IDENTIFICATION OF *LEPTOSPIRA* SPP. USING REP-PCR AND PCR-RFLP TECHNIQUES.

A THESIS

BY

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Presented in partial fulfillment of the requirements for the

Master of Science degree in Molecular Biology

at Srinakharinwirot University

March 2005

The thesis titled

"Identification of *Leptospira* spp. using rep-PCR and PCR-RFLP techniques"

by

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has approved by The Graduate School as partial fulfillment of the requirements for the Master of Science in Molecular Biology of Srinakharinwirot University.

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Acknowledgements

I would like to express my sincere gratitude and appreciation to my advisor, Assistant Professor Dr. Wasana Sukhumsirichart, for her guidance, invaluable advice, continuous supervision and kind attention throughout my study. I am deeply indebted to my co-advisors, Associate Professor Dr. Somchai Santiwatanakul for his criticism and suggestion with kindness for assessment of molecular techniques and Dr. Patcharin Saengjaruk for her valuable advice in culture techniques.

I appreciate Miss. Amornwadee Sungkakam for her kindly supply of clinical samples and also thank to all members in the Wellcome Trust-Mahidol University, Oxford Tropical Medicine Research Programme.

I am deeply grateful to Mr. Sintawee Khuchareontawom and Mr. Montri Yasawong for their great technical assistance, all members in Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, and my graduate studies for their cheerfulness and helpfulness.

I would like express my thanks to School of Graduates, Srinakharinwirot University, The Royal Bangkok Sports Club (R.B.S.C.), The president of Kho-Khae Trading and Construction Co., Ltd.), Associate Professor Dr. Kosum Chansiri (Department of Biochemistry, Srinakharinwirot University) and Dr. Cholvit Jearajit (Faculty of Social Science, Srinakharinwirot University) for their help and financial support.

Finally, I would like to express my deepest gratitude and appreciation to my parents and my friend's for their love, care, support and endless encouragement throughout my graduate studies.

Anchalee Petchuensakul

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Leptospirosis is one of the most widespread zoonotic diseases in the world. It is a common disease in wild life and livestock as well as human infection. This disease is caused by pathogenic bacteria in the genus *Leptospira* divided into two species: *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, comprising the saprophytic strains isolated from the environment. *L. interrogans* is distributed in approximately 160 mammalian species. Nowadays, these are more than 200 antigenically distinct serovars or serologically distinct strains of *L. interrogans* species found in humans (1). Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (2-4).

Leptospirosis is known to be associated with occupations that bring people into direct or indirect contact with contaminated animal urine, soil and water surface (5). Occupational groups at particular risk for leptospirosis include persons employed in agriculture, fish farmers, veterinarians, sewer workers, livestock farmers, abattoir workers and laboratory personnel et cetera (6).

Microbiological characteristics of leptospires

The general structure of *Leptospira* can be distinguished from other bacteria. It is spirochetes with fine spiral of 0.1-0.2 μm in diameter and 6-20 μm in length. One or both end(s) of its cell is usually hooked (7)(FIGURE 1). Leptospires are too slender to be demonstrated by bright-field microscopy but are visible by dark-field or phase-contrast microscopy. Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space (8).

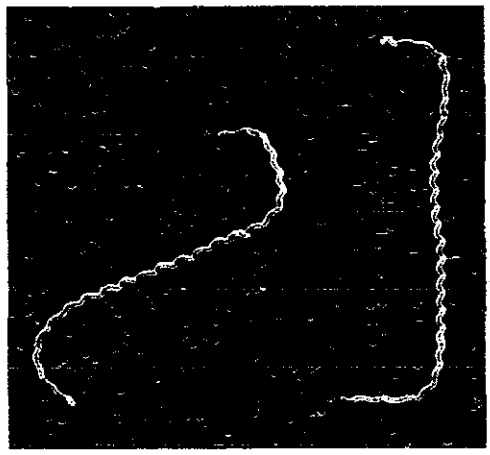

FIGURE 1 Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae strain RGA bound to a 0.2-μm membrane filter (5).

The cell wall of the leptospires is high in lipid content; component fatty acids vary among leptospiral strains. Lipids are utilized as a source of energy by the organism (9). Saprophytic leptospires invariably possess lipase activity, whereas pathogenic leptospires may be lipase-positive or lipase-negative.

On the basis of their ability to attack these lipoproteins, leptospires can be divided into three groups, including: 1) strains that degrade lecithin and sphingomyelin, 2) strains that degrade neither lecithin nor sphingomyelin, and 3) strains that degrade lecithin but not sphingomyelin. Both group 1 and 2 are pathogenic leptospires, whereas group 3 is saprophytic leptospires (10). Leptospires have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane (11). Leptospiral lipopolysaccharide has a composition similar to other gram-negative bacteria (12). Leptospires are obligate aerobes with an optimum growth temperature from 28 to 30°C and their average generation time is about 12 hours. They grow in simple media enriched with vitamins (vitamins B_2 and B_{12} are growth factors), long-chain fatty acids, and ammonium salts (2). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by β-oxidation (13).

Classification of leptospires

Leptospires are spirochetes, the family leptospiraceae includes two genera, *Leptospira* and *Leptonema.* Typically, leptospires were classified according to antigenic determinants. More recently, a molecular classification has been described that divides the *Leptospira* genus into several species on the basis of DNA relatedness.

Serological classification. The distinction between *L. interrogans* and *L. biflexa* are defined by agglutination after cross-absorption with homologous antigen. If more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing, two strains are said to belong to different serovars (14). Over 60 serovars of *L. biflexa* have been recorded (2). Over 200 serovars within *L. interrogans* are recognized. Additional serovars have also been isolated but not published yet. Serovars that are antigenically related have traditionally been grouped into serogroups (4). While serogroups have no taxonomic standing, they have been proven to be useful for epidemiological understanding. The serogroups of *L. interrogans* and some common serovars are shown in (TABLE 1).

Genotypic classification. In a number of genomospecies used to be demonstrated are belong to all serovars of both *L. interrogans* and *L. biflexa* with genetic heterogeneity (15, 16). The 10 genomospecies of *Leptospira* are also demonstrated by DNA hybridization (17). An additional genomospecies is added later and name as *L. kirschneri* (18), which do not correspond to the previous two species (*L. interrogans* and *L. biflexa*). Moreover, pathogenic and nonpathogenic serovars occur within the same species (TABLE 2). The recent studies (19, 20) show leptospiral serovars in multiple species based on their genetic heterogeneity (TABLE 3). Therefore, the reclassification of leptospires on the basis of genotype is taxonomically corrected and provides a strong foundation for future classifications. However, the molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups, which has been served to clinicians and epidemiologists well for many years. Therefore, the confusion of genomic classification still exist.

TABLE 1 Serogroups and some serovars of *L. interrogans* sensu lato (5).

Serogroup	Serovars
Icterohaemorrhagiae	icterohaemorrhagiae, copenhageni, lai, zimbabwe
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

TABLE 2 Genomospecies of *Leptospira* and distribution of serogroups (5).

Species	Serogroups
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. wolbachii	Codice
L. biflexa	Semaranga, Andamana
L. fainei	Hurstbridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadai	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
Genonospecies 1	Ranarum
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini
Genonospecies 3	Holland
Genonospecies 4	Icterohaemorrhagiae
Genonospecies 5	Semaranga
L. parva	Tumeria
L. illini	-

TABLE 3 Leptospiral serovars found in multiple species (5).

Serovar	Species
bataviae	*L. interrogans, L. santarosai*
bulgarica	*L. interrogans, L. kirschneri*
grippotyphosa	*L. kirschneri, L. interrogans*
hardjo	*L. borgpetersenii, L.interrogans, L.meyeri*
icterohaemorrhagiae	*L. interrogans, L. inadai*
kremastos	*L. interrogans, L. santarosai*
mwogolo	*L. kirschneri, L. interrogans*
paidjan	*L. kirschneri, L. interrogans*
pomona	*L. interrogans, L. noguchii*
pyrogenes	*L. interrogans, L. santarosai*
szwajizak	*L. interrogans, L. santarosai*
valbuzzi	*L. interrogans, L. kirschneri*

Transmission

The infections of leptospires occur either from direct exposures to infected animals or their products or indirect contact with water or soil contaminated with urine of a leptospires shedder. The animals that survive from acute infection can harbor the spirochete in their renal tubules for months or even years without suffering from any significant adverse consequences.

Direct transmission: The usual portal of entry is through abrasion or cuts in the skin or penetrate the mucous membranes, including the conjunctiva and vagina (21, 22). The other portal of entry is via milk from a lactating mother to the gut of breast-fed infant (23, 24).

Indirect transmission: Indirect transmission of leptospires to human is from soil or water. The survival of leptospires outside the animal host is essential factor for contaminating soil and water (25). The optimum growth temperature of 28 to 30 ^{o}C, the presence of moisture, and pH values between 6.2 and 8.0 are important for the survival of leptospires (26). Moreover, chemical pollution, salinity, and absorptive properties of clay in the soil are involved to their survival (22). Fresh water, spring

water, and food that are contaminated by rat urine have been associated with transmission to human (22, 27). Inhalation of water or aerosols also may result in infection via the mucous membranes of the respiratory tract.

Venereal transmission: Venereal transmission of leptospirosis is important in rodents and can occur in livestocks. Leptospires have been recovered from the semen of bulls. Transplacental infection of fetus in utero which is well documented in livestocks and other animals may occur also in humans (22, 28). Transmission by sexual intercourse in humans during convalescence has been reported (29).

Epidemiology

Leptospirosis is a worldwide zoonosis with a broad spectrum of animal hosts such as rodents, birds, reptiles, amphibians, mollusks, and helminthes, etc. The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. Domestic animals are also an important source of human infections. Leptospires have been isolated from approximately 160 mammalian species in the temperate zone. The incidence of disease is higher in warmclimate countries than in temperate region (30, 31) due to the leptospires, ability to survive longer in the warm and humid environment.

The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (32) which is a species in which infection is endemic and is usually transferred from animal to animal by direct contact. The prevalence of chronic excretion in the urine increases with the age of the animal.

The extent of transmission of infection depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Different rodent species may be reservoirs of distinct serovars but rats are generally maintenance hosts for serovars of the serogroups Icterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum. Domestic animals are also maintenance hosts. For example, dairy cattle may harbor serovars hardjo, pomona, and grippotyphosa and pigs may harbor pomona (33). Distinct variations in maintenance hosts and the serovars they carry occur throughout the world (34).

Three epidemiological patterns of leptospirosis were defined by Faine (35). The first occurs in temperate climates where few serovars are involved and human infection almost invariably occurs by direct contact with infected animals through farming of cattle

and pigs. The second occurs in tropical wet areas, within which there are many more serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. There are significant reservoirs for human infection in many tropical countries (36) and may be an important source of outbreaks. Human exposure is not limited by occupation but results are more often from the widespread environmental contamination, particularly during the rainy season. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods. The third pattern comprises rodent-borne infection in the urban environment. While this is of lesser significance throughout most of the world, it is potentially more important when the urban infrastructure is disrupted by war or by natural disasters. This type of infection is now rarely seen in developed countries (37).

Clinical manifestations

Clinical manifestations of leptospirosis are associated with a general febrile disease and are not sufficiently characteristic for diagnosis. As a result, leptospirosis often is initially misdiagnosed as meningitis or hepatitis.

Leptospirosis occurs as two clinically recognizable syndromes. The most common syndrome is anicteric leptospirosis, a self-limited illness that occurs in 85% to 90% of the cases. There are two clearly-defined stages, biphasic course (FIGURE 2), in anicteric leptospirosis; the septicemic stage, characterized by fever, headache, severe myalgia, chills with rigors, prostration and sometimes, circulatory collapse and followed by the immune stage, characterized by antibody production and excretion of leptospires in the urine (5, 22, 38).

Icteric leptospirosis or Weil's syndrome is a more serious form of disease characterized by symptoms of hepatic, renal and vascular dysfunction. The biphasic course of the disease is obscured by severe and persistent fever, jaundice and azotaemia. Jaundice appears, but without evidence of hepatocellular destruction. The clinical manifestations of icteric leptospirosis vary in terms of severity and symptomatology. Some patients with jaundice may have no renal manifestation.

