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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Name of Chairman of Supervisory Committee: Professor Dr. Abdul Rani Bahaman

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Name of Member of Supervisory Committee: Associate Professor Dr. Zunita Zakaria

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Abs	Antibodies
Ags	Antigens
Blast	Basic Local Alignment Tool
Bp	Base pair
BSA	Bovine Serum Albumin
Cell/ml	Cell per milliliter
CAAT	Cross Agglutination Absorption Test
°C	Degree Celsius
DFM	Dark Field Microscopy
DNA	Deoxy Ribonucleic Acid
EDTA	Ethelen Diamine Tetra Acetone
Et al .,	And others
EMJH	Ellinghausen McCullough Johnson and Harris
H	Hour
K	Kidney
KL	Kuala Lumpur
L	Leptospira
LPS	Lipo Poly Saccharides
M	Molar
MAT	Microscopic Agglutination Test
M	Marker
MEGA	Molecular Evolutionary Genetics Analysis
Ml	Milliliter
µl/well	Microliter/well

Mg	Milligram
µg	Microgram
µg/ml	Microgram per milliliter
Min	Minute
mPCR	Multiplex Polymerase Chain Reaction
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
OmpL	Outer membrane protein of *Leptospira*
pH	Hydrogen-ion concentration
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
%	Percent
RE	Restriction Enzyme
Rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid
D water	Distilled water
Sec	Second
Spp.	Species
TBE	Tris-Borate buffer
V	Volt
Vol/vol	Volume per volume
Xg	x gravity; measure of centrifuge force

CHAPTER 1

INTRODUCTION

Leptospirosis is a transmissible disease of humans and animals caused through infection with *Leptospira spp.* (Adler& de la Peña Moctezuma, 2010a; Adler, 2015b). It is considered as re-emerging infection that reported by outbreaks in both developing and developed countries including the United States, countries of Latin America and Southeast Asia (Hartskeerl *et al*., 2011; Zhang *et al.*, 2011). Leptospirosis is a zoonotic infection, spread through wide range of domestic mammals and rodents. Leptospires are shed through the urine of the carrier animals which eventually contaminate the environment (Adler& de la Peña Moctezuma, 2010a; Adler, 2015b). Thus, have an occupational risk infection to the farmers, workers; slaughter house workers, butchers, rice field workers and veterinarian.

Leptospirosis has important economic impact on livestock's productivity. Acute leptospirosis is characterized by sudden onset of agalactia (in lactating cows and ewes); jaundice and haemoglobinuria in young animals which may lead to death. Chronic leptospirosis could be diagnosed in cases of abortion, dystocia, still birth and birth of weak or premature fetus and infertility (Levtt, 2001). Human leptospirosis appears as influenza like in mild cases, whilst the severe cases manifests through kidney and liver failure, pulmonary hemorrhage and death (Faine *et al*., 1999). Human's contracts the infection directly through urine or body fluids of infected animals or indirectly through urine contaminated materials, soil and water (Blackwell, 2014).

Leptospirosis is recorded to be endemic in Malaysia since four decades ago. Several outbreaks has occurred exemplifying by the EcoChallenge Sabah 2000 competition, the Lubuk Yu outbreak with co infection of Melioidosis and the recent cases which result from flooding (Bahaman *et al*., 2002; Sapian *et al*., 2012). Moreover, number of human cases has been on increase (Pui *et al*., 2015; Suut *et al*., 2016). Floods, alkaline pH of soil, mild weather and stagnant water are predisposing factors for leptospiral organism's survival. In urban areas, the outdoor recreational activities for example swimming besides, existence of stray dogs and wild rat increases the cases of leptospirosis particularly, in slums where the hygiene is poor (Victoriano *et al.,* 2009). Rats represent main source of leptospirosis (Faine *et al*., 1999; Himsworth *et al*., 2013; Kosoy, 2015).

