UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A RAPID MOLECULAR-BASED DIAGNOSTIC KIT FOR DIAGNOSIS OF LEPTOSPIROSIS

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DEVELOPMENT OF A RAPID MOLECULAR-BASED DIAGNOSTIC KIT FOR DIAGNOSIS OF LEPTOSPIROSIS

By

NARUMON SOMKUNA

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

September 2004

DEDICATION

To my parents, parents in law, husband, uncle, sisters, nieces and nephew

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

DEVELOPMENT OF A RAPID MOLECULAR-BASED DIAGNOSTIC KIT FOR DIAGNOSIS OF LEPTOSPIROSIS

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September 2004

Chairman: **Professor Abdul Rani Bahaman, Ph.D.**

Faculty: **Veterinary Medicine**

Leptospirosis is a worldwide zoonosis, affecting farm animals, wildlife as well as humans particularly in tropical areas. It is a re-emerging infectious disease caused by pathogenic leptospires. Diagnosis of leptospirosis usually depends upon demonstration of serum antibodies by serological tests. These tests are relatively not specific, sensitive and often time-consuming. Early, quick and precise diagnosis is essential so that early and specific treatment of patients can be provided and also allows for suitable control of infection to be implemented. The primary objective of this study to use a polymerase chain reaction (PCR) assay as a major technique to detect and differentiate pathogenic leptospires. In addition, another aim of this study was to develop a diagnostic kit based on PCR assay coupled with DNA hybridization assay. The assay used a nonradioactive leptospiral DNA probe hybridized to target leptospiral DNA in a microtitre plate for the detection of leptospiral DNA in clinical specimens. Twenty-one

reference leptospiral serovars representing four species of *Leptospira* (*L. interrogans*, *L. weilii*, *L. inadai* and *L. borgpetersenii*) and clinical samples were examined. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Low-Stringency-PCR (LS-PCR) were applied to detect and differentiate the leptospires. The results from PCR-RFLP showed that restriction enzymes: *Dde*I, *Nde*II, and *Msp*I could digest the amplified leptospiral DNA with genus specific primers G1/G2 and allowed differentiation of the leptospiral serovars and serogroups into their species level. It is seen that LS-PCR could produce serovar specific DNA fingerprintings. Nonradioactive digoxigenin (DIG)-labelled leptospiral DNA probes and dot blot hybridization (DBH) assay were successfully developed for the detection of leptospiral DNA in cultures and clinical samples. Results showed that these probes had a high sensitivity and specificity. The lowest limit of the DNA concentration in PCR-DBH assay was 10^{-6} µg. A new and rapid diagnostic kit was thus designed. This PCR-MHA detection method has advantages over the conventional PCR assay and DBH assay when handling a large number of samples. The procedure has fewer steps and it is fast, safe and easy to handle. Results showed it has high specificity, sensitivity and reproducibility. Furthermore, the assay could be used to detect leptospiral DNA using reagents and standard laboratory ELISA equipment. Moreover, the entire method could be completed within a day. The technique could be directly applied to clinical samples such as urine and serum for rapid diagnosis of leptospirosis. The technique used in this study will be useful for detection and identification of leptospirosis in

patients. Moreover, it could also be used on farm animals for screening and detection of uninfected animals.

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

PERKEMBANGAN KIT DIAGNOSIS BERASAS MOLEKUL CEPAT UNTUK DIAGNOSIS LEPTOSPIROSIS

Oleh

NARUMON SOMKUNA

September 2004

Pengerusi: **Profesor Abdul Rani Bahaman, Ph.D.**

Fakulti: **Perubatan Veterinar**

Leptospirosis ialah zoonosis sejagat yang melibatkan haiwan ternakan, hidupan liar dan juga manusia erutama sekali dalam kawasan tropika. Ia merupakan penyakit muncul semula yang disebabkan oleh leptospira patogenik. Diagnosis untuk leptospirosis lazimnya bergantung kepada penentuan antibodi serum melalui ujian serologi. Ujian tersebut secara bandingan bukan khusus atau peka, dan kerap kali memakan masa. Diagnosis awal, cepat dan tepat adalah perlu supaya rawatan awal dan khusus terhadap pesakit boleh dilakukan dan juga untuk melaksanakan kawalan yang sesuai terhadap jangkitan ini. Justeru itu, matlamat kajian ini ialah untuk mengguna asai tindak balas rangkaian polimerase (PCR) sebagai suatu teknik utama dalam pengesanan dan pembezaan leptospir patogenik. Sebagai tambahan, matlamat lain ialah untuk mengembangkan kit diagnosis berasaskan asai PCR yang terganding dengan asai