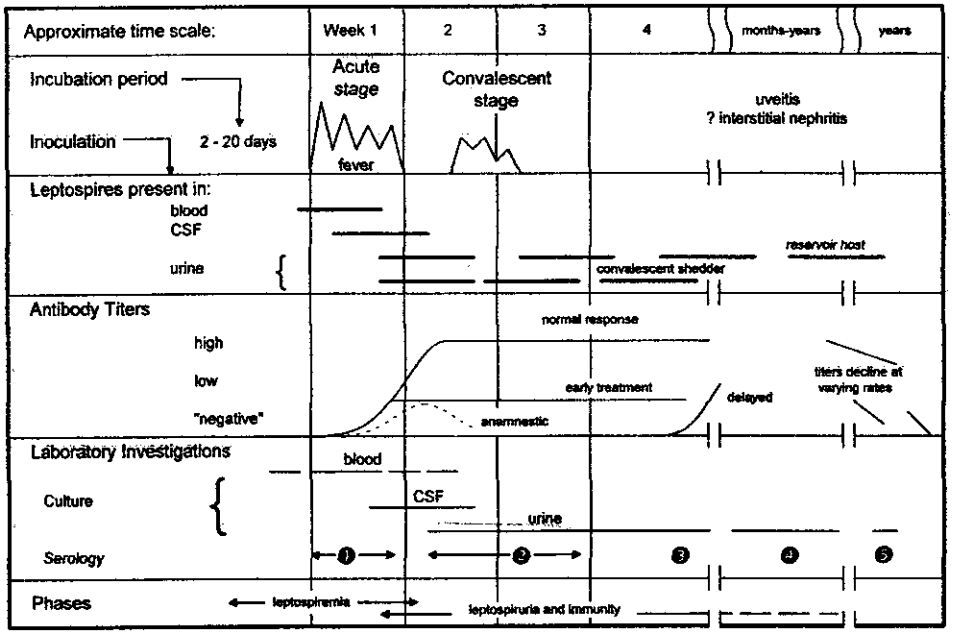

FIGURE 2 Biphasic nature of leptospirosis and relevant to investigations at different stages of disease. Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information (5).

The virulence of leptospires does not necessarily correlate with serovar. For example, a non-virulent hebdomadis serovar in one region of the world would be classified as identical to a virulent hebdomadis serovar from another region. Moreover, identical serovars from different regions may be pathogenic either for humans but not cattle or for cattle but not humans (5).

Pathogenesis

Pathogenic leptospires rapidly invade the blood stream after penetrating skin or mucous membranes. They spread throughout all sites in the body (FIGURE 3). The mechanisms by which leptospires cause disease are not well understood. The production of toxins by pathogenic leptospires in vivo was inferred by Areán (39, 40).

Endotoxic activity has been reported in several serovars (41-44). However, toxin has not been found to occur with all serovars that can cause severe disease (5). Hemolysins from several serovars have been characterized. The hemolysins of serovars ballum, hardjo, pomona, and tarassovi are sphingomyelinases (45, 46). Phospholipase C activity has been reported in serovar canicola (47).

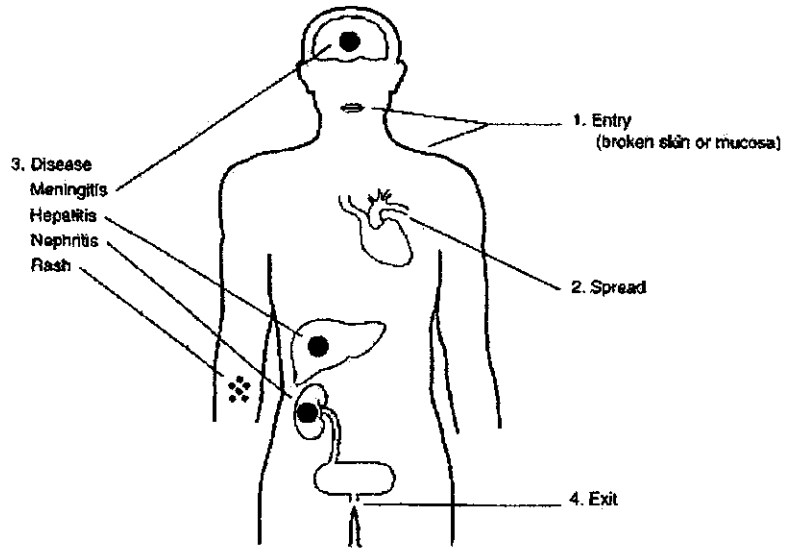

FIGURE 3 Clinical manifestations of leptospirosis.

Strains of serovars pomona and copenhageni elaborate a protein cytotoxin (48-50), and cytotoxic activity has been detected in the plasma of infected animals (51). A glycolipoprotein fraction with cytotoxic activity was recovered from serovar copenhageni (52).

The outer membrane of leptospires contains LPS and several lipoproteins (outer membrane proteins [OMPs]) (11). The LPS is highly immunogenic and is responsible for serovar specificity . An inverse relationship between expression of transmembrane OMPs and virulence is demonstrated in serovar grippotyphosa. Leptospiral LPS preparations exhibit activity in biological assays for endotoxin, but at much lower potencies (41-43). Leptospiral LPS stimulate adherence of neutrophils to endothelial cells (53, 54) and platelets result in aggregation. This bring to the development of thrombocytopenia (54).

Control

Human leptospirosis can be controlled by reducing its prevalence in wild and domestic animals. Although little can be done about controlling the disease in wild animals, leptospirosis in domestic animals can be controlled through vaccination with inactivated whole cells or an outer membrane preparation. If vaccines do not contain a sufficient immunogenic mass, the resulting immune response protects the host against

clinical disease but not against development of the renal shedder state. In spite of the fact that a multiplicity of serotypes may exist in a given geographic region and the protection afforded by the inactivated vaccines is serotype specific. The use of polyvalent vaccines is recommended.

The currently used multivalent vaccine of *Leptospira* in the world is made of dead whole cells of the microorganism (55), but the cross immune protection among different serogroups of *L. interrogans* is weak or little. Owing to large geographical variability among the dominant serovars (56, 57), one kind of vaccine is usually composed of 3-5 dominant serovars particular to the local species. Moreover, the cross-protection of the vaccine is limited, anyone of *L. interrogans* serovars, which does not contain in the vaccine, might be highly possible to cause fulminant epidemic of leptospirosis (58, 59).

CHAPTER 2

LITERATURE REVIEW

Leptospirosis is a zoonotic disease and remains leading health problem worldwide. Therefore, the species identification of leptospires is an important epidemiology for recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of the infection, recognizing particularly virulent strains of organisms, and monitoring vaccination programs.

Nowadays, the classification of leptospires is based on pairwise comparison of cross-absorption of pathogen against with specific antibody. Therefore, the identification of *Leptospira* spp. is tedious and impossible to detect the pathogens during early infection due to the absence of the antibodies against to pathogens. Practically, the detection of leptospires at an early stage of infection increases the chance of successful treatment and will reduce pathology in human infections.

In this review, we have compared some of the most current technologies for genotypic typing with regard to the principles of the methods and their reproducibility and discrimination power, ease of use, and cost in order to give the reader some measure of the clinical practicality and utility of the molecular tools available today.

Isolation of leptospires

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness (60). Therefore, blood cultures should be taken as soon as possible after the patient's presentation. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil at the patient's bedside.

Multiple cultures should be obtained from patients diagnosed leptospirosis because the concentration of organisms in blood at any point in time is low (61). Freshly drawn blood is most desirable, but leptospires may remain visible in anti-coagulated blood for up to 11 days. Inoculation of media with dilutions of blood samples may increase recovery (62). Leptospires survive in conventional blood culture

media for a number of days (63). Rarely, leptospires have been isolated from blood for weeks after the onset of symptoms (64).

Other samples that may be cultured during the first week of illness include CSF and dialysate. Urine can be cultured from the beginning of the second week of symptomatic illness. Survival of leptospires in voided human urine is limited. Therefore, urine should be processed immediately by centrifugation, followed by resuspending the sediment in phosphate-buffered saline (to neutralize the pH) and inoculated into semisolid medium containing 5-fluorouracil. Contaminated cultures may be passed through a 0.2-μm or 0.45-μm filter before subculturing into fresh medium (65).

Laboratory diagnosis of leptospires

Serological diagnosis

Reliability of serological diagnosis is now within the capacity of most general-duty laboratories. Serological tests can be a guide to the infecting serum and this information is useful for prognosis and epidemiology. Serological tests do not react until a few days after infection but reactions persists for months or years. Persistent antibodies allow retrospective diagnosis of leptospirosis (66). The wide range of tests that are available are broadly divided into genus-specific and serogroup/serotype-specific tests.

Microscopic agglutination test (MAT). This test is the procedure for detecting leptospiral antibodies, measuring antibody titer and identifying unknown leptospiral isolates. The MAT is slow, tedious, potentially biohazardous, painstaking and subjective. The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers, and laboratory-acquired infections have been reported (67, 68); whereas, the MAT is a very sensitive and reliable assay when used by skilled people. MAT is carried out with suspensions of living cultures or of cultures killed by the addition of neutralized formaldehyde. The clumps of agglutinated living leptospires differ in appearance from clumps of killed cultures. Living leptospires are agglutinated into highly refractile spheroids of various sizes. By contrast, the agglutinated killed leptospires form looser masses with an irregular often angular, outline; these appear flattened, resembling small piles of threads, or snowflakes, or pieces of cotton wool. The degree of agglutination can only be assessed in terms of the proportion of free

leptospires. The accepted endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are agglutinated (69).

Preparations for MAT require meticulous culture of a collection of the strains used alive as antigen suspensions in the tests. These also require their regular subculture and quality control for authenticity, purity agglutination and skilly educated personnel. A recent advance is the use of standardized preparations of dried leptospires available to accredited diagnostic laboratories from a central reference laboratory (66). Protocols for the MAT have been described in detail (62, 70-72).

Macroscopic agglutination test (MSAT). A rapid macroscopic slide agglutination test (MSAT) can be used to screen human and animal serum samples. These tests are carried out with a dense suspension of leptospires, which agglutinate into clumps visible to the naked eye. The best method is Galton's macroscopic slide agglutination test, in which 12 antigens are originally proposed, and later supplementary antigens are suggested (73). However, positive reactions should be confirmed by complement fixation and microscopic agglutination tests.

Sumathi and college in 1997 (74) showed the correlation of MSAT with IgM ELISA and MAT. Five hundred and ninty two samples were received. They were positive only 317 and 310 samples by IgM ELISA and MSAT, respectively. The 303 samples had MAT positive by the titers of $\geq$80. The 257 samples were negative by either IgM ELISA or MSAT. Thus MSAT had good correlation with both IgM ELISA and MAT. Therefore, MSAT can be used as a valuable and simple screening test. The sensitivity of this test can be enhanced by adding the locally-prevalent serovars (74).

Indirect fluorescent antibody test (IFAT). Immunofluorescence has not been used widely for primary diagnostic tests. It is used to detect leptospirosis in tissues (35). This is a fast and reliable test of which facilities are available. It has a sensitivity of 91.4% (75). In 1995, the IFA assay was compared with MAT, and it was found that the IFA test is moderately sensitive and specific for the initial diagnosis of leptospirosis (76).

Indirect hemagglutination test (IHA). An IHA test offers the advantage of detecting antibodies as early as the fourth day after the onset of illness. The principle of IHA is that the surface hemoglobin of human in "O" group is coated by leptospires antigens and mixed antigens are agglutinated with serum sample. It is genus specific and less time-consuming. It requires only one antigen in the test system. It has an

excellent sensitivity and specificity and may replace the MAT as the screening test of choice (77).

Microcapsule agglutination test (MCAT). MCAT is based on the passive agglutination of synthetic polymer carriers, which are sensitized with mixed antigens of sonicated leptospires, by leptospiral antibody. This test is simple and reproducible and can be employed readily in the routine laboratory (78), but it could not detect antibodies against some serovars, e.g. *sejroe* or the *australis* serogroup in Slovakia. It may not detect antibodies in sera collected more than 1 to 2 months after the onset of disease. Other advantages of the one-point MCAT kit are that it is simple and can be performed by relatively unskilled person with minimum laboratory facilities; it is also very stable and can be kept for long periods without critical storage requirements (79).

Enzyme-linked immunosorbent assay (ELISA). This is now widely used as a genus-specific screening test in man. Both peroxidase and urease labelled conjugates have been used satisfactorily. Stable reagents are available and form the basis of bedside tests, which are read visually. The use of computer assisted automated readers and the appropriate controls improve the reproducibility and could effectively diagnose acute leptospirosis (66).

The ELISA has been compared with MAT for the serologic diagnosis of leptospirosis (80, 81). The comparison of dot ELISA and MAT showed that dot ELISA was more sensitive than MAT in the 1st week of illness but the MAT was more sensitive than dot ELISA in the 3rd week of illness. Dot ELISA required no electrical equipment and only one dilution; whereas, a dark-field microscope and several dilutions were needed for the MAT test (82).

