CHARACTERIZATION OF *Leptospira* SEROVARS BY PULSED-FIELD GEL ELECTROPHORESIS

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CHARACTERIZATION OF *Leptospira* SEROVARS BY PULSED-FIELD GEL ELECTROPHORESIS

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ABSTRACT

Leptospirosis is a wide spread zoonotic disease caused by pathogenic leptospires. This disease is endemic in certain regions, especially in the northeastern region of Thailand. The aim of this study was to investigate the clonal spread of leptospires from rats to humans during the outbreak in the northeastern region of Thailand in 1999-2000.

*Not*I restriction enzyme successfully differentiated 26 PFGE patterns from 27 serovars. The discriminatory power of these strains was 0.99. Each of the 27 serovars gave a unique pattern, except serovars Copenhageni and Icterohaemorrhagiae, which had the same pattern. In addition, all strains had an individual single clone.

All rat isolates were identified by serotypic methods and further characterized by PFGE. The Forty rat isolates consisted of 2 isolates as serovar Australis, 4 isolates as serovar Autumnalis, 8 isolates as serovar Bataviae and 26 as serovar Pyrogenes. Fingerprints of these isolates showed 5 major patterns. Patterns I, II and III were produced for serovars Pyrogenes, Bataviae and Autumnalis. Patterns IV and V were yielded by serovar Australis. Pattern I was similar to serovar Pyrogenes strain Salinem from human isolate, at 70% similarity, and pattern II was similar to reference serovar Bataviae strain Van Tienam, at 100% similarity. Patterns III and IV & V were different from reference serovars Autumnalis strain Akiyami A, at 28% similarity, and Australis strain Ballico, at 37% similarity, respectively. These findings indicated that serovars Autumnalis and Australis among rat isolates were not strains Akiyami A and Ballico, respectively. Moreover, the findings revealed that rat isolates of serovars Autumnalis, Bataviae and Pyrogenes each have their own clone circulating among field rats, excluding serovar Australis which was found to have 2 distinct clones.

The results of serovar characterization among 40 rat isolates by PFGE and ribotyping showed 100% concordance. In addition, the same banding patterns of serovars Pyrogenes and Autumnalis of leptospires isolated from field rats and from human leptospirosis cases were observed. This suggested that there was a clonal spread of pathogenic leptospires among field rats, and field rats may have served as reservoirs for transmission of leptospires to humans during the outbreak.

In conclusion, PFGE is a powerful tool for differentiating clonal difference between *Leptospira* serovars. Moreover, this technique can be used in epidemiological studies as well.

KEY WORDS : LEPTOSPIRA / SEROVAR CHARACTERIZATION / PULSED-FIELD GEL ELECTROPHORESIS / RIBOTYPING

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การศึกษาลักษณะของซีโรวาร์ของเชื้อเลปโตสไปร่าโดยวิธี PULSED-FIELD GEL ELECTROPHORESIS (CHARACTERIZATION OF *Leptospira* SEROVARS BY PULSED-FIELD GEL ELECTROPHORESIS)

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บทคัดย่อ

เลปโตสไปโรซิสเป็นโรคซึ่งเกิดจากเชื้อเลปโตสไปร่าชนิดก่อโรคโดยมีสัตว์เป็นพาหะ โรคนี้เป็นโรคเฉพาะถิ่นโรคหนึ่งในประเทศไทยโดยเฉพาะในภาคตะวันออกเฉียงเหนือ วัตถุประสงค์ของการศึกษาครั้งนี้คือเพื่อสืบค้นการแพร่กระจายของเชื้อเลปโตสไปร่าจากหนูสู่คนของช่วงการระบาดในภาคตะวันออกเฉียงเหนือของประเทศไทยในปีพ.ศ. 2542-2543

เอนไซม์ตัดจำเพาะ *Not*I สามารถจำแนกลักษณะของเชื้อเลปโตสไปร่า 27 ซีโรวาร์ได้เป็น 26 แบบลายพิมพ์ดีเอ็นเอ และมีความสามารถในการจำแนกซีโรวาร์เท่ากับ 0.99 แต่ละซีโรวาร์ใน 27 ซีโรวาร์ให้แบบลายพิมพ์ดีเอ็นเอที่มีลักษณะเฉพาะ ยกเว้นซีโรวาร์ Copenhageni และ Icterohaemorrhagiae ซึ่งพบว่ามีแบบลายพิมพ์ดีเอ็นเอที่เหมือนกัน

เชื้อทุกตัวอย่างจากหนูซึ่งได้จำแนกด้วยวิธีทาง serology นำมาจำแนกต่อไปด้วยวิธี PFGE เชื้อ 40 ตัวอย่างประกอบด้วย 2 ตัวอย่างซีโรวาร์ Australis, 4 ตัวอย่างซีโรวาร์ Autumnalis, 8 ตัวอย่างซีโรวาร์ Bataviae และ 26 ตัวอย่างซีโรวาร์ Pyrogenes แบบลายพิมพ์ดีเอ็นเอของเชื้อเลปโตสไปร่าที่แยกได้จากหนูพบว่ามี 5 แบบ แบบที่ I, II และ III เป็นแบบของซีโรวาร์ Pyrogenes, Bataviae, Autumnalis ตามลำดับ แบบ IV & V เป็นแบบของซีโรวาร์ Australis แบบ I คล้ายกับเชื้อซีโรวาร์ Pyrogenes strain Salinem ที่แยกได้จากผู้ป่วย ที่ similarity ร้อยละ 70 แบบ II ซึ่งเหมือนกับเชื้อซีโรวาร์ Bataviae strain Van Tienam สายพันธุ์มาตรฐาน ที่ similarity ร้อยละ 100 แบบ III พบว่าไม่เหมือนกับ ซีโรวาร์ Autumnalis strain Akiyami A สายพันธุ์มาตรฐาน ที่ similarity ร้อยละ 28 แบบ IV และ V พบว่าไม่เหมือนกับ ซีโรวาร์ Australis strain Ballico สายพันธุ์มาตรฐาน ที่ similarity ร้อยละ 37 แสดงให้เห็นว่า เชื้อซีโรวาร์ Autumnalis ในหนูไม่ใช่ strain Akiyami A เช่นเดียวกันกับเชื้อซีโรวาร์ Australis ในหนูไม่ใช่ strain Ballico ยิ่งไปกว่านี้พบว่าเชื้อซีโรวาร์ Autumnalis, Bataviae และ Pyrogenes ที่แยกได้จากหนูต่างก็มี clone ที่ให้ลักษณะเฉพาะต่อซีโรวาร์นั้นๆ ยกเว้นในซีโรวาร์ Australis พบว่ามี 2 clones ที่แตกต่างกัน

ผลการทดสอบโดย PFGE ให้ผลสอดคล้องกับวิธี ribotyping ร้อยละ 100 นอกจากนี้ยังพบว่าแบบลายพิมพ์ดีเอ็นเอของซีโรวาร์ Pyrogenes และ Autumnalis ที่แยกได้จากหนูมีแบบที่เหมือนกันกับเชื้อที่แยกได้จากผู้ป่วยเลปโตสไปโรซิส แสดงให้เห็นว่าน่าจะมีการกระจายของเชื้อเลปโตสไปร่าที่ก่อโรคได้ในกลุ่มหนูซึ่งเป็นแหล่งรังโรคสำหรับถ่ายทอดเชื้อเลปโตสไปร่าสู่คนได้ในช่วงระบาด

ผลสรุป PFGE เป็นวิธีการศึกษาทางด้านอณูวิทยาที่มีประโยชน์เพื่อใช้ในการจำแนกความแตกต่างของ clone ที่พบในแต่ละซีโรวาร์ของเชื้อเลปโตสไปร่า นอกจากนี้วิธีนี้สามารถนำไปใช้ประโยชน์ในการศึกษาทางด้านระบาดวิทยาได้ดีเช่นเดียวกัน