penghibridan DNA. Asai ini menggunakan kuar DNA leptospira bukan radioaktif yang terhibrid kepada DNA leptospira sasar dalam plat mikrotiter untuk pengesanan DNA leptospira dalam spesimen klinik. Dua puluh satu serovar leptospira yang mewakili empat spesies *Leptospira* (*L. interrogans*, *L. weilii*, *L. inadai* dan *L. borgpetersenii*) dan sample klinik telah diperiksa. Tindak Balas Rangkaian Polimerase-Polimorfisme Panjang Serpihan Pengehadan (PCR-RFLP) dan PCR Keketatan Rendah (LS-PCR) telah diguna untuk mengesan dan membeza leptospira. Hasil daripada PCR-RFLP menunjukkan yang enzim pengehadan: *Dde*I, *Nde*II, dan *Msp*I boleh mencerna DNA leptospira teramplifikasi dengan primer khusus genus G1/G2 dan membolehkan pembezaan serovar leptospira dan serokumpulan dilakukan sehingga pada aras spesies. Apa yang dapat dilihat ialah, LS-PCR boleh menghasilkan sidik jari DNA khusus serovar. Kuar DNA leptospira terlabel digoksigenin (DIG) dan asai penghibridan sap titik (DBH) telah dapat dikembangkan dengan jayanya untuk pengesanan DNA leptospira dalam kultur dan sample klinik. Hasil kajian menunjukkan yang kuar-kuar ini adalah tinggi kepekaan dan kekhususannya. Had paling rendah untuk kepekatan DNA dalam asai PCR-DBH ialah 10^{-6} µg. Satu kit diagnosis baru dan cepat telah direka bentuk. Kaedah pengesanan ini adalah lebih baik daripada asai PCR konvensional dan asai DBH apabila mengendali bilangan sample yang besar. Prosedur ini tidak banyak langkah-langkahnya dan adalah cepat, selamat dan mudah dikendalikan. Hasil kajian juga menunjukkan ia tinggi kekhususan, kepekaan, dan kebolehulangannya. Lagipun, assai ini boleh diguna untuk mengesan DNA leptospira dengan mengguna reagen dan peralatan ELISA makmal standard. Tambahan pula kaedah ini dapat dilengkapkan

dalam tempoh sehari sahaja. Teknik ini boleh diguna secara langsung pada sampel klinik seperti urin dan serum untuk diagnosis cepat leptospirosis. Teknik yang diguna dalam kajian ini adalah berguna dalam pengesanan dan pengenalpastian leptospirosis dalam pesakit. Teknik ini juga boleh diguna terhadap haiwan ternakan untuk penyaringan dan pengesanan haiwan bukan terjangkit.

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I certify that an Examination Committee met on **date of viva** to conduct the final examination of Narumon Somkuna on his Doctor of Philosophy thesis entitled "Development of a Rapid Molecular-Based Diagnostic Kit for Diagnosis of Leptospirosis" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NARUMON SOMKUNA

Date:

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LIST OF ABBREVIATIONS

A	Adenine or adenosine; one- letter code for alanine
Ab	antibody
Ag	Antigen
A_{260}	Absorbance at 260 nm
ABTS	2,2-Azino-bis (3-Ethylbenzthaizoline-6-Sulfonic Acid) Diammonium Salt
AFLPs	Amplified fragment length polymorphisms
AP	Alkaline phosphatase
bp	Base pair
BRENDA	Bacterial restriction endonuclease deoxyribonucleic acid analysis
BSA	Bovine serum albumin
BCIP	5-bromo-4chloro-3-indolyl phosphate
C	Cytosine or cytidine; one-letter code for cysteine
cDNA	Complementary deoxyribonucleic acid
cpm	Counts per minute
cm	centimeter
˚C	Degree Celsius
dATP	Deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DBH	Dot blot hybridization
DIG	Digoxigenin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
EMJH	Ellinghausen, McCullough, Johnson and Harris
G	guanine
HCl	Hydrochloric acid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JS medium	Johnson-Seiter medium
kbp	Kilobase pair
LS-PCR	Low-stringency polymerase chain reaction
LSPs	Low-stringency products
M	Molar
MAT	Microscopic Agglutination Test
mM	Milimolar
ml	Mililiter

mmol/l	Milimol per liter
Mg^{2+}	Magnesium ion
MgCl	Magnesium chloride
MW	Molecular weight
mg/l	Milligram per liter
mol/l	Mol per liter
MHA	Microplate hybridization
NBT	nitroblue tetrazolium salt
ng	nanogram
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffer saline
PCR	Polymerase Chain Reaction
pg	picogram
pH	Puissance hydrogen (Hydrogen-ion concentration)
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
REA	Restriction endonuclease analysis
RE	Restriction enzyme
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
SDS	Sodium dodecyl sulfate
TBS	Tris buffer saline
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TEMED	N′ , N, N, N- tetra-methylethylenediamine
UV	ultraviolet
v/v	Volume per volume
w/v	Weight per volume
µ	micron
µg	microgram
µl	microlitre
µ	micron
µl	microliter
SDW	Sterile distilled water
5-FU	5- fluorouracil

CHAPTER I

INTRODUCTION

Leptospirosis is a worldwide zoonosis that affects animals, wildlife and humans. It is an infectious disease caused by pathogenic spirochaetes of the genus *Leptospira*. It is a disease of great concern for both humans and veterinary medicine because the causative organisms can persist in wildlife and domestic animals such as cattle, swines, and dogs. Moreover, pathogenic leptospires can change host specificity and virulence in response to selective pressure from the environment (Christopher *et al*., 2000). Rats are the most important source of human infection. An infected animal can remain asymptomatic and continue to shed pathogenic leptospires in the urine for a long time (Plank and Dean, 2000). Infected animals with leptospires are also occupational hazards to farmers, butchers, sewer workers, field agricultural workers, fishermen, pet shop owners, slaughterhouse workers, housewives, soldiers and veterinarians (Heath and Johnson, 1994).

Leptospirosis causes abortion, stillbirth, infertility, decreased milk production, and sometimes death in livestock (Thiermann, 1984). Infection in man is not common except in tropical regions and occurs mostly through occupational or accidental contact with infected animals or through the ingestion of water contaminated with their urine (Heath and Johnson, 1994; Farr, 1995).