Direct examination

The typical motility of the leptospires in the clinical sample (blood, CSF, urine or peritoneal fluid) observed with dark-field microscopes or phase-contrast microscopy may aid in early diagnosis. Leptospiremia is rare after the eighth day. It is a simple method but it may not be positive if there are few bacteria in the sample. Double centrifugation of the sample at low speed to separate the cellular elements, and then at high speed, help concentrate the leptospires. Artefacts like lysed RBCs, fibrils, etc. may, however, be mistaken for leptospires. Therefor, it is not recommended as the only diagnostic procedure to be used (75). Laboratory animals may be useful for isolating the organisms from contaminated materials and maintaining recent isolates (5).

Culture methods

The types of media used for the isolation and cultivation of leptospires are media enriched with rabbit serum or bovine serum albumin (BSA) and protein-free synthetic media. The most widely used medium in current practice is based on the oleic acid-albumin medium EMJH (61). This medium is available commercially from several manufacturers and contains Tween 80 and bovine serum albumin. Liquid media are necessary for growing the cultures for serological diagnosis of infection and for typing the isolates. Liquid media are converted to a semisolid form by the incorporation of 0.2% agar and to the solid form by the addition of 1% agar. Solid media are useful for cloning the strains and for isolating leptospires from contaminated sources. Colonies in 1% agar are subsurface and become visible within 7 to 14 days (71). Leptospiral cultures may be maintained by repeated subculture or preferably by storage in semisolid agar containing hemoglobin. Growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil (83). The culture method is definitive diagnosis and should be done together with the other techniques.

Molecular diagnosis

In 1986 Terpestra et al. (84) developed a sensitive and specific diagnostic method for early detection of leptospires using DNA hybridization with ^{32}P and biotin-labelled probes prepared from leptospiral DNA. However, the sensitivity of ^{32}P-labeled probes was approximately 10^3 leptospires (84-86), much lower than the sensitivity of PCR, and probes have not been used extensively for diagnosis since PCR became available.

PCR was first developed for the detection of leptospires in urine samples of infected cattle. Urine samples containing as few as 10 leptospires/ml gave positive results. The development of real-time PCR detection can discriminate between pathogenic and non-pathogenic leptospires and shows the sensitivity as low as 2 cells in blood and 10 cells in urine (87).

Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets (88), most frequently 16S or 23S rRNA genes and repetitive elements (89, 90), while others have been constructed from genomic libraries (91-93). The comparison of PCR, culture and serology, concluded that PCR was a rapid, sensitive and specific method of diagnosing leptospiral infection, especially during the first few days of the disease (94).

DNA restriction enzyme analyses (REA) have highly specific for each type of leptospires. The application of REA for the identification of leptospires was first proposed by Marshall and co-workers in 1981 (95). This technique has been proven to be sensitive enough to differentiate between leptospiral serovars on the basis of genetic difference. The combination of restriction fragment length polymorphism (RFLP) and Southern blotting has been applied successfully in many studies for differentiating bacterial strains (96-98).

Principles and review of molecular typing

Because of the difficulties associated with serological identification of leptospiral isolates, there is great interest in molecular methods for identification and subtyping (99). Methods employed have included pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and repetitive element sequence-based PCR (rep-PCR). Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, to the strain specific level (FIGURE 4). Moreover, each method has its advantages and disadvantages. Some of the most current technologies for genotyping with regard to the principles of the methods and their reproducibility, level of resolution, ease of use, cost-effectiveness and utility of the molecular tools available today are shown in TABLE 4.

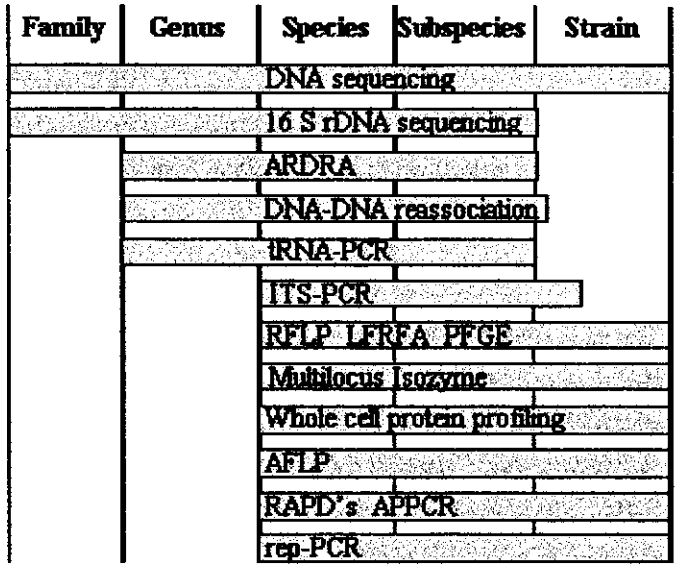

FIGURE 4 Relative resolution of various fingerprinting and DNA techniques (100).

Pulsed-field gel electrophoresis (PFGE). Pulsed field gel electrophoresis (PFGE) is often considered the "gold standard" of molecular typing methods (99). In particular, PFGE resolved extremely large DNA for the first time, raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb.

For Pulsed-field gel electrophoresis (PFGE), bacterial isolates grown either in broth or on solid media are combined with molten agarose and poured into small 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. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. Results of pattern of bands on a gel can be photographed, and the data can be stored by using one of the commercially available digital systems.

Applications of PFGE are numerous and diverse. PFGE has been proven useful to characterize leptospiral serovars (99). In contrast to its application in strain typing of other organisms, PFGE has shown that the genomes of leptospiral serovars are remarkably conserved, both over time and across wide geographical distributions (99-101). Recently, clinical isolates give the same banding patterns as reference strains of the same serovar which have been maintained for many years by repeated subculture (99).

One of the factors that has limited the use of PFGE is the time involved in completing the analysis. While the procedure steps are straightforward, the time needed to complete the procedure can be 2 to 3 days (TABLE 4) (102).

TABLE 4 Summary of the characteristics of the various molecular typing methods (102).

Methodology	Ease of use	Ease of interpretation	Discrimination power	Time to result (days)	Interlaboratory reproducibility	Cost per test
PFGE	Moderate	Easy	High	3	Good	Moderate
RFLP	Easy	Easy	Moderate	1	Good	Low
Rep-PCR	Easy	Easy	High	1	Moderate	Low
RAPD	Easy	Easy	High	1	Poor	Low
AFLP	Moderate	Easy	High	2	Good	Moderate
Sequencing	Difficult	Moderate	High	2	Good	High

Random amplified polymorphic DNA (RAPD). An RAPD method was first described by Williams et al. (103) and Welsh and McClelland (104). RAPD analysis is a technique that utilises a single short oligonucleotide primer, 9 to 10 bases in length, of arbitrary sequence in a low stringency amplification reaction. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis that are shown in a pattern of bands is characteristic of the particular bacterial strain results (103-106).

A number of studies have reported success in using RAPD assays to distinguish bacterial strains among diverse species, including *A. baumannii* (107), *Candida albicans* (108, 109), *H. pylori* (110), *Proteus mirabilis* (111), *Histoplasma capsulatum* (112), *Haemophilus somnus* (113), *Leptospira* species (114), *S. aureus* (115), and *L. pneumophilia* (116).

In comparison to other typing techniques, Vila et al. (117) found that the RAPD assay was more discriminating than RFLP analysis but less discriminating than rep-PCR. Furthermore, a number of problems have been reported for RAPD assays that contribute to a lack of reproducibility and standardization (TABLE 4). Since the primers are not directed against any particular genetic locus, many of the priming events are the result of imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns.

Amplified fragment length polymorphism (AFLP). AFLP is a highly sensitive method for DNA fingerprinting within any organism. AFLPs are based on the selective amplification of a subset of genomic restriction fragments using PCR. Restriction endonucleases such as *MseI* and *EcoRI* are used to digest the DNA before amplification. The restriction fragments are then ligated to linkers containing each restriction site and a sequence homologous to a PCR primer binding site. The PCR primers used for amplification contain DNA sequences homologous to the linker (the procedural steps showed in FIGURE 5. These amplified fragments are then to be analysed using denaturing polyacrylamide gel electrophoresis which generates fingerprints to be compared as polymorphisms.

Originally of AFLP is applied to the characterization of plant genomes. Recently, this technique has been more applied to bacterial typing (118-121). It has good both reproducibility (118, 120, 121) and ability to differentiate clonally derived strains (118 - 121). The differentiation power of AFLP appears to be greater than that of PCR-based ribotyping; however, it has not yet been compared to rep-PCR or PFGE. The AFLP procedure is more labor-intensive than rep-PCR, but results are obtained more rapidly than PFGE.

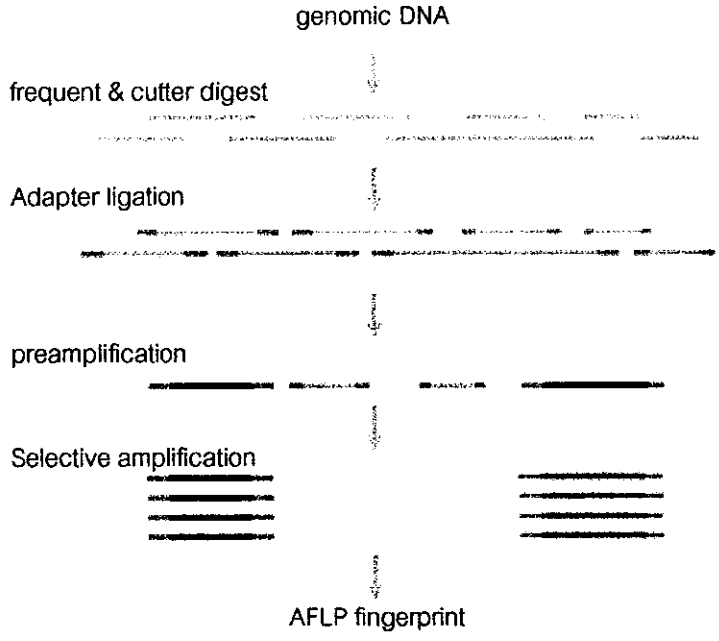

FIGURE 5 Schematic representation of the AFLP fingerprint.

Restriction fragment length polymorphism (RFLP). For RFLP detection, whole chromosomal DNA is digested with a restriction enzyme, and the fragments are separated by electrophoresis through an agarose gel.

Savio and Pacciarini (90) suggested the combination of PCR and restriction enzyme analysis of the amplified products (PCR-restriction fragment length polymorphism [RFLP] analysis) for a more informative diagnosis and for epidemiological studies (90). This technique has been demonstrated to be sufficiently sensitive and rapid to be applied for the detection and characterization of microorganisms in blood and urine samples.

The 16S, 23S, and 16S-23S spacer regions have also been used as targets for locus-specific RFLP (117, 122). RFLP analysis of PCR-amplified 16S and 23S rRNA genes allowed the leptospires grouping of 48 serovars into 16 mapped restriction site polymorphism profiles (114). Using this approach, the genomospecies of *Leptospira* could be identified, and the genotypes hardjobovis and hardjoprajitno of serovar hardjo were clearly distinguished (123). One of the potential advantages of this RFLP approach is the ability to amplify leptospiral DNA from clinical material and to identify the infecting serovar or genomospecies rapidly in the absence of an isolate.

PCR-RFLP has found a significant application in epidemiological studies of hepatitis C virus (HCV). HCV can be divided into six major genotypes, numbered 1 through 6. Davidson et al. (124) have developed a simple method for determining the genotype of an HCV isolate based on PCR amplification and RFLP analysis of the HCV genome.

In a novel application of locus-specific RFLP, Cockerill, et al. (125) have identified point mutations in the *katG* gene of *Mycobacterium tuberculosis* that correspond to various levels of resistance to isoniazid by examination of RFLP patterns of the amplified gene. This method has a good reproducibility. However, the discriminatory power of locus-specific PCR is generally not as good as that of other methods, due primarily to the limited region of the genome which can be examined.

Repetitive element sequence-based PCR (rep-PCR). Repetitive element sequence-based PCR (rep-PCR) is a new typing method that enables the generation of DNA fingerprinting that discriminates bacterial strains. This method uses primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements (102, 126-128). Multiple amplicons of different sizes can be fractioned by

electrophoresis. The result of DNA fingerprint patterns which is specific for individual bacterial clones can be compared.

Repetitive DNA sequences present in multiple copies in the genomes of most Gram-negative (129) and several Gram-positive bacteria. Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp (BOX) element (127).

These sequences appear to be located in distinct, intergenic positions around the genome. REP sequences have been described for numerous enteric bacteria (128, 130-133). The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved and dispersed elements (133, 134). ERIC sequences contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (131, 135). BOX elements are located within intergenic regions and can also form stem-loop structures due to their dyad symmetry. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB, and boxC (136). The three subunit sequences have molecular lengths of 59, 45, and 50 nucleotides, respectively. The BOX elements have no sequence relationship to either REP or ERIC sequences (136).