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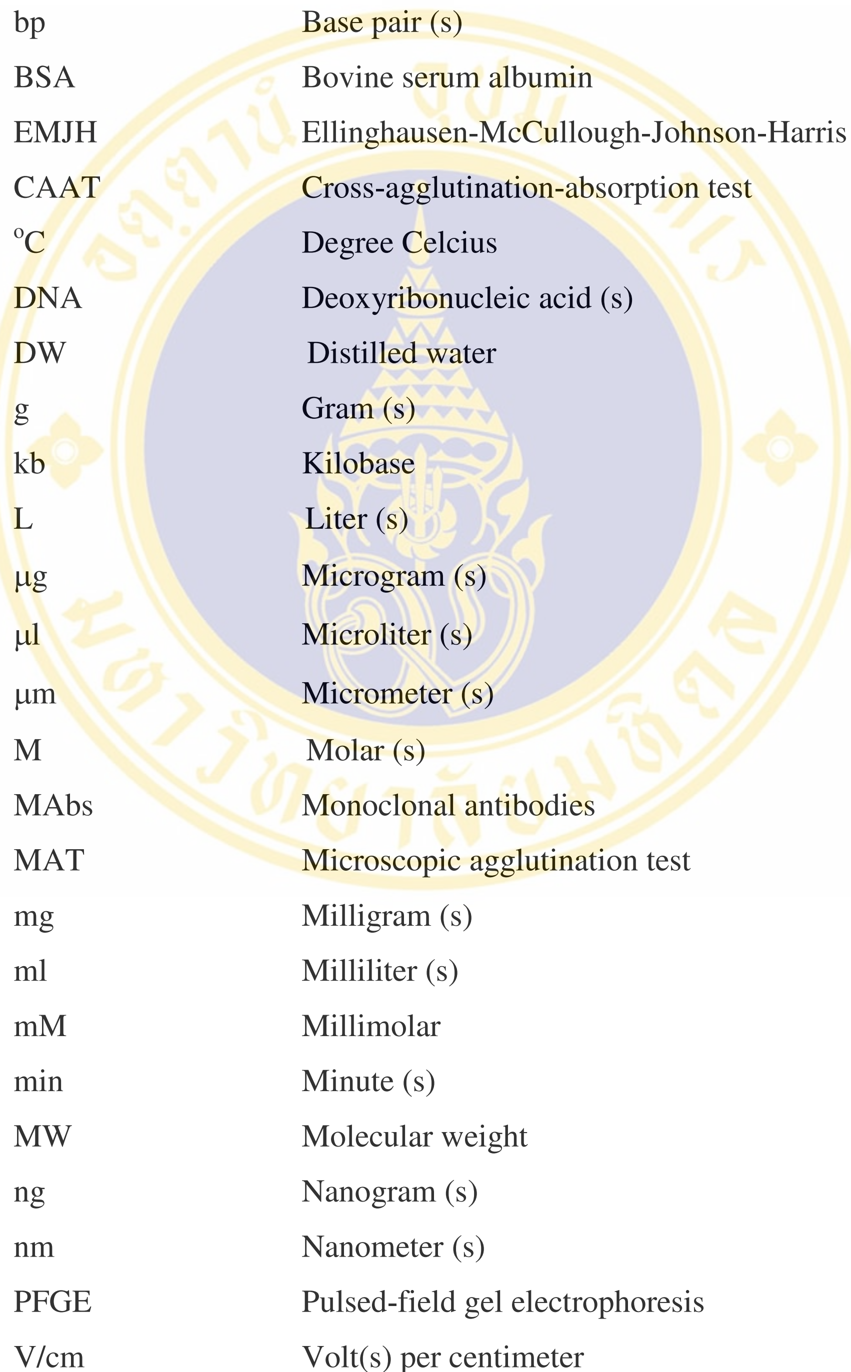

Abbreviation	Meaning
bp	Base pair (s)
BSA	Bovine serum albumin
EMJH	Ellinghausen-McCullough-Johnson-Harris
CAAT	Cross-agglutination-absorption test
^{o}C	Degree Celcius
DNA	Deoxyribonucleic acid (s)
DW	Distilled water
g	Gram (s)
kb	Kilobase
L	Liter (s)
µg	Microgram (s)
µl	Microliter (s)
µm	Micrometer (s)
M	Molar (s)
MAbs	Monoclonal antibodies
MAT	Microscopic agglutination test
mg	Milligram (s)
ml	Milliliter (s)
mM	Millimolar
min	Minute (s)
MW	Molecular weight
ng	Nanogram (s)
nm	Nanometer (s)
PFGE	Pulsed-field gel electrophoresis
V/cm	Volt(s) per centimeter

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CHAPTER I
INTRODUCTION

Leptospirosis is caused by spirochetes belonging to pathogenic members of the genus *Leptospira*. This disease was first recognized by Adolf Weil in 1886 as a clinical entity distinct from other icteric fevers (1). Symptoms in humans range from subclinical mild febrile illness to a very severe fulminating fatal infections with jaundice, hepatitis, nephritis, meningitis and may lead to death (2). Although leptospirosis is a self-limiting disease in 85-90% of cases but mortality remains significant and pulmonary haemorrhage is recognized increasingly as a major, often lethal (2, 3). Leptospirosis is a globally important zoonotic disease that can be transmitted from animals to humans and occurs worldwide, exemplified by large outbreaks in Nicaragua (4), Brazil (5), India (6), the United States (7) and Thailand (8). In developed countries, leptospirosis is associated with recreational activities (1) while in developing countries it produces large urban epidemics mainly during the rainy season (1).

Infection in humans occurs through direct or indirect contact with the urine of infected animals. Rats and other rodents are the most important maintenance hosts, which may transfer infection to domestic farm animals, dogs, and humans. Pathogenic leptospires have the ability to parasitize the proximal renal tubules of the maintenance hosts. Infection with host-adapted leptospires can result in lifelong renal carriage and urinary shedding. In addition, leptospires may survive for days to months in fresh water, soil and mud (1). Therefor, the exposure to contaminated environment may allow subsequent infection in humans. Because of some serovars are known to have preferential animal reservoirs, for example, serovar Copenhageni and Icterohaemorrhagiae occure in rats (2). Thus, a knowledge of the prevalent serovars and their maintenance hosts is essential to understanding the epidemiology of the disease.

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The taxonomy of *Leptospira* is still in progress for diagnosis and epidemiology. There are two systems to classify *Leptospira*. One based on serotypic method, which classify leptospires into serovars by agglutination after cross-absorption with homologous antigen (9). More than 250 leptospiral serovars have been recorded and antigenically related serovars are grouped into 24 serogroups. The other classification based on genotypic method. Recently, leptospires are classified into 10 species by Institut Pasteur Spirochaete Group (10).

Traditionally, leptospires are classified and identified into serovars by microscopic agglutination test (MAT). This technique requires maintenance and handling of live cultures of leptospires, which is laborious (9). Moreover, there is a variation between each laboratory in interpretation depending on the skill of each person and batch control. Therefor, DNA-based methods have been focused for identification and characterization of leptospires. DNA:DNA hybridization method is very useful in differentiation but it is not appropriate for service laboratory due to complicated and used many probes. Hence, the search for alternative methods of diagnosis and identification of isolates has been focused into the other DNA-based techniques.

The restriction endonuclease analysis (REA) and arbitrarily primed PCR (AP-PCR) have been used for characterization of leptospiral serovars but the banding patterns are very complex and difficult to compare (11, 12). Ribotyping, using *Eco*RI for digestion and 16S and 23S rRNA from *Escherichia coli* as the probe gave unique profiles for many serovars, while closely related serovar could not be distinguished by this technique (13). Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) method differentiates few serovars (14). Random amplified polymorphic DNA (RAPD) method have shown some promise for differentiating individual serovars, however, purified DNA samples are required for RAPD fingerprinting (15). Pulsed-field gel electrophoresis (PFGE), although provides a reliable and reproducible identification of *Leptospira* serovars but time-consuming and requires specialized process and equipment. Most leptospiral reference strains, about 100 serovars have been tested, could be differentiated by PFGE technique with some exception of serovar Icterohaemorrhagiae and Copenhageni (16). Insertion sequence elements have been introduced to typing *Leptospira* serovars such as IS1500

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(17) and IS1533 (18). Genomic DNA from some serovars tested with IS1500-base assay (17) failed to yield detectable hybridization pattern while IS1533-base assay showed small unique banding pattern because of several serovars have a few full or partial copies of IS1533 (18). Recently, multiple-locus variable number of tandam repeats (MLVA) assay is a new PCR-based technique for serovar identification among leptospiral. Seven loci of variable number tandam repeats (VNTR) were found to be a powerful markers for serovar identification, epidemiology and phylogenetic studies of *L. interrogans* (sensu stricto) serovars (19).

