UNIVERSITI PUTRA MALAYSIA

DETECTION OF LEPTOSPIRAL DNA IN RAT KIDNEY AND ENVIRONMENTAL SAMPLES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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DETECTION OF LEPTOSPIRAL DNA IN RAT KIDNEY AND ENVIRONMENTAL SAMPLES USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

By

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Leptospirosis is regarded as one of the most widespread zoonotic disease which is caused by the pathogenic *Leptospira spp*. Rodents are important reservoirs for human leptospirosis while environmental samples plays an important role as source of infection to human and animals. A rapid and robust detection of *Leptospira* is essential to reduce avoidable leptospirosis death substantially. Hence, this study aims to detect the presence of *Leptospira* targeting *secY* gene in rat kidney and environment samples using loop-mediated isothermal amplification (LAMP). Rat kidney samples were obtained from leptospirosis outbreak areas and hot spot area while environmental samples were obtained from leptospirosis outbreak areas. A total of 150 rat kidney samples and 46 environment samples consisting of 22 water samples and 24 soil samples were used in this study. DNA extraction was performed in rat kidney samples using commercially available extraction kit while direct boiling method was used for environment samples. All isolated DNA samples were subjected to LAMP assay targeting *secY* gene which was performed for 30 minutes at 65°C. PCR was performed on all isolated DNA samples using LAMP outermost primers (F3 and B3) to compare the analytical sensitivity of these two nucleic acid detection methods. The presence of *secY* gene was confirmed by sequencing on selected PCR positive samples. Leptospiral DNA was detected from 56 out of 150 (37.3%) rat kidney samples using LAMP while 28 out of 150 (18.7%) were detected using PCR. For environment samples, leptospiral DNA was detected from 4 out of 22 (18.9%) water samples and 14 out of 24 (58.3%) soil samples using LAMP and none of the environment samples were detected positive using PCR. LAMP system targeting *secY* gene showed higher detection rate compared to PCR in rat kidneys particularly for environment samples. The *Bst* DNA polymerase used in the LAMP system was reported to be more tolerable to inhibitors than *Taq* DNA polymerase used

in PCR. LAMP also showed to have better sensitivity compare to PCR and these may explain the higher detection rate in LAMP compared to PCR in this study. The LAMP assay was further modified to investigate the stability of the premixed LAMP stored at room temperature. The premixed LAMP was stable up to 45 days without losing the activity when stored at room temperature in the presence of sucrose. In conclusion, LAMP offered better detection compared to PCR for its simplicity, rapidity and higher detection rate. The addition of sucrose in reaction mixture allowed the premixed reagent to be stored at room temperature which is helpful in field testing during outbreak where cold storage is not available.

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PENGESANAN LEPTOSPIRAL DNA DI BUAH PINGGAN TIKUS DAN SAMPEL ALAM SEKITAR DENGAN MENGGUNAKAN AMPLIFIKASI ISOTERMA DENGAN PENGANTARAN GELUNG

Oleh

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Leptospirosis dianggap sebagai salah satu penyakit zoonosis yang berleluasa. Penyakit ini disebabkan oleh *Leptospira spp.* patogenik. Tikus adalah reservoir penting bagi leptospirosis manusia manakala sampel alam sekitar memainkan peranan penting sebagai sumber jangkitan kepada manusia dan haiwan. Pengesanan pantas dan teguh *Leptospira* adalah penting bagi mengurangkan kematian leptospirosis secara ketara. Oleh itu, kajian ini bertujuan untuk mengesan kehadiran *Leptospira* yang menyasarkan gen *secY* dalam sampel buah pinggang tikus dan persekitaran menggunakan amplifikasi isotermal dengan pengantaran gelung (LAMP). Sampel buah pinggan tikus didapati dari kawasan wabak leptospirosis dan tempat yang lazim dikunjingi tikus. Sejumlah 150 sampel buah pinggang tikus dan 46 sampel persekitaran yang terdiri daripada 22 sampel air dan 24 sampel tanah telah digunakan dalam kajian ini. Pengekstrakan DNA dilakukan pada sampel buah pinggang tikus menggunakan kit pengekstrakan komersial sementara kaedah mendidih digunakan untuk sampel persekitaran. Semua sampel DNA diuji dengan reaksi LAMP yang menyasarkan gen *secY* dengan inkubasi selama 30 minit pada 65^{o}C. PCR dilakukan ke atas semua sampel DNA dengan menggunakan primer luaran LAMP (F3 dan B3) untuk membandingkan kepekaan analisis kedua-dua kaedah pengesanan asid nukleik. Gen *secY* disahkan dengan penjujukan DNA diatas sample positif PCR. DNA Leptospiral dikesan dari 56 daripada 150 (37.3%) sampel buah tikus menggunakan LAMP sementara 28 daripada 150 (18.9%) dikesan menggunakan PCR. Bagi sampel persekitaran, DNA leptospiral dikesan dari 4 daripada 22 (18.9%) sampel air dan 14 daripada 24 (58.3%) sampel tanah menggunakan LAMP dan tiada sampel persekitaran yang dikesan positif menggunakan PCR. Sistem LAMP yang menyasarkan gen *secY* menunjukkan kadar pengesanan yang lebih tinggi berbanding dengan PCR

