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DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR A SIMPLE AND RAPID DETECTION OF LEPTOSPIRAL DNA IN HUMAN SAMPLES

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By

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Leptospirosis is a zoonotic disease caused by bacteria of genus *Leptospira* that affects both humans and animals worldwide. Early detection of leptospirosis is crucial to provide appropriate treatment and control the progression of the disease to a severe state. Hence, this study aims to develop a simple and rapid LAMP system for detection of *Leptospira* in suspected leptospirosis patients. LAMP primer set was specifically designed to target the *secY* gene of *Leptospira*. Recombinant plasmid containing the target gene was constructed and used as template in optimization procedure. Optimization of the LAMP reaction was done on the incubation temperature and reagents concentration. It was found that the designed LAMP primer set works best at 65°C and optimum concentration of betaine and $MgSO_4$ was optimized at 0.4 M and 8 mM respectively. Sensitivity of the LAMP system was assessed based on the lower limit of detection using serially diluted genomic DNA of *Leptospira interrogans* serovar Pomona. The results showed that as low as 2×10^4 copies of genomic DNA per reaction (equivalent to 0.1 ng) could be detected within 40 min of reaction. The specificity of the system was tested by using DNA extracted from 15 leptospiral and nine non-leptospiral bacteria. None of the non-leptospiral DNA was amplified in the reaction indicating a highly specific system. Spiking assay was performed to mimic the clinical situation for determining the clinical sensitivity of the LAMP system. Pure culture of *L. interrogans* serovar Pomona were spiked into blood and urine samples donated by healthy donors. In spiked blood samples, 1×10^2 leptospires/ml was found to be the detection limit. Two methods of DNA isolation from spiked urine samples; column purification and direct boiling were performed to compare the efficiency. It was observed that samples from column purification results in higher LAMP amplification rate and more sensitive compared to direct boiling where the detection limit was found to be 1×10^2 leptospires/ml and 1×10^3 leptospires/ml respectively. As a proof-of-concept, the optimized LAMP system was performed on samples of blood and urine from suspected leptospirosis patients. The results were compared with conventional *secY* polymerase chain reaction (PCR). In 30 min LAMP reaction, 28 of 69 blood samples collected during admission was found to be positive. Of these, only 26 samples were able to be detected

using PCR. As for admission urine samples, of 34 tested, 16 were positive by LAMP against 14 for PCR. These positive urine samples include those collected as early as on the third day of clinical symptoms onset suggesting urine could be use as well for diagnosis of leptospirosis apart of using blood samples during admission. In conclusion, the developed LAMP system can serve as an alternative rapid diagnosis of leptospirosis considering its robustness, sensitivity and specificity as demonstrated in this study.

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sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMBANGUNAN AMPLIFIKASI ISOTERMA DENGAN PENGANTARAAN GELUNG UNTUK PENGESANAN DNA *LEPTOSPIRA* YANG RINGKAS DAN PANTAS DALAM SAMPEL MANUSIA

Oleh

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Leptospirosis merupakan penyakit bawaan haiwan yang boleh menjangkiti manusia. Penyakit ini disebabkan oleh bakteria daripada genus *Leptospira* yang boleh memberi kesan kepada manusia dan haiwan di seluruh dunia. Pengesanan awal penyakit leptospirosis adalah penting bagi membolehkan rawatan yang sewajarnya diberikan dan mengawal penyakit ini daripada merebak ke tahap yang lebih serius. Oleh itu, kajian ini telah dijalankan bertujuan untuk membangunkan satu sistem amplifikasi isoterma dengan pengantaraan gelung (LAMP) yang ringkas dan pantas bagi pengesanan DNA *Leptospira* dalam pesakit yang disyaki menghidap leptospirosis. Satu set primer LAMP yang mensasarkan gen *secY* daripada *Leptospira* telah direka. Plasmid rekombinan yang mengandungi gen sasaran digunakan sebagai templat dalam prosedur pengoptimuman. Beberapa parameter telah dioptimumkan termasuklah suhu inkubasi dan kepekatan reagen. Set primer yang digunakan telah didapati berfungsi dengan sempurna pada suhu 65°C dan kepekatan optimum bagi *betaine* dan $MgSO_4$ masing-masing adalah pada 0.4 M dan 8 mM. Tahap kepekaan sistem ini telah diuji berdasarkan had kepekaan pengesanan dengan menggunakan DNA genomik daripada *Leptospira interrogans serovar Pomona* yang dicairkan secara bersiri. Keputusan menunjukkan bahawa had kepekaan pengesanan adalah 2×10^4 salinan DNA genomik bagi setiap tindak balas (bersamaan 0.1 ng) untuk reaksi selama 40 minit. Kekhususan sistem ini pula diuji dengan menggunakan DNA yang diekstrak daripada 15 *Leptospira* dan sembilan bakteria bukan *Leptospira*. Sistem ini terbukti khusus untuk pengesanan DNA *Leptospira* apabila tiada satu pun DNA dari bakteria bukan *Leptospira* yang berjaya dikesan. Sampel darah dan air kencing yang diperoleh daripada penderma yang sihat telah dicampurkan dengan kultur tulen *L. interrogans serovar Pomona.* Proses ini dilakukan untuk meniru keadaan klinikal yang sebenar bagi menentukan had kepekaan klinikal sistem ini. Dalam sampel darah yang telah dicampurkan dengan kultur *Leptospira*, 1×10^2 *Leptospira* / ml telah didapati sebagai had pengesanan. Dua kaedah pengasingan DNA daripada sampel air kencing telah dibandingkan kecekapannya iaitu purifikasi dengan menggunakan kolum dan pendidihan. Sampel DNA hasil daripada purifikasi dengan menggunakan kolum dilihat memberikan keputusan yang lebih baik dari segi masa pengesanan yang lebih cepat dan kepekaan yang lebih tinggi berbanding