In this study, PFGE is used to differentiate leptospiral serovars, which are commonly found in Thailand. Fingerprinting pattern results of 40 leptospires isolated from field rats are compared with ribotyping methods. In addition, to compare fingerprints for leptospiral isolates between patients and rats.

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CHAPTER II
OBJECTIVES

The specific objectives of this study are:

1. To differentiation of 27 *Leptospira* serovars including 17 serovars which commonly found in Thailand
2. To characterize 40 leptospires isolated from field rats trapped from the northeastern region of Thailand during 1999-2000
3. To compare fingerprints for leptospiral isolates between patients and rats
4. To compare results between PFGE and ribotyping for characterization of 40 rat isolates

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CHAPTER III
LITERATURE REVIEW

Leptospirosis is a globally important zoonotic disease that affects for both humans and animals. In human, symptoms of infection vary from subclinical to potentially fatal with multiorgan involvement (2). The disease is caused by a pathogenic spirochetes of the genus *Leptospira* which belong to the *Leptospiraceae* family and *Spirochaetales* order. This family are divided into two genera, *Leptonema* and *Leptospira* (20). *Leptospira* includes several *Leptospira* species that are associated with diseases of humans and animals (2). Phylogenetic analyses based on 16S rDNA sequences of *Leptospira* and *Leptonema* as an outgroup, show that *Leptospira* is split into two well-supported monophyletic groups, one of them formed by the pathogenic strains (e.g., *L. interrogans*) and the other formed by the non-pathogenic strains (e.g., *L. biflexa*) (20).

1. Taxonomy and classification

1.1 Serological classification

Genus of *Leptospira* based on serology is divided into two species, which are pathogenic *L. interrogans* and the non-pathogenic *L. biflexa* containing the saprophytic strains isolated from the environment. The standard method for determining the serovars is microscopic agglutination test (MAT) with the cross-agglutination absorption of homologous and heterologous strains. 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 (21). Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by MAT after cross-absorption with homologous antigen. More than 230 serovars are currently recognized within the species *L. interrogans* sensu lato and over 60 serovars of *L. biflexa* sensu lato, arranged into 24 serogroups by antigenically related among serovars (21). The

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serogroups of *L. interrogans* sensu lato and some common serovars are shown in **Table 1.**

1.2 Genotypic classification

Serological typing is so complicated and confusing in the interpretation and maintenance of live organisms. It has been replaced by a genotypic one, in which a number of genomospecies including all serovars of both *L. interrogans* and *L. biflexa*. The application of DNA hybridization based on the definition of >70% hybridization under defined high stringency conditions, with less than 5% divergence has been defined as 12 species of *Leptospira* by Brenner et al. (22), with at least four additional species that have not yet been named **(Table 2).** More recently, leptospires were reclassified by Institut Pasteur Spirochaete Group (10) as 10 species **(Table 3).** The reclassification of leptospires using genotypic method provides useful taxonomic information, but is incompatible with serological classification which has served clinical and epidemiological as well (1). Moreover, genetic heterogeneity within serovars occurs, resulting in strains of some serovars being classified in multiple species. For example, serovar bataviae are placed into *L. interrogans* and *L. santarosai* (2).

In addition, some confusion arises owing to the retention of the species names *L. interrogans* and *L. biflexa* for one of the genetically defined pathogenic and saprophytic species, respectively. Recently, the term of "sensu stricto" is used to refer to the genomospecies by genotypic classification. The term of "sensu lato" is used to refer to leptospires which differentiated by serotypic classification.

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Table 1 Serogroups of *Leptospira interrogans* sensu lato of clinical importance with some associated serovars (2)

Serogroup	Serovar(s)
Australis	Australis, Bratislava
Autumnalis	Autumnalis, Fortbragg, Bim
Ballum	Ballum, Arborea
Bataviae	Bataviae
Canicola	Canicola, Portlandvere
Celledoni	Celledoni
Cynopteri	Cynopteri
Djasiman	Djasiman
Grippotyphosa	Grippotyphosa
Hurstbridge	Hurstbridge
Hebdomadis	Jules
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai
Javanica	Javanica
Louisiana	Lanka
Lyme	Lyme
Manhao	Manhao
Mini	Georgia
Panama	Panama
Pomona	Pomona
Pyrogenes	Pyrogenes, Zanoni
Sejroe	Sejroe, Hardjo
Tarassovi	Tarassovi

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Table 2 Genotypic classification of *Leptospira* species (22)

Pathogenic genomospecies	Non-pathogenic genomospecies
L. alexanderi	*L. biflexa*
L. borgpetersenii	*L. wolbachii*
L. fainei	*Genomospecies 3*
L. inadai	
L. interrogans	
L. kirschneri	
L. meyeri	
L. noguchii	
L. santarosai	
L. weilii	
Genomospecies 1	
Genomospecies 4	
Genomospecies 5	

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Table 3 Genotypic classification of *Leptospira* species with some associated serogropus (10)

Genomospecies	Serogroups
L. interrogans (pathogenic)	Australis, Autumnalis, Ballum, Bataviae Canicola, Cellidoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis Icterohaemorrhagiae, Javanica, Louisiana, Lyme, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum Sarmin, Sejroe, Shermani, Tarassovi
L. biflexa (non-pathogenic)	Andamana, Codice, Semaranga
L. borgpetersenii	
L. inadai	
L. noguchii	
L. santarosai	
L. weilii	
L. kirshneri	
L. meyeri	
L. wolbachi	

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2. Molecular typing

Molecular typing method have been described as many techniques. These techniques have included digestion of chromosomal DNA by restriction endonucleases (REA), ribotyping, PFGE, and a number of PCR-based assays.

REA was applied for identification of leptospires by Marshall et al. (23). They reported that serovars Hardjo and Balcanica were clearly distinguished from each other by REA using *Eco*RI restriction enzyme. This method successfully differentiated among closely antigenically related serovars Icterhaemorrhagiae strain RGA and Copenhageni strain M20 by using *Eco*RI (24). However, these two strains differed slightly in the high-molecular-weight bands. Moreover, REA was used for classification of leptospiral isolates belonging to serogroup Pomona (25). Furthermore, Thiermann et al. demonstrated genotype of Harjobovis to be subtyping A, B and C, which was isolated from bovine in North America, can discriminate between Hardjobovis and Hardjoprajitno, which belong to the same serovar (26). Although REA seems to be easy to performed and not require specialised equipment, however, the restriction patterns that obtained from this method were very complex and difficult to compare.

The database of ribotyping was constructed by using *Eco*RI for digestion genomic DNA and hybridized with 16S and 23S rRNA probes from *Escherichia coli* (13, 27). Many serovars gave unique profiles, however, some closely related serovars such as Icterhaemorrhagiae and Copenhageni could not be distinguished from each other by ribotyping. Use ribotyping also shown to discriminate accurately between the serovar Hardjo genotypes Hardjobovis and Hardoprajitno as well as REA (28). 16S rDNA from *Escherichia coli* was used as probes in hybridization with genomic DNA of *L. interrogans* serogroup Icterhaemorrhagiae and the genetic variation was found among these strains (29).

Pulse-field gel electrophoresis (PFGE) is a useful tool for characterization of leptospiral serovars. This method was used to determine the size of genome of pathogenic and saprophytic *Leptospira* species (30). Hermann et al. developed PFGE to differentiate 72 reference strains following *Not*I digestion of chromosomal DNA (16). Most of all serovars gave unique PFGE patterns, however, *L. interrogans* serovars Bratislava, Lora, Jalna, and Muenchen gave identical patterns but could be

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differentiated by *Sgr*AI restriction enzyme. Moreover, *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae were indistinguishable by PFGE including *L. borgpetersenii* serovars Arborea and Castellonis (16). This technique was also useful for epidemiological investigation of leptospiral serovars among small rodents in Croatia (31). PFGE is technically require some specialised equipment such as contour-clamped homogeneous electricfield electrophoresis systems, but this method produces highly reproducible restriction profiles that typically show distinct well resolved fragments.