REP or ERIC amplification can be performed with a single primer or multiple sets of primers. ERIC patterns are generally less complex than REP patterns but both give good discrimination at the strain level. Application of both REP and ERIC PCR to samples to be typed increases the discriminatory power over that of either technique used alone (102).

Rep-PCR is become the most widely used method of DNA typing. Rep-PCR with primers based on REP and ERIC sequences has been successfully used to differentiate strains of *Bartonella* (137), *Bacillus subtilis* (126), *Citrobacter diversus* (138, 139), *Enterobacter aerogenes* (140), *Rhizobium meliloti* (141), methicillin-resistant *Staphylococcus aureus* (142), *Streptococcus pneumoniae* (143), *Acinetobactor baumanii* (107, 118), *Burkholderia cepacia* (144), *Legionella pneumophilia* (145), *Helicobacter pylori* (146), *Neisseria gonorrhoeae* (147), and *N. meningitidis* (148).

The presence of many copies of the leptospiral insertion sequence such as IS*1500* and IS*1533* has been exploited for serovar identification (90, 149, 150). Methods based on IS*1533* have limited application because of the absence of this insertion sequence in *L. interrogans* (sensu stricto) and *L. noguchii* (149).

Rep-PCR shows broader species applicability. Furthermore, studies which have compared rep-PCR to other typing methods such as multilocus enzyme electrophoresis (139), biochemical characterizations (151), or ribotyping (117, 152) have shown rep-PCR to be superior to these methods. Finally, several studies have shown rep-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power (153).

Application of molecular typing methods

Tenover, et al. (154) have identified criteria to aid in the selection and interpretation of molecular typing methods for epidemiological studies (154). Each of these methods has its advantages and disadvantages with regard to ease of application, reproducibility, requirement for equipment, and level of resolution. The choice of a molecular typing method will depend upon the needs, skill level, and resources of the laboratory as summarized in TABLE 4.

The characteristics of a molecular typing method into a clinical laboratory situation are summarized in TABLE 4. This criterion may not necessary for some clinical laboratories. However, routine laboratories that involve in large epidemiological studies may be crucial. For high throughput, simple techniques with high discriminatory power, good reproducibility and low cost, such as rep-PCR and PCR-RFLP, may be most suitable.

Therefore, in this experiment is designed to develop an identification of leptospires in Thailand. Here we propose two techniques, including repetitive element sequence-based PCR (rep-PCR) and PCR coupled with restriction fragment length polymorphism (PCR-RFLP) for the differentiation and identification of *Leptospira* spp. in Thailand.

Objectives

1. To identify of *Leptospira* spp. in Thailand using rep-PCR and PCR-RFLP typing techniques.

2. To differentiate pathogenic and non-pathogenic serovars of *Leptospira* spp. in Thailand.

CHAPTER 3

MATERIALS AND METHODS

The strategies of this research were emphasized on 3 main parts.

1. Cultivation of leptospires samples.
2. Identification of leptospires serovars by using rep-PCR and PCR–RFLP.
3. Phylogenetic tree construction using band-based analysis of RFLP patterns.

Cultivation of leptospires samples

The all of leptospires were cultivated at 30°C in EMJH liquid medium (culture media preparation see in appendix) and these were confirmed the serovars by the microscopic agglutination test (MAT).

Samples of leptospires

Reference strains

Twenty samples of leptospires were derived from National Institute of Health, Department of Medical Sciences during the year 2001 to 2003; the names of serovars were shown in TABLE 5. The reference strains of leptospires were divided into 2 parts, one kept in semisolid medium and the another were grown in EMJH liquid medium at 30°C. Stock laboratory cultures were maintained by regular subculture into fresh medium (every 10 days for the liquid medium and every month for the semisolid medium).

Clinical samples

Clinical samples used in this study were kindly provided by the Wellcome Trust-Mahidol University, Oxford Tropical Medicine Research Programme. These were obtained during the year 2000 to 2003, and then were confirmed by the microscopic agglutination test (MAT).

TABLE 5 List of Reference strains were used for study.

Serovar	Species
1. bratislava	*L. interrogans*
2. bangkok	*L. interrogans*
3. bataviae	*L. interrogans*
4. canicola	*L. interrogans*
5. copenhageni	*L. interrogans*
6. icterohaemorrhagiae	*L. interrogans*
7. pyrogenes	*L. interrogans*
8. hebdomadis	*L. interrogans*
9. autumnalis	*L. interrogans*
10. rachmati	*L. interrogans*
11. patoc	*L. biflexa*
12. andamana	*L. biflexa*
13. CH11	*L. biflexa*
14. djasiman	*L. kirschneri*
15. cynopteri	*L. kirschneri*
16. saigon	*L. noguchii*
17. javanica	*L. borgpetersenii*
18. poi	*L. borgpetersenii*
19. sejroe	*L. borgpetersenii*
20. hyos/tarassovi	*L. borgpetersenii*

DNA extraction

The leptospires were cultured in EMJH medium at 30°C for 7-10 days. The bacterial cells were collected by centrifugation at 13,000 rpm for 15 minutes. Leptospires DNA was extracted using DNAzol reagent (Gibco BRL); briefly, 1 ml of DNAzol was added to cells pellet and repeated inverting to completely lyse the cell. DNA was precipitated from the lysate by the addition of 0.5 ml of absolute ethanol per 1 ml of DNAzol. Mix samples by inverting the tubes for 5-8 times and store at room temperature for 1-3 minutes.

The DNA was centrifuged at 10,000 rpm for 10 minutes at 5°C. The DNA precipitate was washed 2 times by using 1.0 ml of 75% ethanol. The DNA pellet was sedimented at 10,000 rpm for 5 minutes at 5°C, then ethanol was removed by pipetting or decanting. DNA pellet was air-dried and re-dissolved in sterile distilled water. The genomic DNA was stored at 4°C until used. DNA concentration was determined by UV spectrophotometer at the absorbent of 260 nm.

$$20\ O.D._{260} \quad = \quad 1\ mg/ml\ of\ DNA$$

Identification of leptospires

rep-PCR method

Primer for rep-PCR

Primer of rep-PCR technique was designed from repetitive DNA elements present within bacterial genomes. The sets of primers were used for rep-PCR amplification were shown in TABLE 6.

TABLE 6 List of primers were used for rep-PCR.

Sequence Name	Sequence (5'⟶3')	Tm (°C)	References
iRep1	GCG-GAC-TCA-TAC-CCG-CT	56	129
EPR2	CTC-GCA-TCT-AAC-CCA-CGT-TT	60	149
EPL2	AGA-TTT-ACT-GCT-CCG-GAT-GG	60	149
BOX1	CTA-CGG-CAA-GGC-GAC-GCT-G	64	127
ERIC1R	ATG-TAA-GCT-CCT-GGG-GAT-TC	60	126
ERIC2	GTA-AGT-GAC-TGG-GGT-GAG-C	60	126

rep-PCR amplification

The amplification of repetitive elements were performed with a single primer, a single set of primers, or multiple sets of primers. The PCR mixtures contained of 50 ng of genomic DNA in 1x PCR buffer, 2.0 -2.5 mM of $MgCl_2$, 0.3 mM of dNTPs, 1-2.5 µM of (each) primer and 1 unit of *Taq* DNA polymerase (Fermentas). Sterile distilled water was added to make volume to 10 µl. The PCR amplification was performed using MJ

Research PTC-200 thermocycler and following conditions: (i) 1 cycle of pre-heating at 94°C for 2 minutes; (ii) 36-40 cycles (numbers of cycles vary depend on each primer), each cycle consisted of denaturation at 94°C for 1 minute, annealing (temperature vary depend on each primer) for 1 minute and extension at 72°C (time vary depend on each primer); (iii) post-extension 1 cycle at 72°C for 2 minutes. The PCR products were analyzed using electrophoresis in 1.5-1.6 % agarose gel at 100 volts for approximately 60 minutes prior to ethidium bromide staining and observed under ultraviolet light.

PCR-PFLP method

Primer for PCR-PFLP

Two sets of primers were tried for PCR-RFLP, sequences name and melting temperatures were shown in TABLE 7. The primers for PCR-RFLP were designed based on the conserved region of leptospires. LepA/LepB primers were used for detected *Leptospira* DNA by the (16S) gene as a target sequence (330 bp band was expected from PCR amplification using LepA/LepB primers). The inHF/inHR primers were used for detected *L. interrogans* but no amplification the nonpathogenic such as *L. biflexa* (PCR products of 590 bp in sizes was expected).

TABLE 7 List of primers were used for PCR-RFLP.

Sequence Name	Sequence (5' ⟶ 3')	Tm (°C)	References
LepA	GGC-GGC-GCG-TCT-TAA-ACA-TG	64	88
LepB	TTC-CCC-CCA-TTG-AGC-AAG-ATT	62	88
inHF	CGT-TGT-CAG-AGG-TCT-AAA	52	90
inHR	TAT-TCG-CCA-GAG-TTT-CGG	54	90

PCR amplification

The PCR mixtures consisted of genomic DNA in 1x PCR buffer, 2.0 mM of $MgCl_2$, 0.3 mM of dNTPs, 1 µM of each primer and 1 unit of *Taq* DNA polymerase (Fermentas). Sterile distilled water was added to make total volume to 10 µl and the PCR reaction was performed using DNA thermal cycler apparatus (MJ Research PTC-200). Conditions following: 1 cycle of pre-denaturation at 95°C for 3 minutes; 35 cycles, each cycle consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute of inHF/inHR primers or at 60°C for 1 minute of LepA/LepB primers and extension at 72°C for 1 minute; post-extension 1 cycle at 72°C for 3 minutes. The PCR products were confirmed by electrophoresis in 1.2 % agarose gel at 100 volts for 30 minutes.

Restriction endonuclease analysis of PCR-amplified products

The sequence of PCR product was searched for restriction mapping using Bio-Edit program. The restriction enzymes, *Hinf* I, *Sau* 3AI, *Hha* I, *Alu* I and *Dde* I were then selected from the restriction map of 16S sequence (PCR products from LepA/LepB primers) and tested. PCR products (10 µl) were digested in 1x of restriction enzyme buffer and 1 unit of restriction enzyme. Sterile distilled water was added to make total volume to 20 µl and digested at 37°C for 3 hours. The DNA fragments were fractionated by electrophoresis at 80 volt in 1.6-2% agarose gel.

Phylogenetic tree construction

The pattern of DNA fragments were translated to binary characters and analyzed by using MIX (Phylip program version 3.6b). The tree was calculated by using Wagner model and phylogenetic tree was reconstructed by consense and treeview for explained evolutionary relationship of leptospires.

Agarose gel electrophoresis

Various concentrations of agarose gel (1.0-2.0%) were prepared in 1x TBE buffer [89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA] according to the sizes of DNA to be analyzed. After heating to dissolve completely, the gel was poured onto the gel electrophoresis tray and a comb of a desired thickness was placed at the top of the tray. After the gel became solid, the comb was removed and transferred the agarose

gel to the chamber gel electrophoresis. DNA samples were mixed with loading dye [0.1% bromophenol blue, 40% ficoll 400-DL, 5 mM EDTA] previous to application onto the gel slots under submarine condition. The electrophoresis was achieved by using the voltage of 80 – 110 volts for 30 minutes to 2 hours (vary depend on the percentage of agarose and the voltage that used) and staining the agarose gel in 2.5% ethidium bromide solution for 1 minute. The excess amount of ethidium bromide was eliminated upon de-staining the agarose gel in distilled water for 30 minutes or until the band products were observed. DNA bands were visualized under the UV-light.

CHAPTER 4

RESULTS

Leptospires genomic DNA extraction

The genomic DNA of all *Leptospira* serovars was extracted and analysed by agarose gel electrophoresis. A single band of high molecular weight DNA sized more than 23 kb was observed after ethidium bromide staining. The DNA concentration was estimated by spectrophotometric measurement at wavelength of 260 nm. The ratio of OD_{260}/OD_{280} was used to indicate the purity of DNA. The results of DNA yield and its purity were shown in TABLE 8. The DNA concentration was diluted to 50 ng/ml before used.

TABLE 8 The DNA concentration and purity of genomic DNA.