dengan sampel dari proses pendidihan di mana had pengesanan masing-masing adalah 1 X 10^2 *Leptospira* / ml dan 1 x 10^3 *Leptospira* / ml. Seterusnya, sistem LAMP ini diuji dengan menggunakan sampel darah dan air kencing yang diperolehi daripada pesakit yang disyaki menghidap leptospirosis. Hasilnya telah dibandingkan dengan keputusan tindak balas berantai polymerase (PCR) konvensional. Dalam reaksi LAMP selama 30 minit, 28 daripada 69 sampel darah yang diambil semasa kemasukan ke wad telah didapati positif. Daripada jumlah ini, hanya 26 sampel dapat dikesan sebagai positif dengan menggunakan PCR. Selain itu, daripada 34 sampel air kencing yang diuji, 16 didapati positif oleh LAMP manakala 14 positif untuk PCR. Ini termasuk sampel air kencing yang diperoleh seawal hari ketiga penunjukan simptom penyakit. Melalui dapatan kajian ini, adalah dicadangkan agar air kencing turut sama digunakan sebagai sampel bagi diagnosis penyakit leptospirosis semasa kemasukan ke wad. Konklusinya, sistem LAMP yang telah dibangunkan melalui kajian ini boleh dijadikan sebagai alternatif diagnosis bagi penyakit leptospirosis yang pantas berdasarkan kecekapan, kepekaan dan kekhususan yang telah ditunjukkan.

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

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LIST OF ABBREVIATIONS

BIP	Backward inner primer
BLAST	Basic local alignment search tool
CSF	Cerebrospinal fluid
DENV	Dengue virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immune sorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
FIP	Forward inner primer
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMR	Institute of Medical Research
LAMP	Loop-mediated isothermal amplification
LB	Loop backward
LF	Loop forward
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
MGSO4	Magnesium sulphate
$MnCl_2$	Manganese chloride
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center of Biotechnology Information
NCR	Non-coding region
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEA	South east asia
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Leptospirosis is a disease caused by bacteria of the genus *Leptospira* that affect both humans and animals worldwide. Infection in humans occur through contact with water, food or soil containing urine from infected animals (1). Wide range of symptoms may appear including high fever, headache, muscle aches and vomiting which often mistaken for other diseases. In some infected persons, the symptoms may occur at the later stage therefore late detection can lead to death due to multi-organ system complications indicating how serious the disease is (2). Due to this problem, early detection of leptospirosis is crucial in order to provide appropriate treatment and control the progression of the disease to a severe state. Furthermore, it is important for this fatal disease to be detected at early stage simply because it can be treated by using antibiotics.

To date, there are a few alternatives for leptospirosis diagnosis. Serology method is the most widely used diagnostic tool in the detection of this disease. Microscopic agglutination test (MAT) is known as the gold standard for diagnosis of leptospirosis (3). However, this approach does not contribute to early diagnosis because anti-*Leptospira* antibodies only become detectable in the late acute phase, 3-5 days after the onset of the disease (4). Dark field microscopy is used to view the organism in urine or blood and this is an example of a low cost diagnosis tool but this method requires the specimen to be prepared from the culture which is impractical since the bacteria are fastidious to be cultured (5). Besides, this method has a low specificity as misinterpretation of fibrin or protein threads often reported (6). Genomic methods including polymerase chain reaction (PCR), multiplex PCR and real-time PCR are one of the most reliable tools in terms of sensitivity and rapidity of detection. Nevertheless, the need of expensive thermocycler which may not be readily available in many laboratories of resource-limited countries has become a major drawback of this diagnosis method.