All above methods that analyze the chromosomal DNA has the limitation in which required the large amount of purified DNA or leptospiral culture. As a result, several methods based on analysis of PCR products have been developed. Randomly amplified polymorphic DNA (RAPD) techniques have been used to differentiate among a few serovars such as serovars Hardjo strain Hardjobovis and Hardjoprajitno, Pomona, Balcanica, Tarassovi, Swajizak, Kremastos, Australis, and Zanoni (32). Arbitrarily primed-polymerase chain reaction (AP-PCR) was used to derive species-specific probes for identification of *L. interrogans* (sensu stricto), *L. borgpetersenii*, and *L. kirschneri* by dot blotting (33). In addition, this method successfully differentiated at the strain level among the Hardjobovis group (28). Twenty-two mer primer has been found to give good resolution between different serovars and strains belonging to the same serovar of leptospires isolates from Andaman (34).

Recently, fluorescence amplified fragment length polymorphism (FAFLP) is used for genotyping and epidemiological study among the outbreak and sporadic cases in Andaman Islands (35). Although FAFLP have shown promise in rapid detection and identification of leptospires, however, this techniques need to be standardized and validated against the others molecular typing methods in the future. Insertion sequence elements (IS)-based PCR assay were used in serovar identification among leptospires. However, this method is limited because there are few full or partial copies of IS*1533* among several serovars. Thus some serovars could not be differentiated by this target (18). A new target as IS*1500*-based assay was developed for identification of *L. interrogans* (sensu stricto) isolates. The new target successfully differentiated numeroues genetic subgroups within serovar Pomona type kennewickii while IS*1533*-base assay could not distinguish (17).

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RFLP analysis of PCR-amplified 16S and 23S rRNA genes allowed the grouping of 48 serovars into 16 mapped restriction site polymorphism profiles (36). Using this approach, the genomospecies of *Leptospira* could be identified and the genotypes Hardjobovis and Hardjoprajitno of serovar Hardjo were clearly distinguished (28). A new repetitive DNA sequence, Rep1, was used as a target for develop PCR assay based on using a single oligonucleotide primer (iRep1). This method could differentiate among strains belonging to different species and serogroups of leptospires in Salvador, Brazil (37). One disadvantage of this technique was its inability to distinguish among isolates at the serovar level. However, it may be useful in discriminating serogroups from different animal reservoirs during an outbreak. More recently, multiple-locus variable number of tandam repeats (MLVA) assay is a new PCR-based technique for serovar identification among leptospiral. Seven loci of variable number tandam repeats (VNTR) were found to be a powerful marker for serovar identification, epidemiology and phylogenetic studies of *L. interrogans* (sensu stricto) serovars. However, serovars Australis and Bratislava, serovars Copenhageni and Icterohaemorrhagiae and serovars Romanica and Wolffi could not be distinguished to each others by this technique (19).

3. Microbiology

Leptospires are obligate aerobic spirochetes that share features of both Gram-positive and Gram-negative bacteria. This organism are a thin spiral about 0.25 x 6–25 µm in size and active motility by rotating (spinning) or bending. Because of their narrow diameter, the leptospires are best visualized by dark-field illumination or phase contrast microscopy (1). Moreover, There are flexible and able to pass through 0.2 µM pore size membrane filters. The helical conformation is right-handed with more than 18 coils per cell and coils have amplitude of 0.10-0.15 µm and a wavelength of approximately 0.5 µm **(Figure 1).** Leptospires are stained poorly but may be stained using carbol fushsin counterstain (2).

Electron microscopy shows a cylindrical cell body (protoplasmic cylinder) wound helically around an axistyle (0.01–0.02 µm in diameter), which comprises two axial filaments (a spirochetal form of a modified flagellum) inserted subterminally at the extremities of the cell body, with their free ends directed towards the middle of the

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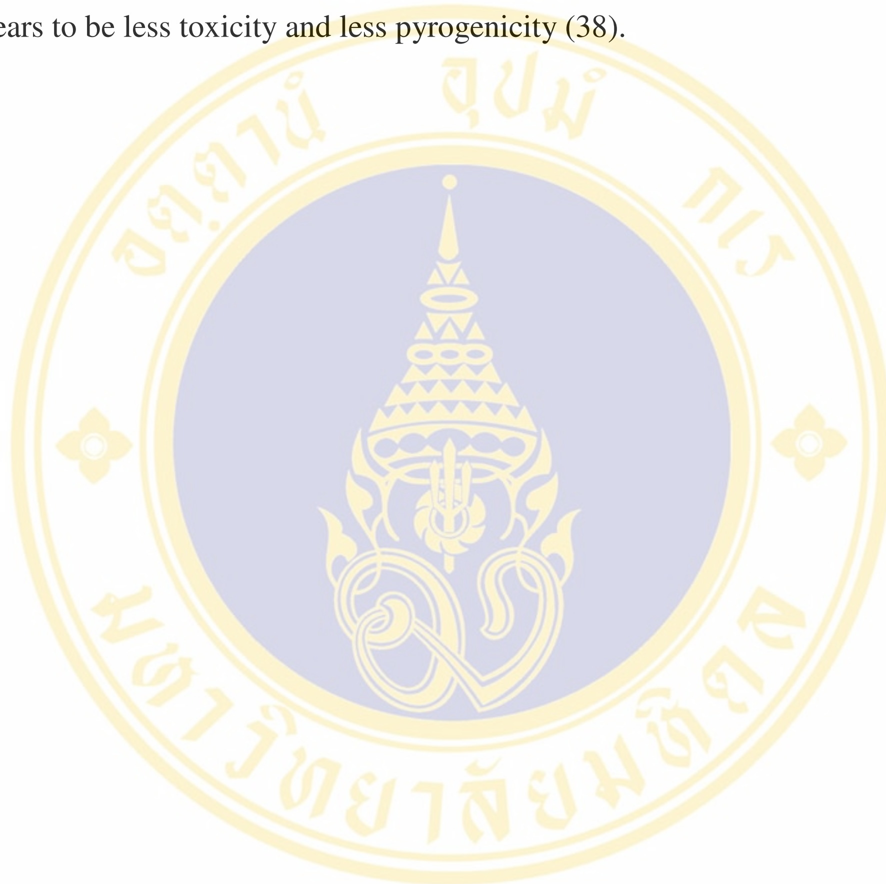
cell. An external sheath envelops the axistyle and protoplasmic cylinder, which is demarcated by a cytoplasmic membrane **(Figure 2)**. The axial filaments with the hook- and spiral-shaped end are thought to be a cytoskeletal element that enables movement (2). The leptospiral lipopolysaccharide (LPS) has a chemical and biological properties different from those of the classical LPS of gram-negative bacteria and appears to be less toxicity and less pyrogenicity (38).



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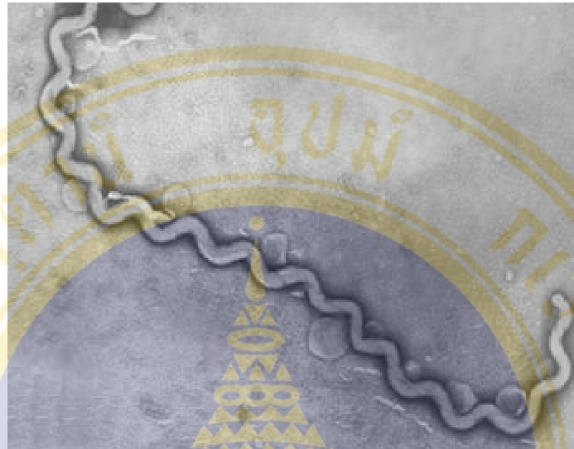

Figure 1 Electron micrograph of *Leptospira* spp.
(www.medicinapreventiva.com.ve/articulos/leptospira.htm)

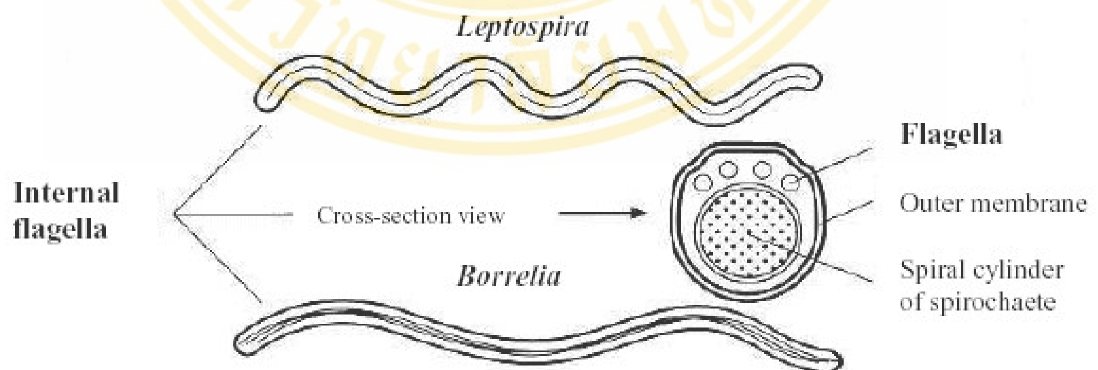

Figure 2 Longitudinal and cross-sectional view of *Leptospires* structure
(www.gsbs.utmb.edu/microbook/ch035.htm)