SEROVARS	OD260	OD280	OD260/OD280	DNA concentration (ug/ml)
1. bratislava	0.034	0.014	2.429	1.70
2. bangkok	0.028	0.013	2.154	1.40
3. bataviae	0.025	0.009	2.778	1.25
4. canicola	0.021	0.010	2.100	1.05
5. cynopteri	0.026	0.009	2.889	1.30
6. copenhageni	0.025	0.011	2.273	1.25
7. icterohaemorrhagiae	0.028	0.014	2.000	1.40
8. poi	0.018	0.009	2.000	0.90
9. pyrogenes	0.033	0.017	1.941	1.65
10. hebdomadis	0.011	0.005	2.200	0.55
11. patoc	0.022	0.011	2.000	1.10
12. andamana	0.033	0.016	2.063	1.65
13. CH11	0.024	0.009	2.667	1.20
14. djasiman	0.020	0.008	2.500	1.00
15. javanica	0.019	0.008	2.375	0.95
16. saigon	0.015	0.007	2.143	0.75
17. autumnalis (Akiyami-A)	0.023	0.011	2.091	1.15
18. rachmati	0.018	0.008	2.250	0.90
19. sejroe	0.013	0.006	2.167	0.65
20. hyos	0.020	0.010	2.000	1.00

Identification of leptospires by repPCR

Optimization of the rep-PCR amplification condition

The optimum condition was required for efficient discrimination among different serovars of *Leptospira* spp. In this study, primers designed from repetitive sequences of bacterial DNA were examined. Each of primer was tested for optimum annealing temperatures, primer concentrations, magnesium ion concentrations, extension times and cycling number. The optimum condition for each primer was reported in TABLE 9.

TABLE 9 The optimum condition of rep-PCR primer.

Sequence Name	Primer concentration	Magnesium ion concentration	Annealing temperature	Extension times	Cycling numbers
iRep1	2.5 μM	2.5 mM	50 °C	1.5 min.	40
EPR2	1.0 μM	2.5 mM	50 °C	2.0 min.	36
EPL2	1.0 μM	2.5 mM	50 °C	2.0 min.	36
BOX1	1.6 μM	2.0 mM	60 °C	1.5 min.	40
ERIC1R	1.6 μM	2.5 mM	55 °C	1.5 min.	40
ERIC2	1.6 μM	2.5 mM	55 °C	1.5 min.	40

Repetitive element sequence-based PCR (rep-PCR) patterns of leptospires

The primers of repetitive element, the iRep primer (GCG-GAC-TCA-TAC-CCG-CT) and the BOX1 (CTA-CGG-CAA-GGC-GAC-GCT-G) were selected to amplify variable DNA fragments from all serovars of *Leptospira* spp. After amplification, the products were electrophoresed on agarose gel and stained with ethidium bromide. FIGURE 6 and FIGURE 7 showed DNA patterns resulted from BOX1 and iRep1 amplification, respectively.

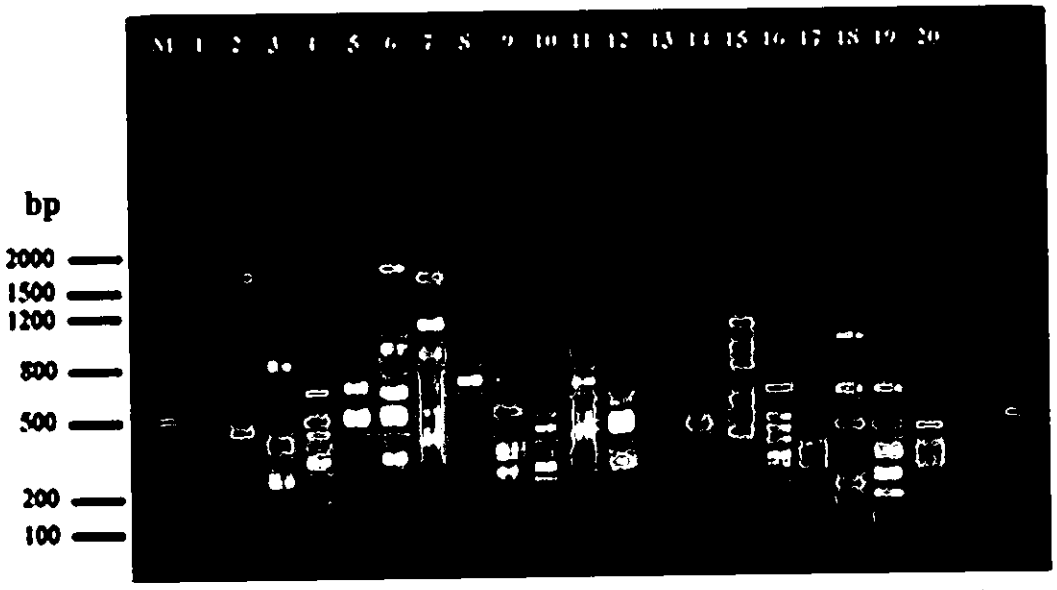

FIGURE 6 Electrophoretic separation of PCR products obtained from amplification of genomic DNA of leptospires by using BOX1 primer.

Lane M = 100bp Ladder Plus (Fermentas)
Lane 1 = bataviae
Lane 2 = copenhageni
Lane 3 = cynopteri
Lane 4 = hebdomadis
Lane 5 = djasiman
Lane 6 = bratislava
Lane 7 = poi
Lane 8 = pyrogenes
Lane 9 = javanica
Lane 10 = canicola
Lane 11 = patoc
Lane 12 = icterohaemorrhagiae
Lane 13 = bangkok
Lane 14 = CH11
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

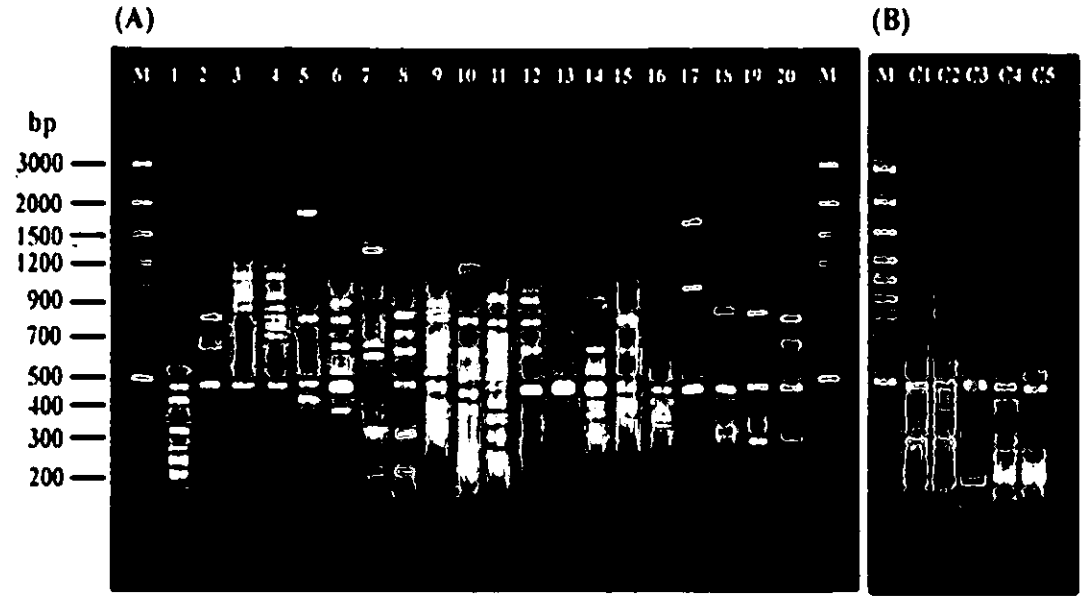

FIGURE 7 Electrophoretic separation of PCR products obtained after amplification of references serovars (A) compared with clinical samples (B) of leptospires by using iRep1 primers.

Lane M = 100bp Ladder Plus (Fermentas)
Lane 1 = bataviae
Lane 2 = copenhageni
Lane 3 = bangkok
Lane 4 = bratislava
Lane 5 = poi
Lane 6 = pyrogenes
Lane 7 = cynopteri
Lane 8 = hebdomadis
Lane 9 = sejroe
Lane 10 = rachmati
Lane 11 = saigon
Lane 12 = javanica
Lane 13 = canicola
Lane 14 = icterohaemorrhagiae
Lane 15 = djasiman
Lane 16 = patoc
Lane 17 = andamana
Lane 18 = CH11
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

Lane M = 100bp Ladder Plus
Lane C1 = clinical sample (autumnalis)
Lane C2 = clinical sample (autumnalis)
Lane C3 = clinical sample (hebdomadis)
Lane C4 = clinical sample (bataviae)
Lane C5 = clinical sample (bataviae)

Identification of leptospires by PCR-RFLP

Two sets of primers were examined for PCR-RFLP analysis. The first set, inHF/inHR, amplified 600 bp DNA fragment from bataviae, copenhageni, bratislava, pyrogenes, icterohaemorrhagiae, bangkok, canicola, hebdomadis, autumnalis, rachmati and hyos/tarassovi serovars which are member of pathogenic leptospire (*L. interrogans*). No amplification product was detected from the other *Leptospira* species. The result of inHF/inHR amplification was shown in FIGURE 8.

The second set of primer, LepA/LepB, was specific for all *Leptospira* species it amplified 330 bp DNA fragment from both of pathogenic and non-pathogenic leptospires. The results of LepA/LepB primers amplification was shown in FIGURE 9.

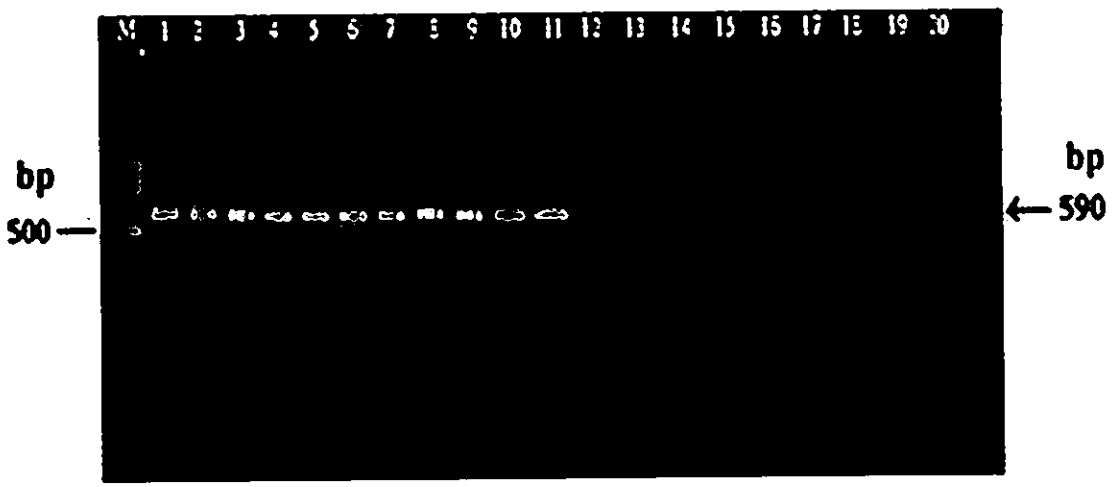

FIGURE 8 Ethidium bromide staining of 1.2% agarose gel electrophoresis of PCR products amplified by using inHF/inHR primers.

Lane M = 100bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = bangkok
Lane 3 = bataviae
Lane 4 = canicola
Lane 5 = copenhageni
Lane 6 = icterohaemorrhagiae
Lane 7 = pyrogenes
Lane 8 = hebdomadis
Lane 9 = autumnalis
Lane 10 = rachmati
Lane 11 = hyos/tarassovi
Lane 12 = patoc
Lane 13 = andamana
Lane 14 = CH11
Lane 15 = djasiman
Lane 16 = cynopteri
Lane 17 = saigon
Lane 18 = javanica
Lane 19 = poi
Lane 20 = sejroe

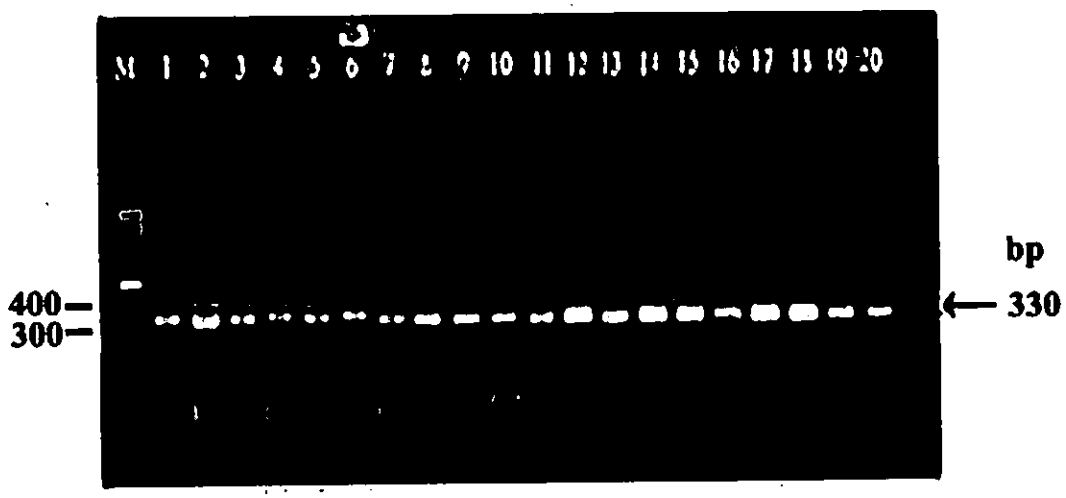

FIGURE 9 Ethidium bromide staining of 1.2% agarose gel electrophoresis of PCR product amplified by using LepA/LepB primers.