In recent years, development of capitals and towns has created proper environment for rats to arise within human residency as these rats feed on human food (Davis, 2005; Zain *et al*., 2012; Haake & Levett, 2015). Rats are generally found living closely to human habitation and nurturing on human wastes of food, water and space for nesting their shelters (Tung *et al*., 2013;

Fang *et al*., 2014). Rats serve as renal carrier; reservoirs or maintenance host. Leptospires colonizes renal tubules of infected host with long period persistent and consistently being shed in the urine thereby contaminating the environment (Nakamura *et al*., 2013; Costa *et al*., 2014). The infection can transmit directly or indirectly to humans and domestic animals (Schoonman and Swai, 2010).

Leptospirosis continues to be a major health challenge in Malaysia. The impacts of the infection on the economic well-being are evidenced cost of medication and low livestock productivity. It is a significant public health problem as it is a zoonotic infection. In addition, it has occupational and recreational related risk.

To date, the prevalence of leptospirosis in Malaysian urban area is not yet to be comprehensively studied. Besides, there is need to investigate the role of the rats in spreading of leptospires specifically in urban settings. Collection of relevant data on *Leptospira* infection incidence rate and serovars circulating among urban rat population is essentially. Particularly, to understand the role of rats as host and the potential risk they pose to both human and susceptible animals inhabiting urban environment. Recently, in Malaysia (Mohamed-Hassan *et al.*, 2010; Ridzlan *et al.*, 2010; Shafei *et al.*, 2012; Benacer *et al.*, 2013; Thayaparan *et al.*, 2015; Azali *et al*., 2016) reported several serovars isolated from animal specimens, water, soil and humans related to events of floods. Lately, the rate of human leptospirosis incidence was estimated to be 2-5 per 100,000 in a population. Many human fatal cases were reported in the last four years in some areas in Malaysia for example Kedah during July 2011 and in Sungai Siput during March 2012 (Thayaparan *et al*., 2013a). Misdiagnosed case of human leptospirosis with febrile illness causes delay in treatment and control of leptospirosis. Therefore, laboratory decision and rapid diagnosis are significant for effective therapy and epidemiological surveillance. Economic losses in livestock's and possibility to human contacting leptospirosis stressed the importance of prompet, sensitive analytical tools that can assist in proposing effective treatment and control.

Currently, detection of small number of leptospires in the clinical specimens is possible within a short period of time through use of Polymerase chain reaction (PCR) assay (Merien *et al*., 1992). Then, use of Multiplex polymerase chain reaction (mPCR) assay for detection and identification of leptospires in different specimens through use of two sets of primers including genus specific and pathogenic primer provides high sensitive and specific tool for rapid diagnosis of *Leptospira* infection (Haake*,* 2000; Ahmed *et al.,* 2012c). Developing PCR- Restriction fragment length polymorphis (PCR-RFLP) for characterization of *Leptospira spp.* through unconventional technique has focused on specific DNA amplification by PCR. This method has been established to be appropriately sensitive and rapid to practice for the detection and characterization of pathogen in different specimens (Ou *et al*., 1988). Phylogenetic analysis assist in classification of *Leptospira spp.* in three major

groups, based on the pathogenicity such as; pathogenic, saprophytic and intermediate strains of unclear pathogenicity (Matthias *et al*., 2008).

Even though molecular methods offer sensitivity, specificity and time, still this study to prevalence of leptospirosis in rats has some limitations. The limitations represented by difficulty of wild rats trapping and handling, high probability of contracting infection besides, high contamination of collected organs which require accurate procedures for purification of tissues from another contaminants then, culturing and isolating of *Leptospira ssp*. Isolation of leptospires is a hazardus, complex and expencive procedure which lasted more than three months. Besides, isolation requires expert laboratory technichian to do.

Since almost all rats are infected with *Leptospira spp.* and they are the main source of leptospirosis to humans and animals. On the other hand, there is not much information available about the renal carriage in rats from poor hygiene urban areas in the capital Kuala Lumpur. This study aimed at using sensitive, specific and rapid molecular methods for detection and characterization of the circulating *Leptospira* serovars from rat populations in urban of Kuala Lumpur.

Objectives of this study are:

1. To detect *Leptospira spp.* infection in the rat populations in Kuala Lumpur using mPCR and MAT.
2. To isolate and identify the pathogenic *Leptospira* strains using conventional and molecular methods.
3. To characterize the *Leptospira* isolates using molecular methods; Restriction fragment length polymorphism RFLP and phylogenetic analysis.

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