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4. Cultivation

Leptospires grow in the optimal temperature of 28°C to 30°C and optimal pH of 7.2 to 7.6. Culture of the leptospires are usually incubated for 7 to 14 days which average generation time varies from 7 to 12 hours (2). Vitamin B1, B12 and long chain fatty acids are the only organic compounds known to be necessary for growth of the leptospires. Vitamin B1 and B12 serve as growth factors. Fatty acid is their main source of energy and carbon. The nonessential nutrient pyruvate enhances the initiation growth of fastidious leptospires. Ammonium salts are an effective source of cellular nitrogen. Because of there are resistant to the antibacterial activity of the pyrimidine analogue, 5-fluorouracil, this compound is used in selective media for the isolation of leptospires from contaminated sources. Types of media used for the isolation and cultivation of leptospires are usually enriched with rabbit serum or bovine serum albumin (BSA) such as Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, Korthof's and Fletcher's (1). Leptospires usually not grow on solid media in colonies, which are in any event hard to see. Nonpigmented diffuse to discrete subsurface colonies commonly form on 1% agar and clear to turbid surface colonies occur on 2% agar. Some strains may grow as both surface and subsurface colonies on 1% or 2% agar (1). Pathogenic leptospires differs from saphophyts by its ability to grow in the presence of 8-azaguanine and its inability to grow at 13°C. Non-pathogens and avirulent strains of pathogens grow in media containing 0.85% NaCl at 30°C, but not at 37°C (39).

5. Molecular Biology

Recently, the completion of the genome sequences of *L. interrogans* serovar Lai and *L. interrogans* serovar Copenhageni has been achieved. The *L. interrogans* genome consists of two circular chromosomes with a total of 4,627 to 4,690 kilobase pairs (kb). Chromosome I is 4,277 to 4,332 kb and chromosome II is 350 to 359 kb. Moreover, The average percent identity of the nucleotides between the two genomes is 95% and the extrachromosomal elements have not been reported. The complete genome sequences revealed an average G+C content of 36% and approximately 4,500 predicted open reading frames (ORFs), among which more than 50% failed to exhibit similarities to proteins of known function or any protein in other organisms (40, 41).

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The comparative general genomic features of the *L. interrogans* serovars Copenhageni and Lai genomes are shown in **Table 4** (40). The small replicon of *L. interrogans* suggested being a second chromosome based upon the localization of the *metF* gene that encodes an essential methionine biosynthetic pathway enzyme, methylenetetrahydrofolate reductase (41). Moreover, the genes encoding enzymes for metabolic pathways, such as glycolysis and the tricarboxylic acid cycle, as well as the enzymes for biosynthesis pathways of amino acid and co-factor are also distributed between the two chromosomes (42). Unlike most other bacteria, rRNA genes in *L. interrogans* are not organized into operons but are scattered over the chromosome. Most leptospires contain two sets of 16S and 23S rRNA genes whereas in serovar Lai has only one 23S rRNA gene (41). For 5S rRNA gene, pathogenic leptospires contain only a single copy within the genome whereas non-pathogenic leptospires have two sets of the gene located several kb apart on the chromosome (40, 41). The extraordinarily low number of tRNA and rRNA genes might well account for the fastidious growth of *L. interrogans*.

A number of insertion sequences (IS) has been identified, IS*1500*, IS*1501*, IS*1502*, IS*1533* and IS*lin1* which scatter on both chromosomes by varying copy number in each serovar (40, 41, 43). Both IS*1500* and IS*1533* are found in many serovars (40, 41), but the copy number varies widely between different serovars and among strains within the same serovar. The number and distribution of IS*1501* and IS*lin1* differed dramatically between serovar Lai and Copenhageni, highlighting the impact these elements have in *Leptospira* species strain genome differentiation (40).

Lipopolysaccharides (LPSs) on the leptospiral surface distinguish from those of the other invasive spirochetes. The 40-kb *rfb* or O antigen biosynthesis gene locus of *L. interrogans* is likely to acquire through lateral transfer (44). Changes in genes involved in the LPS polysaccharide biosynthesis apparatus are thought to account for serovar diversity among leptospires. Flexibility in leptospiral LPS biosynthesis is thought to be a mechanism of adaptation to new animal host species and probably accounts for the remarkable diversity of pathogenic leptospiral serovars (40, 44). The varying number of genes in the *degT* family in *L. interrogans* has been found. DegT proteins are involved in the biosynthesis of the LPS O side chain and may account for serological characteristics (40). *L. interrogans* has three toxin-antitoxin systems that

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may mediate global gene regulation during nutritional stress (45). Moreover, leptospires have over 70 genes with putative regulatory roles (40) .

Table 4 General features of the *L. interrogans* serovars Copenhageni and Lai genomes (40)

Feature	Copenhageni		Lai	
	CI	CII	CI	CII
Size (bp)	4,277,185	350,181	4,322,241	358,943
G+C content (%)	35.1	35.0	36.0	36.1
Protein-coding genes				
With assigned function	1,811	161	1,901	159
Conserved and Hypothetical	1,643	113	2,459	208
Total	3,454	274	4,360	367
Transfer RNA	37	0	37	0
Ribosomal RNA genes				
23S	2	0	1	0
16S	2	0	2	0
5S	1	0	1	0
Insertion sequences	26	0	48	9
tmRNA[a]	0	0	0	0

tmRNA[a], transfer-messenger RNA

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6. Epidemiology

Leptospirosis is a zoonotic disease of worldwide distribution. The highest prevalence rates remain in tropical, developing countries where leptospirosis cases are increasing (1, 2). The rise of leptospirosis cases are associated with urban population growth, urban decay and flooding. Outbreaks have been related to heavy rainfall in various parts of the world including India (6) and Brazil (5). Leptospirosis is considered being an occupational disease such as farmers, sewer workers, miners, fisherman and veterinary medicine. The occupational risks have decreased since protective measures have been implemented. However, changing modes of human interaction with the environment may allow for additional routes of exposure and subsequent infection. In developed countries, outdoor leisure activity provides opportunity for infection such as wading, swimming, rafting and water-related activities in endemic countries (46, 47). In developing countries where leptospirosis occurs both endemically and epidemically, risk of exposure is not limited to those with circumscribed occupational or recreational risks but is generalized to the population at large.

The proximal renal tubules are the target for leptospires to maintain inside the maintenance animal host. An infected animal can remain symptom-free and shed infectious organisms in the urine for its entire lifetime (2). Human infection results from exposure to infected urine of carrier mammals, either directly or indirectly via contamination of soil or water. Although excretion of leptospires in human urine for months after recovery has been recorded (1), direct transmission between humans has been demonstrated rarely. The most important maintenance hosts are small mammals such as mice, rats and mongooses, which may transfer infection to domestic farm animals, dogs, and humans. In addition, some leptospiral serovars are commonly associated with particular animal reservoirs **(Table 5)** (2). Knowledge of the prevalent serovars and their maintenance hosts is essential to understanding the epidemiology of the disease in any region.

Leptospirosis in Thailand was first reported by Yunibandhu *et al* in 1943 (8). Before 1996, the occurrence of leptospirosis appeared sporadically with some outbreaks usually associated with flooding (48). In 1996, the epidemic began after flooding in Nakhon Ratchasima province of the northeast region (49). In 1997, it

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expanded to 16 out of 19 provinces in this region and spread to other regions of the country in 1998 (50). During the epidemic period, leptospirosis showed the same seasonal variations especially in the rainy season which the peak number of cases occures in September to October each year. Moreover, most affected persons have been farmers. After that, the incidence in Thailand was high and was considered as an important re-emerging infectious disease. Although the majority of cases have been found in the rural areas particularly in the northeastern part of Thailand but patients who live or work in urban areas like Bangkok and never go outside the city within 2 weeks have been recorded (51), remark to the physicians should be aware. In addition, most causes of acute undifferentiated febrile illnesses such as rickettsial infection, influenza, dengue fever, scrub typhus, malaria infections and leptospirosis can not be identified, due to the limitation of laboratory diagnosis. Thus, the actual incidence of leptospirosis is not yet fully known.