Lane M = 100bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

The PCR product of LepA/LepB was separately digested with *Hinf* I, *Hha* I, *Alu* I, *Sau* 3AI and *Dde* I. The restriction patterns obtained from *Hinf* I, *Hha* I, *Alu* I, *Sau* 3AI and *Dde* I digestion was shown in FIGURE 10-14, respectively.

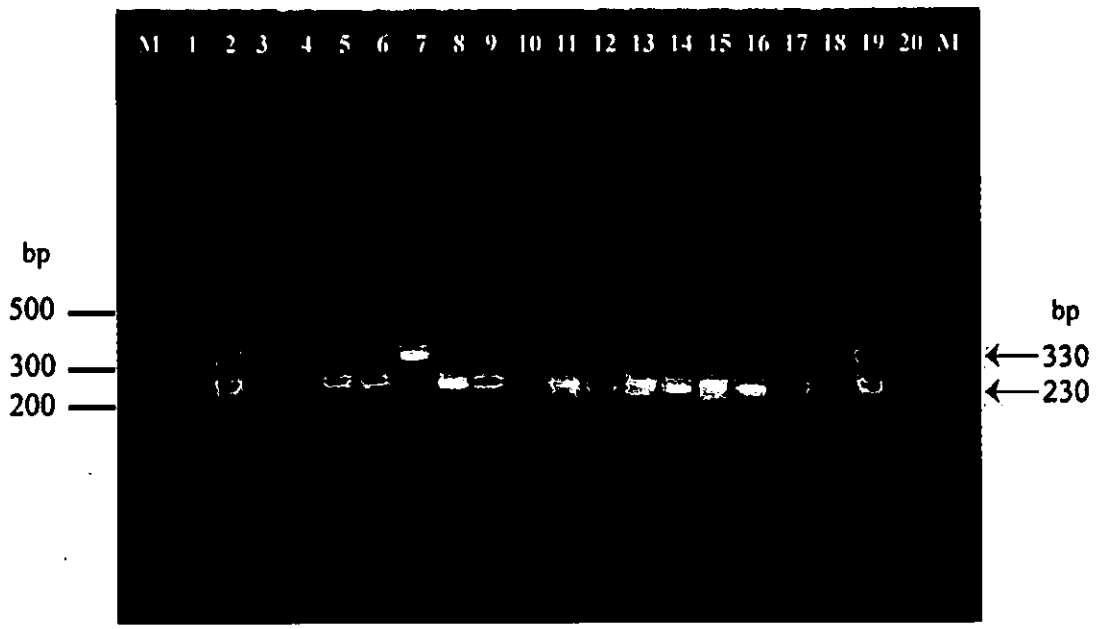

FIGURE 10 Restriction patterns of PCR product amplified by using LepA/LepB primers and digested with *Hinf* I.

Lane M = 100bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

FIGURE 11 Restriction patterns of PCR product amplified by using LepA/LepB primers and digested with *Hha* I.

Lane M = 50bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

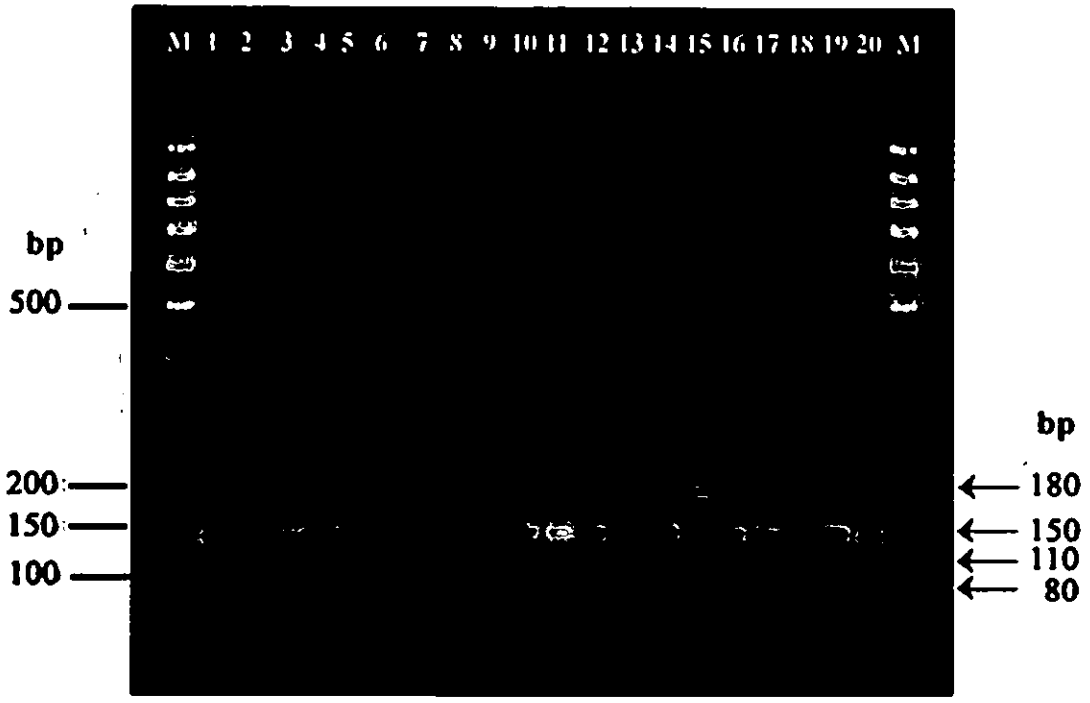

FIGURE 12 Restriction patterns of PCR product amplified by using LepA/LepB primers and digested with *Alu* I.

Lane M = 50bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

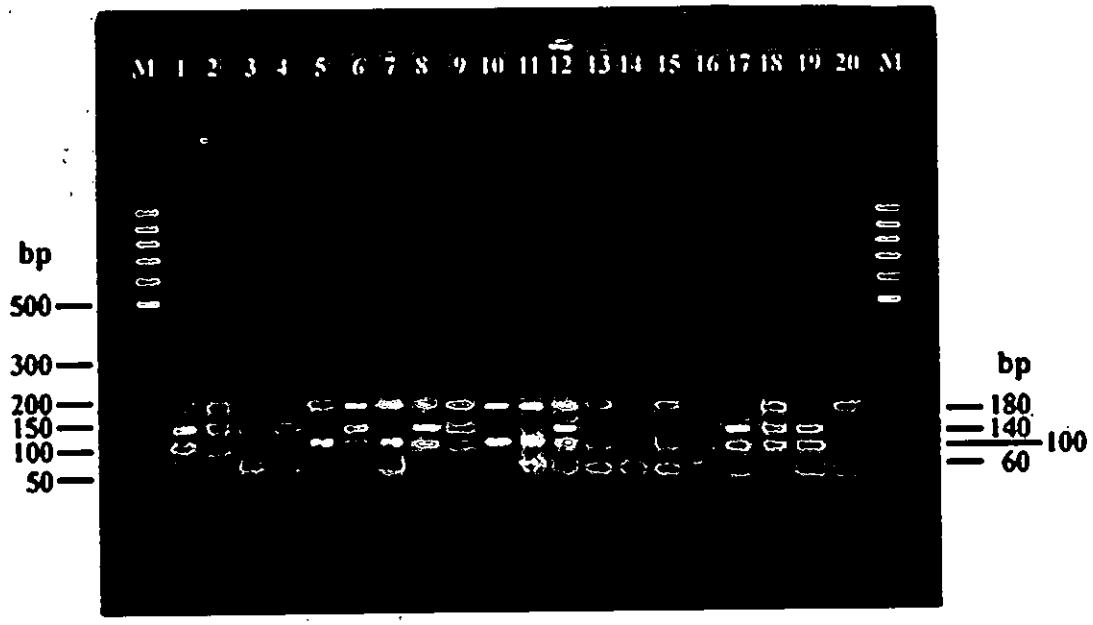

FIGURE 13 Restriction patterns of PCR product amplified by using LepA/LepB primers and digested with *Sau 3*AI.

Lane M = 50bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

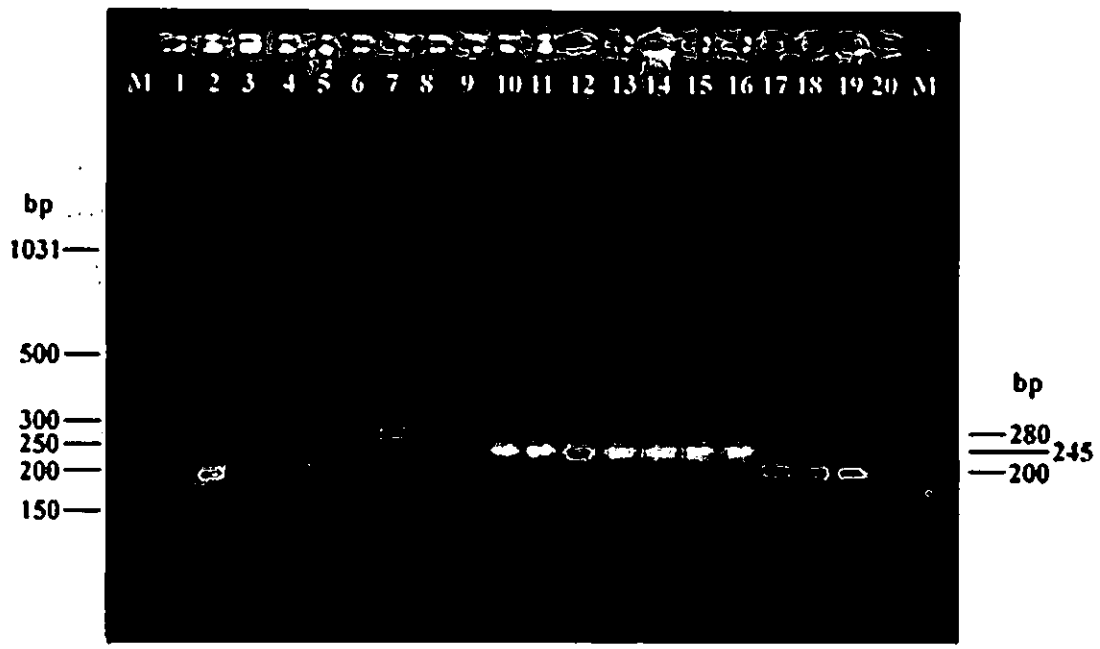

FIGURE 14 Restriction patterns of PCR product amplified by using LepA/LepB primers and digested with *Dde* I.

Lane M = 50bp marker (Fermentas)
Lane 1 = bratislava
Lane 2 = pyrogenes
Lane 3 = copenhageni
Lane 4 = icterohaemorrhagiae
Lane 5 = CH11
Lane 6 = canicola
Lane 7 = patoc
Lane 8 = bangkok
Lane 9 = bataviae
Lane 10 = poi
Lane 11 = djasiman
Lane 12 = cynopteri
Lane 13 = javanica
Lane 14 = hebdomadis
Lane 15 = andamana
Lane 16 = sejroe
Lane 17 = rachmati
Lane 18 = saigon
Lane 19 = autumnalis
Lane 20 = hyos/tarassovi

Phylogenetic tree construction using restriction patterns

The overall results of restriction patterns were described as a matrix of binary variables; band present = 1 and band absent = 0. The matrices of restriction patterns from all serovars of leptospires were shown in FIGURE 15.

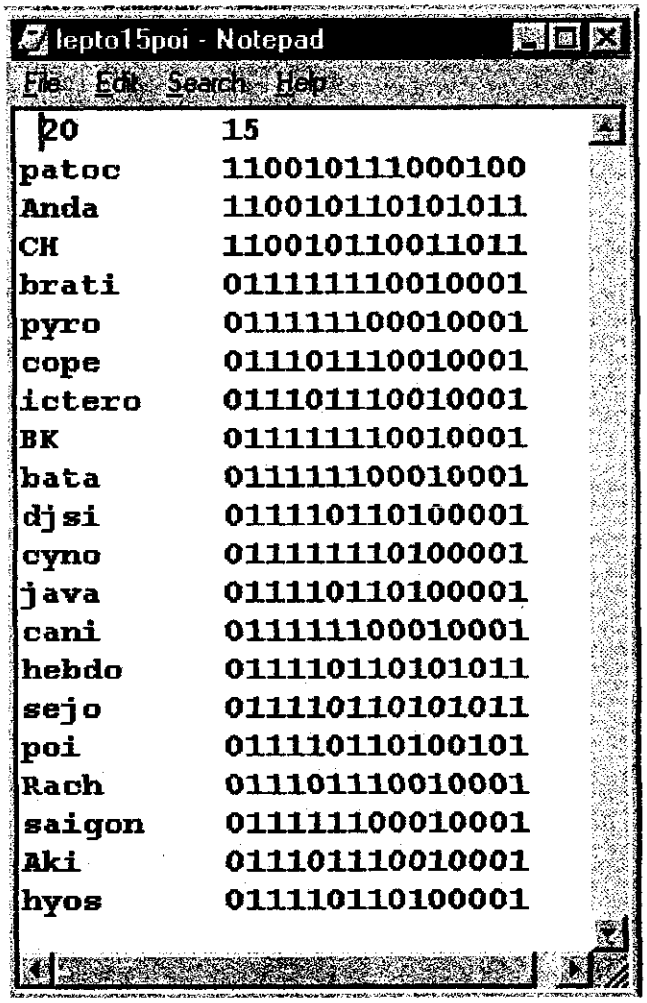

FIGURE 15 The matrices of restriction patterns from all serovars of leptospires saved in text file form.