dalam buah pinggang tikus dan terutamanya untuk sampel persekitaran. Polimerase DNA *Bst* yang digunakan dalam sistem LAMP dilaporkan lebih tahan dengan kehadiran perencat berbanding dengan polimerase *Taq* DNA yang digunakan dalam PCR. LAMP juga menunjukkan kepekaan yang lebih baik berbanding dengan PCR dan ini mungkin menjelaskan kadar pengesanan yang lebih tinggi dalam LAMP berbanding PCR. LAMP diubah lagi untuk menyiasat kestabilan premix LAMP yang disimpan pada suhu bilik. Premix LAMP stabil hingga 45 hari tanpa kehilangan aktiviti apabila disimpan pada suhu bilik dengan kehadiran sukrosa. Sebagai kesimpulan, LAMP menawarkan pengesanan yang lebih baik berbanding dengan PCR kerana kesederhanaan, kepantasan dan kadar pengesanan yang lebih tinggi. Penambahan sukrosa dalam ujian LAMP membolehkan ujian disimpan pada suhu bilik serta membantu dalam ujian lapangan semasa wabak di mana penyimpanan sejuk tidak didapati.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
B3	Backward outer primer
BIP	Backward inner primer
Bst	Bacillus stearothermophilus
BLAST	basic local alignment search tool
dNTPs	Nucleotide triphosphate
EMJH	Ellinghausen-McCullough-Johnson-Harris
F3	Forward outer primer
FIP	Forward inner primer
DFM	Dark field microscopy
DNA	Deoxyribonucleic acid
HNB	Hydroxy napthol blue
IM	Inner membrane
LAMP	Loop-mediated isothermal amplification
LB	Loop backward
LEG	leptospirosis burden epidemiology reference group
LF	Loop forward
LPS	lipopolysaccharide
MAT	Microscopic agglutination test
mM	millimolar
Mg^{2+}	Magnesium ions
$Mg_2P_2O_7$	Magnesium pyrophosphate
NCBI	National Center for Biotechnology Information
ng	nanogram
OM	Outer membrane

PCR	Polymerase chain reaction
PF	Periplasmic flagella
PG	Peptidolycan layer
qPCR	Real-time polymerase chain reaction
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of study

Leptospirosis or more generally known as “rat urine disease” is an infectious disease provoked by spirochetes that belongs to the genus of *Leptospira sp.* (Chai, Lesley, Su, & Chong, 2017). *Leptospira* are divided into three groups including pathogenic, non-pathogenic and intermediate group that differ in pathogenicity (Adler & Moctezuma, 2010). Pathogenic *Leptospira* is responsible for causing disease to human varying from sub-clinical to severe disease such as respiratory distress, renal failure and may lead to mortality (Chiriboga et al., 2015). As for reservoir animals such as rat, pathogenic *Leptospira* may not cause the rat to exhibit disease however the rat may experience long-term colonization which plays a significant role in transmission of leptospirosis to human (Fouts et al., 2016). In contrast, non-pathogenic *Leptospira* are free-living environment microorganisms and do not cause disease while for intermediate *Leptospira*, although it is not well-characterized however several studies have showed that intermediate group may also causes mild to severe disease to human (Matthias et al., 2008; Tsuboi et al., 2017; Zakeri et al., 2010) and causes *Leptospira* renal colonization in animals (Fouts et al., 2016).