Rodents and shrews trapped in urban areas were found to be the reservoirs of leptospires as same as those trapped in rural areas (52). There are a number of serogroups presented in Thailand. In the past, rodents isolated leptospires which were commonly found in Bangkok was serovar Bataviae, whereas in rural areas especially in north and northeast Thailand were Autumnalis and Hebdomadis as detected by microscopic agglutination test (MAT) (48). Recently, the most common serotypes have found to be Pyrogenes, Sejroe and Bataviae, respectively (52). As exemplify by during 1999 and 2000, rodent isolated leptospires trapped from northeastern regions were found most predominantly in serovars Bataviae (77.6%), Pyrogenes (36.6%), Autumnalis (17.8%), Australis (3.9%), Javanica (2.9%), and Sejroe (0.9%), repectively. Morover, *Rattus norvegicus* was a major reservoir of serovar Pyrogenes whereas *Bandicota indica* was major reservoir of serovar Autumnalis, Bataviae, Pyrogenes, Javanica and Australis (53). Serological survey of leptospiral antibody among a variety of other animal species in epidemic provinces of the northeast region found in cattle, buffalo and swine as 77.2%, 86.1% and 60.4%, respectively. In addition, seroreactivity in cattle and buffalo was observed mainly with serogroups Sarmin, Ranarum, Sejroe. In swine, reactivity was observed mainly with serovars Sarmin, Ranarum, Bratislava and Pomona (54). There are only some reports about correlation between type of leptospires in patients and animals have identified. So far,

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the knowledge for identified the exactly animals for spreading of leptospirosis in human still unclear but most of researchers have believed that rodents are the mainly maintenance host of leptospirosis in human.

Table 5 Typical reservoir hosts of common leptospiral serovars (2)

Reservoir host	Serovar(s)
Pigs	Pomona, Tarassovi
Cattle	Hardjo, Pomona
Horses	Bratislava
Dogs	Canicola
Sheep	Hardjo
Racoon	Grippotyphosa
Rats	Icterohaemorrhagiae, Copenhageni
Mice	Arborea, Ballum, Bim
Marsupials	Grippotyphosa
Bats	Cynopteri, Wolffi

7. Pathogenesis

Host infection by pathogenic *Leptospira* produces a diverse array of clinical manifestations ranging from subclinical infection to undifferentiated febrile illness to jaundice, renal failure and potentially lethal pulmonary haemorrhage. Disease determinants for leptospirosis presumably relate to exposures that influence the inoculum size during infection, host factors and the pathogen's virulence characteristics. To date, several putative virulence factors have suggested. However, the pathogenetic mechanism of leptospirosis is yet to be clarified.

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7.1 Mobility and attachment

The unique feature of *Leptospira* pathogenesis is the ability of the pathogen to rapidly penetrate and disseminate during host infection and establish persistent colonization in the renal tubules. Motility is one virulence factor of leptospires and probably important in initial infection and dissemination of organisms from the site of entry to sites of end-organ damage such as lung, liver, kidney, eye, and brain (2). Associated with motility, twice methyl-accepting chemotaxis proteins, which are likely to confer selective advantages in adapting to and migrating through host tissues, were also identified (41, 55). Virulent *Leptospira* strains, but not avirulent or saprophytic strains, have been shown to exhibit chemotaxis towards haemoglobin (56). Adherence of leptospires to host mammalian cells plays an essential role in pathogenesis of leptospirosis. Host cell entry may play a role in the rapid dissemination of leptospires as *L. interrogans* has been shown to rapidly translocate across polarized MDCK monolayers without disrupting tight junctions (57). Electron microscopy studies found that leptospires tightly adhere to host cell plasma membrane during the translocation process, suggesting that this type of association may be required early step for translocation (57). In addition, leptospires attach tigthly to the luminal surface of the renal epithelium in order to establish persistent carriage in animal reservoirs (58).

Surface-exposed proteins are presumably the factors that mediate adherence of the leptospiral pathogen to mammalian host cells. A 36 kDa surface exposed protein which potentially involved in fibronectin binding is identified in virulent *L. interrogans* but absent in the culture-attenuated form of that strain (59). More recently, leptospiral immunoglobulin-like protein, LigA and LigB, was identified which has tandem repeats of the bacterial immunoglobulin-like (Big) domain. This repeat motif is found in bacterial virulence factors that mediate mammalian host cell adherence and invasion, such as *Escherichia coli* intimin and *Yersinia pseudotuberculosis* invasin. In addition, *lig* genes are present in pathogenic but not saprophytic *Leptospira* species. The expression of *lig* gene is significantly reduced or lost as virulent strains are attenuated during culture passage (60). Interestingly, Lig proteins may play an analogous role to intimin and invasin in mediating host cell interactions during leptospiral pathogenesis.

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7.2 Toxicity

Several cellular components of *L. interrogans*, such as hemolysins (61), lipopolysaccharide (62), glycolipoprotein (63) and peptidoglycan (64), have been described as toxic and may participate in the pathogenesis of the disease. Leptospiral lipopolysaccharide showed greater cytotoxicity and aggregation of platelets (62). A glycolipoprotein (GLP) fraction extracted from *Leptospira interrogans* is demonstrated by the blocking sodium-potassium ATPase (Na^{+},K^{+}-ATPase) pump activity in the rabbit renal tubule epithelial cells (63). This may be one of the mechanisms of electrolytical disorders such as renal dysfunction and cardiac arrhythmia that is characteristic of the disease. Other toxic effects exerted by GLP may be observed in cultures of Vero and L929 cells, leading to cell membrane perforations followed by cell death (63) . Moreover, GLP can be detected in the damaged tissues of patients and experimental animals model (65).

Haemolytic, sphingomyelinase, and phospholipase activities have been described *in vitro* (66). Specific genes have been characterized including haemolysins, sphingomyelinase C, sphingomyelinase H, and haemolysis associated protein-1 (Hap1, also known as LipL32) (67). Sphingomyelinase H (SphH) of *L. interrogans* serovar Lai neither showed sphingomyelinase nor phospholipase activities, whereas SphA was known to have sphingomyelinase activity. In addition, SphH was shown to be a cytotoxic pore-forming protein on several mammalian cells (68). Lipoproteins, such as LipL 32 and LipL 41, are also potential toxic factors in leptospirosis and may be involved in disease pathogenesis through their ability to trigger the host inflammatory response (69).

8. Immune response

The knowledge of mechanisms of host immunity to *Leptospira* or the role of host immunity in pathogenesis is still unclear. Naturally acquired immunity that protects against reinfection by *Leptospira* does occur and has been assumed to be humorally mediated (2). Protective immunity may be engenderd by antibodies directed against serovar-specific leptospiral lipopolysaccharide. Leptospiral lipopolysaccharide stimulates the innate immune system via a Toll-like receptor 2 (TLR2)–dependent mechanism, another potential mechanism of either protective immunity or

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immunopathogenesis (70). There is also evidence that antibodies specific to *Leptospira* membrane-associated proteins may play a role in host defense, but such evidence is not definitive (71).

The role of cell-mediated immunity in host defense to *Leptospira* remains poorly understood in both animal models and human disease. Insight into protective humoral and cellular immune responses against *Leptospira* has been gained from studies of cattle given a killed *L. borgpetersenii* vaccine. A recent study has shown that cattle immunized with a killed *Leptospira* vaccine have $CD4^+$ T cells and γδT cells that give in vitro proliferative responses and produce IFN-γ following stimulation with a *Leptospira* antigens preparation (72). Glycolipoprotein from *L interrogans* has been shown to induce in vitro production of TNF- α, IL-10 and IL-6 as well as up-regulate the expression of CD69 and HLA DR on PBMCs (73, 74). Human peripheral blood mononuclear cells from leptospirosis-naive individuals are stimulated to produce large quantities of IFN-γ producing $TCR\gamma\delta^+$ T cells during *in vitro* stimulation by pathogenic leptospires (75), and those PBMC-derived dendritic cells, when stimulated by leptospires, secrete IL-12 and IFN-γ-inducing cytokines IL-12p40 (76). Moreover, $TCR\gamma\delta^+$ T-cell concentrations are increased in peripheral blood of patients with acute leptospirosis (75). Bacterial stimulation of TCR $\gamma\delta^+$ T cells would also have important consequences, such as prompt release of proinflammatory cytokines and chemokines capable of facilitating the onset of local inflammation. However, the functional and phenotypic characteristics of T cell populations in human leptospirosis in term of pathogenesis and protection remain to be elucidated.