The bootstrap was performed from discrete morphology with the number of bootstrap = 1000 replicates. Parsimony tree was constructed by using MIX (Phylip, version 3.6b). This tree was calculated by using Wagner model and tree was reconstructed by using Consense and Treeview. The phylogenetic tree was shown in FIGURE 16.

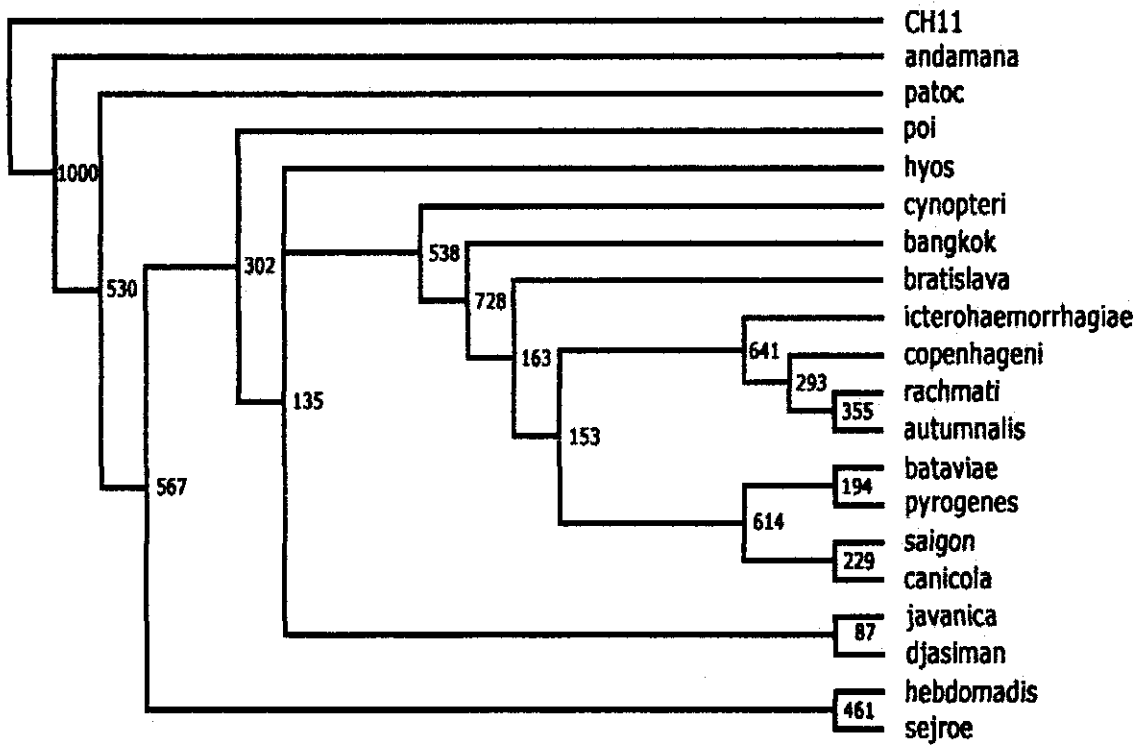

FIGURE 16 Phylogenetic tree of leptospires from discrete morphology was shown (restriction patterns) by using MIX in Phylip, version 3.6b. The number at nodes represented the percentage of times and the group occurred out of 1000 trees.

CHAPTER 5

DISSCUSSION

Leptospirosis is presumed to be the most widespread zoonosis in the world. It is caused by pathogenic spirochaetes of the genus *Leptospira*, while the genus *Leptospira* was divided into numerous serovars. Therefore, the species identification of leptospires is an important epidemiology for recognizing outbreaks of infection, determining the source of the infection, recognizing particularly virulent strains of organism and monitoring vaccination programs.

Confirmatory serological diagnosis of leptospires is usually performed based on pair-wise comparison of cross-absorption of pathogen against with specific antibody. Therefore, the identification of leptospires is tedious and impossible to detect the pathogens during early infection due to the absence of the antibodies against to pathogens. Practically, the detection of leptospires at an early stage of infection increases the chance of successful treatment and will reduce pathology in human infections.

In this experiment two molecular biological techniques was developed including repetitive element sequence-based PCR (rep-PCR) and restriction fragment length polymorphism (PCR-RFLP) for the differentiation and identification of *Leptospira* spp.

Repetitive element sequence- based PCR (rep-PCR) technique

Rep-PCR is a new typing method that enable the generation of DNA fingerprinting that can be discriminated bacterial strains. This method uses primers complementary to highly conserved of repetitive DNA sequences in the bacterial genome. Rep-PCR fingerprinting has been successfully used to differentiate many strains of bacteria such as *Bacillus subtilis* (126), methicillin-resistant *Staphylococcus aureus* (142) and *S. pneumoniae* (143). Therefore, in this study rep-PCR was used for discrimination of *Leptospira* species. Universal primers, BOX1 element and ERIC sequences, the repetitive regions as review by Versalovic, et al. (126, 127) were selected for testing the discriminating power for leptospires.

The primary result showed that BOX1 primer generated higher number of bands discriminated than the ERIC primer. The DNA patterns from ERIC primer could not

distinguished some serovars. Therefore, the BOX1 primer was selected to amplify DNA from twenty samples of leptospires (FIGURE 6). The DNA pattern from BOX1 primer showed the high discriminative, twenty individual serovars were clearly distinguished. But it was highly complex pattern and did not showed any relationship among the serovars of *Leptospira* spp.

The other primers including EPR2, EPL2 and iRep1 were examined. The results from EPR2 and EPL2 primers gave low sensitivity and could not distinguished some serovars of leptospires; whereas, the results from iRep1 provided a good trend for differentiation. Therefore, iRep1 primer was selected for amplify the twenty references strains of leptospires (FIGURE 7).

PCR fingerprinting patterns of the twenty references strains of leptospires by using iRep1 primer could distinguish the variation among repetitive sequence in the genome of each serovars of *Leptospira* spp. Sizes of the amplified products observed were ranged from 200 bp to 2,000 bp. The number and size of visible bands were varied among serovars. However, most of them have the major visible bands at 500 bp, 800 bp and 900 bp. The examination for reproducibility of iRep1 primer for discrimination of leptospires was performed by repeating the experiment for three times. Similarity DNA patterns were obtained every times of testing. This result confirmed that iRep1 posses high reproducibility.

Therefore, iRep1 primer was used for identification of leptospires in clinical samples by comparing their DNA patterns with reference strains of leptospires. The results of five clinical samples were divided into three patterns; two samples showed the same pattern as autumnalis, one sample showed the same pattern as hebdomadis and the others showed the same pattern as batavia. The results of iRep1 identification agree with the results of MAT test.

In conclusion, the rep-PCR technique could be performed by using single-oligonucleotide primer such as BOX1 and iRep1 primer. The PCR fingerprints produced by these two primers are able to distinguish a variation among twenty serovars of leptospires. The serotyping of *Leptospira* spp. by this method has a low cost per test, high discrimination power and high reproducibility (in a stringency amplification reaction). This method can be applied for discrimination of leptospires from field. However, the contamination of other bacteria may interfere the discrimination power of rep-PCR. In this case, selection experiment using specific media for culture may be necessary before performing rep-PCR experiment.

PCR coupled with restriction fragment length polymorphism (PCR-RFLP) technique

PCR has been an advanced technique for the detection of leptospires. Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets (88), 16S rRNA and repetitive elements (89, 90), while others have been constructed from genomic libraries (91-93).

DNA restriction enzyme analyses (REA) have highly specific for each type of leptospires. The application of REA for the identification of leptospires was first proposed by Marshall and co-workers in 1981 (95). This technique has been proven to be sensitive enough to differentiate between leptospiral serovars on the basis of genetic difference (95).

In this study, the PCR technique combined with restriction enzyme analysis for the differentiation *Leptospira* species was developed. This method was performed by using two set of primers (inHF/inHR primers) as described by Savio, et al. (90) and LepA/LepB primers which were designed from specific gene target (88) for amplification of leptospires DNA.

The inHF/inHR primers could be amplified eleven serovars of *L. interrogans* from the twenty serovars tested. However, there were no amplification product from the other *Leptospira* species, except *L. borgpetersenii* serovar (hyos/tarassovi) which gave a band product (FIGURE 8).

The LepA/LepB primer set was designed on the basis of amplification of 16S DNA of *Leptospira.* This primer set was specific for all *Leptospira* species. It amplified both of pathogenic and non-pathogenic leptospires (FIGURE 9), but did not amplified the DNA from other spirochetes, such as *Treponema* spp., *Spirocheta aurentia,* and *E. coli* (a more distant organisms).

The sensitivity of PCR amplification using inHF/inHR primers was 1 ng of genomic DNA; whereas, the LepA/LepB primer set detected only 1 pg of DNA. These results revealed that PCR amplification by using LepA/LepB had higher sensitivity than inHF/inHR.

By comparison of DNA sequence of PCR products resulted from amplification of DNA of icterohaemorrhagiae serovar (used as a model) by using inHF/inHR and LepA/LepB primers with the data in GenBank revealed less variation within DNA sequence from inHF/inHR amplification than LepA/LepB primers. Therefore, LepA/LepB primers were used for PCR-RFLP analysis of twenty reference strains. The restriction

enzyme analysis was preformed by using five restriction enzymes *Hinf* I, *Hha* I, *Alu* I, *Sau* 3AI and *Dde* I.

The pattern of *Hinf* I digestion of PCR product distinguished patoc from the other serovars. The pattern of *Hha* I digestion can be divided the leptospires into 3 groups; group 1 with CH11, hebdomadis, andamana and sejroe; group 2 with patoc and poi, and group 3 with bratislava, pyrogenes, copenhageni, icterohaemorrhagiae, canicola, bangkok, bataviae, djasiman, cynopteri, javanica, rachmati, saigon, autumnalis and hyos. *Alu* I pattern divided leptospires into 2 groups; group 1 with CH11, patoc, and andamana; group 2 with bratislava, pyrogenes, copenhageni, icterohaemorrhagiae, canicola, bangkok, bataviae, poi, djasiman, cynopteri, javanica, hebdomadis, sejroe, rachmati, saigon, autumnalis and hyos. The *Sau* 3AI digestion gave DNA patterns which showed the most polymorphism among serovars. The samples were able to divide into 4 groups; group 1 with bratislava, bangkok and cynopteri; group 2 with pyrogenes, bataviae, canicola and saigon; group 3 with copenhageni, icterohaemorrhagiae, rachmati and autumnalis; group 4 with CH11, patoc, poi, djasiman, javanica, hebdomadis, andamana, sejore and hyos. For the *Dde* I digestion can be separated into 3 groups; group 1 with patoc; group 2 with poi, djasiman, cynopteri, javanica, hebdomadis, andamana, sejore and hyos; group 3 with bratislava, pyrogenes, copenhageni, icterohaemorrhagiae, CH11, canicola, bangkok, bataviae, rachmati, saigon and autumnalis.

Because of PCR-RFLP provided an easy interpreted result, it was selected to study the relationship between leptospires serovars by phylogenetic tree construction. The restriction DNA patterns were translated into binary character (FIGURE15) which then used for construction phylogenetic tree by using Phylip, version 3.6b.

Phylogenetic reconstruction using restriction fragment length and restriction site polymorphism is a simple method for estimating evolutionary rates. Mathematical formulas based on this model are presented for estimating the number of nucleotide substitutions between populations or species. The phylogenetic tree reconstruction from band characters has been successfully used to study evolutionary distances in a wide populations such as differentiate mud crab species of genus *Scylla* (155), *Penaeus monodon* (156), *Apis cerana* (157) and genetic diversity of cupped oyster (158). Therefore, this method was used for study the relationship between *Leptospira* spp.