In Malaysia, leptospirosis in a re-emerging disease that affect both human and animals causing in mortality and morbidity (Tilahun, Reta, & Simenew, 2013). Transmission of leptospirosis occurs through direct contact with urine of infected animals or indirect contact of environmental water and soil contaminated with leptospires (Evangelista & Coburn, 2010). The pathogenic leptospires are usually excreted from the colonized renal tubule of the rat and shed through rat urine into the environment (Wojcik-Fatla et al., 2014). Majority of the human infection have been attributed by rats and other rodent species. Rapid urbanization of cities and improper waste management have created a suitable environment favoring the presence of rodents. As a result, the spreading of rats in human populations have subsequently increased the risk of leptospirosis infection in human (Tilahun et al., 2013). Leptospirosis encompassed a wide range of clinical symptoms from mild symptoms such as fever, headache and vomiting to severe signs of multi-organ dysfunction. Due to the overlapping of clinical presentations of febrile illness with other diseases such as dengue, malaria and arboviral disease, it is hard to predict leptospirosis based on clinical signs and symptoms in infected patients (WHO, 2003).

The lack of appropriate laboratory diagnostic has led to an urgent need to develop a rapid and highly sensitive detection tool to detect the presence of *Leptopsira spp.* in order to control the transmission of leptospirosis

(Thayaparan et al., 2013). Bacteria culture which is the gold standard for detecting *Leptospira spp*. was found to be very challenging as leptospires may take up to several weeks to growth and it may also overgrowth with co-existing microorganisms (Saito et al., 2013). Apart from bacterial culture, microscopic agglutination test (MAT) is also considered as a "gold standard" for diagnosis of leptospirosis. To date, MAT remains one of the reference serological method used to detect *Leptospira* however this technique is laborious, time consuming and required highly trained personnel for preservation of panel *Leptospira* live cultures (Monahan, Miller, & Nally, 2009). On the other hand, nucleic acid-based methods have significantly shortened turnaround time for pathogen identification and detection. Polymerase chain reaction (PCR) and real-time PCR (qPCR) are among the most common molecular technique for detecting *Leptospira* as it allows rapid diagnosis. However, PCR are known to have low sensitivity while qPCR requires sophisticated instrument which is not readily available in many laboratories although this technique is known to have a high sensitivity (Lin, Chen, Lu, Yan, & Yan, 2009). Therefore, it has been a timely need to establish an economical, less sophisticated testing methods with higher sensitivity and specificity (Yang et al., 2010).

In 2000, Notomi et al., reported a molecular technique termed as loop-mediated isothermal amplification (LAMP) which employed DNA polymerase and a set of four primers binding to six distinct regions on the target DNA which enables LAMP reaction to be highly specific. In addition, the amplification time of LAMP reaction can be further reduced using loop primers which provides a rapid molecular diagnostic tool as the test can be done in less than an hour (Nagamine, Hase, & Notomi, 2002). The use of *Bst* DNA polymerase with high displacement activity allowed LAMP reaction to be carried out in isothermal condition (Notomi et al., 2000). To date, LAMP has become a forerunner in molecular diagnostic technology and has been widely applied in many fields (Barkway, Pocock, Vrba, & Blake, 2011; Batra et al., 2015).

A previous study by Shuhaidah et al (2019) reported a developed LAMP system for detection of leptospiral DNA targeting *secY* gene in clinical samples of patient suspected with leptospirosis (Shuhaidah, 2018). The present study strives to fully utilized the potential of the developed LAMP system by testing on various types of samples. Rats were known to be the primary reservoir host of *Leptospira* (Sumanta et al., 2015) while environmental water and soil are recognized as an important source of leptospirosis infection (Benacer et al., 2013). A retrospective study was conducted in Malaysia and found that most of the leptospirosis cases in human are mostly those who come in contact with rodents as well as related to their leisure activities such as swimming (Garba et al., 2017). Detection of reservoir animals particularly rat and environmental water and soil samples is crucial to reduce the risk of transmission through animals reservoirs and environmental sample (Chai et al., 2015; Paixão et al., 2014) Henceforth, the present study aims to detect leptospiral DNA in rat kidney and environmental water and soil samples by targeting *secY* gene of *Leptospira* using LAMP.

1.2 Objectives

1.2.1 General Objective

To detect the presence of leptospiral DNA targeting *secY* gene in rat kidney and environmental samples using loop-mediated isothermal amplification (LAMP).

1.2.2 Specific Objective

1) To detect the presence of leptospiral DNA targeting *secY* gene in rat kidney samples using LAMP and PCR.
2) To detect leptospiral DNA targeting *secY* gene in water and soil using LAMP and PCR.
3) To compare the rate of detection of leptospiral DNA targeting *secY* gene using LAMP and PCR.
4) To investigate the stability of premixed LAMP reaction mixture stored in room temperature.

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