9. Clinical features

Human is an accidental host of leptospirosis. The clinical manifestation of leptospirosis in human mimics to many others diseases and is highly variable. Typically, leptospirosis occurs as two from of clinically recognizable syndromes. The most common form of syndrome is anicteric leptospirosis (biphasic illness) that usually self-limited and occurs in 85% to 90% of the cases. There are two clearly defined stage in anicteric leptospirosis, the first phase is commonly referred to as the septicemia phase which followed by the immune phase of illness. Other form is icteric leptospirosis, or also known as Weil's syndrome, is characterized by a fulminant

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course with rapid onset of hepatic and renal failure and high mortality that occurs in 5% to 10% of the cases (77).

9.1 Anicteric Leptospirosis

In the biphasic illness, the initial acute or septicaemic phase is characterized by bacteraemia that typically lasts about 1 week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine. Fever, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting, and prostration are usually characterized in the septicemia phase. The most common physical finding is conjunctival suffusion in the absence of purulent discharge. Other signs include masculopapular skin rash, pharyngeal injection, lymphadenopathy, splenomegaly, hepatomegaly and muscle tenderess. Moreover, leptospires can be isolated from the blood and the CSF during this phase. The resolution of symptoms may coincide with the immune phase when antibodies begin to be produced, accompanied by excretion of spirochetes in the urine. However, fever may recur after a remission of 3–4 days, producing a biphasic illness. In most cases, the biphasic disease is not clinically distinguishable from other undifferentiated febrile illness syndromes. Less than one-quarter of all leptospirosis cases may be seen aseptic meningitis, which neurological features include headache, vomiting, and signs of meningeal irritation. Examination of the CSF shows increased opening pressure, raised protein, normal glucose, and lymphocytic pleocytosis. Antibodies can detect during this phase but *Leptospira* cannot be isolated. It is uncommon for leptospirosis to present as a primary neurological disease (77).

9.2 Icteric Leptospirosis

Icteric leptospirosis or Weil's disease is a form of disease characterized by symptoms of hepatic, renal and vascular dysfunction. The clinical manifestations vary in terms of severity and symptomatology. Some patients with jaundice may have no renal manifestation. This syndrome can develop after the acute phase as the second phase of a biphasic illness, or simply present as a single, progressive illness. Between 5 and 10% of all patients with leptospirosis have the icteric form of the disease. The jaundice occurring in leptospirosis but there is not associated with hepatocellular destruction. Hepatic dysfunction occurs but it resolves and it is rarely the cause of death. Serum bilirubin levels may be high, and many weeks may be required for

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normalization. There are moderate rises in transaminase levels, and minor elevation of the alkaline phosphatase level usually occurs. Leptospirosis is a common cause of acute renal failure (ARF). ARF is frequently leading to multiple organ failure and to death in one-third of the patients. Oliguria is a significant predictor of death. Hypokalemia frequently occurs in ARF. Vasculitis is observed in the acute phase of the disease. Tubular necrosis and interstitial nephritis are responsible for renal failure. Thrombocytopenia is common around 50 to 93% of cases, is transient, and does not result from disseminated intravascular coagulation. Hyperamylasemia has also commonly found in patients with severe leptospirosis (1).

9.3 Other complications

Severe hemorrhagic pneumonitis may be a prominent manifestation of infection (78). Three radiographic patterns have been described in patients with pulmonary involvement during leptospiral infection; small nodular densities, diffuse ground glass densities and rarely, confluent areas of consolidation (79). Epidemic leptospirosis with pulmonary haemorrhage without jaundice or renal failure has been reported (4). Severe pulmonary involvement in leptospirosis has been reported from Andaman and Nicobar islands. Serovar Grippotyphosa (80) and more recently, serovar Valbuzzi (81), have caused such outbreaks. The cardiac involvement is common in any form of leptospirosis and can manifest as myocarditis, congestive heart failure and nonspecific electrocadiogram (EKG) abnormalities. Arrhythmias have considered a poor prognostic indicator in severe leptospirosis cases and have shown more common in patients dying of leptospirosis than in the survivors (82). Pathological findings of cardiac involvement include interstitial myocarditis with lymphocytes and plasma cells infiltration, mononuclear infiltration and petechial hemorrhages in the epicardium, pericardial effusions and coronary arteritis (1).

Ocular manifestations of leptospirosis can lead to significant morbidity. During the acute phase of illness conjunctival congestion is a common clinical finding. Uveitis is a rare sequel of the disease. It usually presents as a panuveitis with or without hypopyon. Visual damage can occur in as many as 35% of patients. Furthermore, uveitis is an important late complication that can cause reversible or irreversible blindness in people and in horses. Nongranulomatous uveitis, hypopyon,

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vitreous inflammatory reaction more specifically, membranous veil-like opacities, disc edema, and retinal periphlebitis are characteristic features of leptospiral uveitis (83).

10. Diagnosis

Diagnosis of leptospirosis depends on simple diagnostic tests, which are often not done because of a low index of clinical suspicion. Therefore, specific microbiological tests are necessary for confirmation of the diagnosis. Several non-specific findings may include increased Erythrocyte Sedimentation Rate (ESR, mild increase in transaminases, alkaline phosphatase, amylase and bilirubin. Urinalysis shows proteinuria, pyuria and microscopic haematuria. Moreover, hyaline and granular casts may also be present during the first week of illness. CSF examination may initially show a predominance of polymorphs or lymphocytes, but later examination almost show that lymphocytes predominate. CSF protein may be normal or elevated, while CSF glucose is usually normal. In patients with severe jaundice, xanthochromia may occur. Renal function impairment is indicated by raised plasma creatinine levels. Thrombocytopenia is common and may be marked (2).

10.1 Direct examination

Dark-field microscopy is most often used to observe leptospires in clinical specimens such as blood and urine (1). However, artefacts like lysed red blood cells or fibrils may be mistaken for leptospires. For this reason, direct dark-field microscopy is not recommended as the only diagnostic procedure to be used.

10.2 Culture

Leptospires can be isolated from blood and CSF samples during the first 10 days of infection, and from urine after the second week up to 3 months (77). Urine sample should be collected by aseptically and inoculated within 2 hours after voiding because leptospires die quickly in urine (1). The optimum temperature for culture is 28-30 °C in the most wildly used EMJH medium and examined weekly by dark-field microscopy for up to 13 weeks before being discardrd. Contaminated cultures may be passed through a 0.2 μm or 0.45 μm filter before subculture into fresh medium (1). Because of culture of leptospires is very tedious, complicated, time consuming and low sensitivity. It is rarely used for diagnostic confirmation.

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10.3 Antigen detection

Detection of leptospiral antigens in clinical materials would offer greater specificity and sensitivity than dark-field microscopy. Enzyme-linked immunosorbent assay (ELISA) method could detect 10^5 leptospires/ml and radioimmunoassay (RIA) was more sensitive than dark-field microscopy but less sensitive than culture when applied to porcine urine (84). A double sandwich ELISA could detect 10^4 leptospires of serovar Hardjo but less sensitive of other serovars (85). A chemiluminescence immunoassay was applied to human blood and urine but was no more sensitive than earlier ELISA (86). Immunomagnetic antigen capture was combined with fluoroimmnoassay to detect as few as 10^2 leptospires/ml in urine of cattle infected with serovar Hardjo (87). Monoclonal antibody based dot ELISA could detect *Leptospira* antigen in urine samples of patients whose collected on the first day of hospitalization whereas IgM antibody detection assays showed seronegative (88).