The result of phylogenetic tree placed leptospires into five clusters. The cluster 1 with hebdomadis and sejroe; cluster 2 with javanica, djasiman and hyos serovars;

cluster 3 with bangkok and Bratislava serovar; cluster 4 with copenhageni, icterohaemorrhagiae, rachmati and autumnalis; and cluster 5 with pyrogenes, bataviae, canicola and saigon serovar. Five individual serovars including patoc, andamana, CH11, poi and cynopteri were clearly discriminated into single serovar; whereas, poi was shown to be closely related to leptospires in the cluster 2. The result of phylogenetic tree constructed from band character of PCR-RFLP can be differentiate between non-pathogenic and pathogenic leptospires and agrees with the result of sero-groups identification using MAT method previously reported by Brenner et al. (19).

Phylogenetic tree construction using discrete band morphology is an easy method for study evolution and has a lower cost than sequencing. However, the result from band character has a lower discrimination power than sequencing method.

The differentiation of serovars in genus *Leptospira* by PCR amplification and restriction analysis had several advantages including easy for interpretation, low cost per test and higher reproducibility between laboratories than rep-PCR technique. However, PCR-RFLP technique had lower discrimination power than rep-PCR. It could not clearly discriminated among twenty serovars of leptospires.

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APPENDIX

APPENDIX

Culture media preparation

Culture media were prepared to 2 portions.

1. EMJH basal medium

1.1 Liquid medium

EMJH (Difco)	2.3	g
Glycerol (Sigma)	1.0	ml

The EMJH was dissolved in 800 ml of distilled water. One milliliter of glycerol was added to the solution. Then the solution was adjusted to pH 7.4. Distilled water was added to make 900 ml. The medium was sterilized by autoclaving for 15 minutes at 15 psi pressure at 121 ^{o}C.

1.2 Semisolid medium

EMJH (Difco)	2.3	g
Glycerol (Sigma)	1.0	ml
Bacto agar (Gibco BRL)	1.8	g

The EMJH was dissolved in 800 ml of distilled water. One milliliter of glycerol was added to the solution. The pH and volume of the solution was adjusted to pH 7.4 and final volume was made to 900 ml, respectively. Thereafter, Bacto agar was added to the medium. The medium was heated and stirrer until the agar was completely dissolved. The medium was autoclaved for 15 minutes at 15 psi pressure at 121 ^{o}C.

2. Supplement solution

The supplement solution was prepared as solution A and B:

2.1 Solution A

Bovine serum albumin Fraction V (Sigma)	10.0	g
Sodium acetate (Sigma)	0.1	g
Sodium pyruvate (Sigma)	0.2	g

Bovine serum albumin, sodium acetate and sodium pyruvate were dissolved in 50 ml sterile distilled water. Then the following stock solutions were added slowly to the albumin solution while it was being stirred:

2.2 Solution B

0.4% $ZnSO_4.7H_2O$ (Sigma)	1.0	ml
1.0% $CaCl_2.2H_2O$ (Sigma)	0.5	ml
1.0% $MgCl_2.6H_2O$ (Sigma)	0.5	ml
0.5% $FeSO_4.7H_2O$ (Sigma)	10.0	ml
0.3% $CuSO_4.5H_2O$ (Sigma)	0.1	ml
0.1% $MnSO_4.4H_2O$ (Sigma)	1.0	ml
10% Tween 80 (Sigma)	12.5	ml
0.02% Vitamin B12 (Sigma)	1.0	ml

Solution A and B were mixed together and adjusted to pH 7.4 and the final volume was made up to 100 ml. The complete solution was sterilized by filtering through a 0.22 µm steriled Millipore membrane.

3. Complete EMJH medium

The complete medium was prepared by adding 1 volume of the supplement solution to 9 volumes of EMJH basal medium.

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IDENTIFICATION OF *LEPTOSPIRA* SPP. USING REP-PCR AND PCR-RFLP TECHNIQUES.

AN ABSTRACT

BY

ANCHALEE PETCHUENSAKUL

Presented in partial fulfillment of the requirements for the

Master of Science degree in Molecular Biology

at Srinakharinwirot University

March 2005

Anchalee Petchuensakul. (2005). *Identification of Leptospira spp. using rep-PCR and PCR-RFLP techniques.* Master thesis, M.Sc. (Molecular Biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assist. Prof. Dr. Wasana Sukhumsirichart, Dr. Patcharin Saengjaruk, Assoc. Prof. Dr. Somchai Santiwatanakul

Leptospirosis is a zoonotic disease and remains health problem worldwide. This disease is caused by pathogenic bacteria in the genus *Leptospira* which have been classified into more than 200 serovars. In this study rep-PCR and PCR-RFLP techniques were developed for typing twenty serovars of leptospires and the patterns of PCR-RFLP fingerprinting was used for elucidation the relationships of leptospires.

DNA fingerprinting patterns of rep-PCR by using BOX1 and iRep1 primers could distinguish a variation among serovars of *Leptospira* spp. However, the pattern obtained from BOX1 primer was highly complex and difficult to interprete. In contrast, the pattern resulted from iRep1 amplification provided more easier interpretation and high reproducibility. Therefore, iRep1 primer was then used for identification of *Leptospira* spp. in clinical samples by comparing the patterns with reference strains. The DNA fingerprints in clinical samples showed similar pattern to serovar autumnalis, hebdomadis and batavia.

PCR-RFLP technique was performed by using LepA/LepB primers which designed from 16S rDNA of leptospires. Restriction enzyme analysis was preformed by using five restriction enzymes *Hinf* I, *Hha* I, *Alu* I, *Sau* 3AI and *Dde* I. Although the pattern from *Sau* 3AI digestion gave more polymorphic bands than the others enzymes. However, the bands from five enzymes were used for construction phylogenetic tree. The tree placed leptospires into five clusters that included: cluster 1 with hebdomadis and sejroe; cluster 2 with javanica, djasiman and hyos serovars; cluster 3 with bangkok and bratislava serovar; cluster 4 with copenhageni, icterohaemorrhagiae, rachmati and autumnalis; and cluster 5 with pyrogenes, bataviae, canicola and saigon serovar. Five individual leptospires including patoc, andamana, CH11, poi and cynopteri were discriminated into single serovar. The classification by phylogenetic tree agrees with MAT method.

The differentiation of *Leptospira* spp. using rep-PCR was successful to detect a variation among twenty serovars of leptospires. This method has several advantages such

as: low cost, high discrimination power and reproducibility. However, the DNA used as target for PCR amplification should not contaminated with other bacterial DNA. Therefore, selection experiment using specific media in culture may be necessary before performing rep-PCR. The PCR-RFLP technique also provided several advantages included: easy interpretation, low cost and high reproducibility between laboratories than rep-PCR technique. Nevertheless, PCR-RFLP technique had lower discrimination power than rep-PCR.

การจำแนกเชื้อเลปโตสไปร่าโดยใช้เทคนิค rep-PCR และ PCR-RFLP

บทคัดย่อ

ของ

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เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาวิชาอณูชีววิทยา
มีนาคม 2548

อัญชลี เพชรชื่นสกุล. (2548). *การจำแนกเชื้อเลปโตสไปร่าโดยใช้เทคนิค rep-PCR และ PCR-RFLP.* ปริญญานิพนธ์ วท.ม.(อณูชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: ผู้ช่วยศาสตราจารย์. ดร. วาสนา สุขุมศิริชาติ, ดร. พัชรินทร์ แสงจารึก, รองศาสตราจารย์. ดร. สมชาย สันติวัฒนกุล

โรคเลปโตสไปโรซิส (Leptospirosis) หรือโรคฉี่หนู เป็นโรคที่ติดต่อจากสัตว์สู่คน และเป็นปัญหาสุขภาพ ของคนทั่วโลก โรคนี้เกิดจากเชื้อแบคทีเรียก่อโรคใน genus *Leptospira* ซึ่งมีอยู่มากกว่า 200 ซีโรว่า ในการศึกษานี้เทคนิค rep-PCR และ PCR-RFLP ได้ถูกพัฒนาขึ้นเพื่อนำไปใช้ในการจำแนกกลุ่มของเชื้อเลปโตสไปร่า และรูปแบบลายพิมพ์ดีเอ็นเอที่ได้จากวิธี PCR-RFLP ได้ถูกนำมาใช้หาความสัมพันธ์ทางวิวัฒนาการของเชื้อเลปโตสไปร่า

รูปแบบลายพิมพ์ดีเอ็นเอที่ได้จากวิธี rep-PCR โดยใช้ไพรเมอร์ BOX1 และ iRep1 นั้น สามารถ แยกความแตกต่างระหว่างซีโรว่าของเชื้อเลปโตสไปร่าได้ แต่อย่างไรก็ตามรูปแบบที่ได้จากไพรเมอร์ BOX1 นั้นมีความซับซ้อนมาก และยากต่อการแปลผล ในทางตรงกันข้าม รูปแบบของผลที่ได้จาก iRep1 นั้นง่ายต่อการแปลผลมากกว่า และมีประสิทธิภาพการทำซ้ำที่สูง ดังนั้นจึงเลือกใช้ไพรเมอร์ iRep1 สำหรับทดสอบการจำแนกเชื้อเลปโตสไปร่าในตัวอย่างจากคนไข้โดยเปรียบเทียบรูปแบบที่ได้กับเชื้ออ้างอิง ผลของรูปแบบลายพิมพ์ดีเอ็นเอจากตัวอย่างคนไข้ที่ได้ เหมือนกับรูปแบบลายพิมพ์ดีเอ็นเอของเชื้อซีโรว่า autumnalis, hebdomadis และ batavia

เทคนิค PCR-RFLP ได้ถูกดำเนินการโดยใช้ไพรเมอร์ LepA/LepB ซึ่งออกแบบมาจาก 16S rDNA ของเชื้อเลปโตสไปร่า แล้วทำการตัดด้วยเอนไซม์ตัดจำเพาะ 5 ชนิด คือ *Hinf* I, *Hha* I, *Alu* I, *Sau* 3AI และ *Dde* I โดยรูปแบบที่ได้จากการตัดด้วย *Sau* 3AI ให้รูปแบบที่มีความหลากหลายมากกว่าเอนไซม์อื่นๆ อย่างไรก็ตาม ชิ้นดีเอ็นเอที่ได้จากการตัดด้วยเอนไซม์ตัดจำเพาะทั้ง 5 ชนิด ได้ถูกนำมาใช้เป็นข้อมูลในการสร้าง phylogenetic tree ซึ่ง tree ที่ได้สามารถแบ่งเชื้อเลปโตสไปร่าออกได้เป็น 5 กลุ่ม คือ: กลุ่ม 1 ประกอบด้วยซีโรว่า hebdomadis และ sejroe; กลุ่ม 2 ประกอบด้วยซีโรว่า javanica, djasiman และ hyos; กลุ่ม 3 ประกอบด้วยซีโรว่า bangkok และ bratislava; กลุ่ม 4 ประกอบด้วยซีโรว่า copenhageni, icterohaemorrhagiae, rachmati และ autumnalis; กลุ่ม 5 ประกอบด้วยซีโรว่า pyrogenes, bataviae, canicola และ saigon นอกจากนี้ยังมีเชื้อที่แยกเป็นตัวเดี่ยวๆ อีก 5 ชนิด คือ patoc, andamana, CH11, poi และ cynopteri ซึ่งผลที่ได้จากการจัดกลุ่มด้วย phylogenetic tree มีความสอดคล้องกับผลการจำแนกโดยวิธี MAT

การจำแนกความแตกต่างของเชื้อเลปโตสไปร่าโดยใช้เทคนิค rep-PCR นั้นประสบผลสำเร็จโดยสามารถบอกความแตกต่างระหว่างเชื้อเลปโตสไปร่าทั้ง 20 ซีโรว่าได้ วิธีนี้มีข้อดีหลายประการ เช่น ราคาถูก, มีประสิทธิภาพการจำแนกสูง และสามารถทำซ้ำได้ อย่างไรก็ตาม ดีเอ็นเอที่

ใช้เป็นต้นแบบในการเพิ่มจำนวนจะต้องไม่มีการปนเปื้อนจากดีเอ็นเอของเบคทีเรียอื่น ดังนั้น จึงจำเป็นต้องมีการคัดเลือกโดยการใช้อาหารที่จำเพาะในการเลี้ยงเชื้อก่อนการทำ rep-PCR ส่วนของเทคนิค PCR-RFLP มีข้อดีหลายประการ เช่น ง่ายในการแปลผล, ราคาถูก และมีประสิทธิภาพการทำซ้ำสูงกว่าเทคนิค rep-PCR อย่างไรก็ตาม เทคนิค PCR-RFLP นั้นมีประสิทธิภาพในการจำแนกต่ำกว่า rep-PCR