Alternatives for rapid detection with high degree of reliability, sensitivity and cost effective may become the ultimate solution for the control of this infectious disease (7). Notomi et al (2000) reported a novel molecular technique of nucleic acid amplification termed loop-mediated isothermal amplification (LAMP) where a set of four (or six) different primers binding to six (or eight) different region on the target gene making it to be highly specific (8). Besides, the use of *Bst* DNA polymerase with high-displacement activity enables the LAMP reaction to be performed at a constant temperature simply by using a water bath (8). Various LAMP detection methods such as turbidimeters, fluorescent agents, colorimetric agents, lateral flow dipstick, and lab-on-a-chip devices have been developed (9). As reported by Suwancharoen et al (2012), LAMP method provides a specific assay of 10 – 100 times more sensitive than standard PCR in the detection of *Leptospira* spp. (10). Even though it was reported that real-time PCR could results in detection of as low as 1 copy of leptospiral DNA (11), this method is not cost effective as it requires advanced and expensive instruments like thermocycler (12).

In this study, *secY* gene of *Leptospira* was selected as the target gene. *secY* is a house keeping gene that present in all *Leptospira* species and strains (11,13). Previously, a number of studies had reported the development of LAMP system for detection of *Leptospira* but focusing more on the pathogenic and intermediate group of this pathogen (10,12,14). However, a rising fact about leptospirosis that was recently reported was the presence of *Leptospira* saprophytic strains detected in leptospirosis suspected patients (15–17). Development of leptospirosis diagnostic test that only focused on detection of pathogenic strains may cause a nonresponsive results or false negative outcome (18). Hence, this study aims to develop a LAMP system for detection of *Leptospira* in suspected leptospirosis patients regardless of its pathogenicity group.

1.2 Objectives of the study:

1.2.1 General objective:

To develop a LAMP system for detection of *Leptospira* in human clinical samples

1.2.2 Specific objectives:

1. To optimize a LAMP system for detection of leptospiral DNA using newly designed LAMP primer sets targeting *secY* gene of *Leptospira*.
2. To optimize DNA isolation method and detection of leptospiral DNA from blood and urine samples spiked with leptospires.
3. To test the established LAMP system for detection of leptospiral DNA in blood and urine samples from suspected leptospirosis patients

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BIODATA OF STUDENT

Shuhaidah Othman was born and raised in Kuala Lumpur, Malaysia. She is the third child in the family of five children. She started school in Sekolah Kebangsaan La Salle (2) Jinjang followed by Sekolah Menengah Kebangsaan Jinjang. Throughout her primary and secondary education, she developed a great interest for science, particularly biology. Her interest in this field remains undiminished, as she continued her post-secondary education in the field of biology at Selangor Matriculation College. She then attended Universiti Putra Malaysia from 2011 to 2015 and graduated with a second-class upper degree in Bachelor of Science in Cell Biology and Molecule. During her undergraduate study, she was involved in a research project of plant molecular biology mainly on the identification of putative monolignol transporter gene homolog in *Oryza sativa*. Apart from that, during her industrial training in Forest Research Institute Malaysia (FRIM) she conducted a research project on the screening of anti-inflammatory effects of traditional plant extracts. It was during that time; she discovered her passion toward scientific research and began to embrace the beauty of it. Her interest grew even stronger with the knowledge she gained throughout her undergraduate journey. In year 2016, she entered the Master program at her alma mater, Universiti Putra Malaysia, in the field of medical microbiology where she performs a research on development of molecular detection for *Leptospira* by using loop mediated isothermal amplification method. Her curiosity and perseverance roots from the thought she always lives by – "You will never know until you try, and however difficult the journey may seem, one should not give up because there will always be something one can do and succeed at."

LIST OF PUBLICATIONS

Othman S, Philip N, Taib N-M, Neela V-S, Chee H-Y. Detection of Leptospiral DNA in Urine Sample Following Prolonged Hospitalization: A Case Report. Mal J Med Health Sci. 2019

Wong Y-P, Othman S, Lau Y-L, Son R, Chee H-Y. Loop Mediated Isothermal Amplification (LAMP): A Versatile Technique for Detection of Microorganisms. J Appl Microbiol. 2017

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