10.4 Serology

The reference method for serological diagnosis of leptospirosis is the microscopic agglutination test (MAT), in which patient sera are reacted with live antigen suspensions of leptospiral serovar. After incubation, the serum-antigen mixtures are examined microscopically for agglutination and the titers are determined. The accepted endpoint of agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are agglutinated (1). A fourfold increase in antibody titer of paired (acute and convalescent phase) sera, or a conversion from seronegativity to a titer of 100 or above, is usually accepted in confirming a positive MAT (1). The results of this assay provide useful epidemiologic data in the form of presumptive serogroup and serovar. However, this assay is not suitable for routine laboratories since it is technically demanding, costly, requires the maintenance of live, hazardous stock serovar cultures and also requires analyses of paired sera to verify the seroconversion which delays the diagnosis (2).

Several other alternatives of antibody detection assays have subsequently been developed for early diagnosis of leptospirosis. These include the indirect hemagglutination assay (89), the microcapsule agglutination test (90), the enzymes-linked immunosorbent assay (ELISA) for immunoglobulin M (IgM) antibodies (91), dot blot-enzyme-linked immunosorbent assay (dot-ELISA) (92) and the Lepto

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Dipstick (93). Several studies have shown that assays which detected IgM were more sensitive than MAT which gave positive results earlier in the acute phase of the disease and permitted the use of a single serum sample (94). Among these assays, IgM-ELISA has been widely used, because IgM antibodies usually become detectable during the first week of illness. One commercial IgM-ELISA was found to be 100% sensitivity and 93% specificity when compared with the MAT (95). Dot-ELISA was developed to detect IgM, IgG, and IgA. In acute phase leptospirosis, dot-ELISA with sera from patients collected until the 14th day of illness detected IgM in 98%, IgG in 70% and IgA in 76% (96). A commercially available indirect hemagglutination assay has been demonstrated to have 100% sensitivity and 94% specificity for diagnosing acute leptospirosis. This assay can detect both IgM and IgG (89). A microcapsule agglutination test is more sensitive than either the MAT or an IgM-ELISA in early-acute-phase samples, but fails to detect infections caused by some serovars (90). Lepto Dipstick for detection of *Leptospira*-specific IgM antibodies in human sera was evaluated in 1997 (93). The sensitivity and specificity of this assay agree as well with IgM-ELISA. However, The dipstick assay revealed cross-reactivity with sera from patients with other diseases such as HIV and malaria (93). Due to this assay ease to use and storage, Lepto Dipstick may prove a valuable diagnostic tool especially where laboratory resources are limited.

10.5 Molecular methods

In 1987, dot blot hybridization with ^{32}P- and biotin-labelled probes (97) and *in situ* hybridization assays (98) were firstly demonstrated as early detection leptospires in clinical specimens. Specific probes to Hardjobovis strain were developed and applied to detect leptospires in bovine urine (99). After that, alternative molecular techniques based mainly on polymerase chain reaction (PCR) showed the higher sensitivity than DNA hybridization (100). Then PCR has become a widely used in direct detection of leptospires in clinical samples for early diagnosis. Detection of leptospires in serum of patients with leptospirosis by using PCR has been reported by Gravekamp et al (101). Using a mixture of primer sets G1/G2 and B64-I/B64-II derived from genomic DNA libraries of leptospiral serovars Icterohaemorrhagiae (RGA) and Bim (strain 501), all pathogenic leptospires such as serovars Icterohaemorrhagiae, Copenhageni, Hardjo, Pomona, Grippotyphosa and Bim were

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detected in serum samples collected from patients during the first 10 days after the onset of illness (101). Merien et al. (102) developed PCR for detection of leptospires in urine samples. A 331-bp fragment of the *rrs* (16S rRNA) gene of both pathogenic and nonpathogenic leptospires was amplified. Unfortunately, false positive may be occur when the sample has the contamination of non-pathogenic leptospires (102). A real-time quantitative PCR assay using TaqMan chemistry which targeted an 87 bp section of the 16S rRNA gene of leptospires was developed in clinical and environmental samples (103). Real-time PCR is sensitive and can differentiate between pathogenic and non-pathogenic species. Moreover, this method can be used in patients who started on antibiotics already. More recently, real-time PCR by SYBR green fluorescence was developed using a 423 bp target on lipL32 gene, which is conserved among pathogenic leptospires. This technique show successfully detected leptospiral DNA from serum and urine samples of patients with leptospirosis, with the exception of *L. fainei* serovar Hurstbridge (104). Rugsasuk developed PCR for differentiation among pathogenic and non-pathogenic leptospires by using 23S rRNA gene as a target. This assay successfully differentiated between pathogenic and non-pathogenic *Leptospira* serovars which commonly found in Thailand (105).

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CHAPTER IV
MATERIALS AND METHODS

1. *Leptospira* reference strains

Of twenty-seven reference serovars, 26 were pathogenic. The pathogenic *Leptospira* species included 17 *L. interrogans,* 4 *L. borgpetersenii*, 2 *L. weilii* and one of each *L. kirscheneri*, *L. noguchii* and *L. meyeri.* A non-pathogenic serovar was identified as *L. biflexa*. These serovars were obtained from National Institute of Health (NIH), Thailand and Armed Forces Research Institute of Medical Sciences (AFRIMS) as listed in **Table 6**.

2. Rat *Leptospira* isolates

Forty leptospires isolated from field rats in the northeastern regions of Thailand during 1999-2000 were provided by LTC. Duangporn Phulsuksombati, AFRIMS. Serovars of these rat isolates were identified and characterized by microscopic agglutination test (MAT), monoclonal antibody coated latex test and cross absorbtion agglutination test (CAAT). Forty leptospiral strains were isolated from rat species including *Rattus norvegicus* (33 isolates), *Rattus exulans* (1 isolate), *Rattus losea* (1 isolate), *Bandicota indica* (4 isolates) and *Bandicota savilei* (1 isolate). Results of serovar identification by serological tests were obtained from AFRIMS. Detail of rat species and *Leptospira* serovars of rat isolates are shown in **Table 7**.

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Table 6 Serogroup, serovar, strain, and species used in this study

	No.	Serogroup	Serovar	Strain	species
Pathogenic *Leptospira* (n = 26)	1	Australis	Australis	Ballico	*L. interrogans*
	2		Bangkok	Bangkok D 92	*L. interrogans*
	3		Bratislava	Jez Bratislava	*L. interrogans*
	4	Autumnalis	Autumnalis	Akiyami A	*L. interrogans*
	5		New	Heusden P2062	*L. interrogans*
	6		Rachamati	Rachmat	*L. interrogans*
	7	Ballum	Ballum	MUS 127	*L. borgpetersenii*
	8	Bataviae	Bataviae	Van Tienam	*L. interrogans*
	9	Canicola	Canicola	Hound Utrech IV	*L. interrogans*
	10	Cellidoni	Cellidoni	Celledoni	*L. weilii*
	11	Djasiman	Djasiman	Djasiman	*L. interrogans*
	12	Grippotyphosa	Grippotyphosa	Moskva V	*L. kirscheneri*
	13	Hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*
	14	Icterohaemorrhagiae	Copenhegeni	M 20	*L. interrogans*
	15		Icterohaemorrhagiae	RGA	*L. interrogans*
	16	Javanica	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*
	17	Louisiana	Saigon	L 79	*L. interrogans*
	18	Panama	Panama	CZ 214 K	*L. noguchii*
	19	Pomona	Pomona	Pomona	*L. interrogans*
	20	Pyrogenes	Zanoni	Zanoni	*L. interrogans*
	21	Ranarum	Ranarum	ICF	*L. meyeri*
	22	Sarmin	Sarmin	Sarmin	*L. weilii*
	23	Sejroe	Hardjo	Hardjoprajitno	*L. interrogans*
	24		Sejroe	M84	*L. borgpetersenii*
	25		Wolffi	3750	*L. interrogans*
	26	Tarassovi	Tarassovi	Perepelicin	*L. borgpetersenii*
Non-pathogenic *Leptospira*	27	Semaranga	Patoc	Patoc I	*L. biflexa*

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Table 7 Rat species and *Leptospira* isolated from field rats in the Northeastern regions of Thailand during 1999-2000

No.	Lab Code	Province of isolate	Rat species	Serovar identification		
				MAT	CAAT	MAb coated latex test
R1	050	Buri Ram	*B. indica*[1]	Autumnalis, Djasiman	Autumanalis	Autumnalis
R2	526	Khon Kaen	*B. indica*	Autumnalis, Djasiman	Autumanalis	Autumnalis
R3	571	Khon Kaen	*R. exulans*[2]	Pyrogenes	ND*	Pyrogenes
R4	574	Khon Kaen	*R. norvegicus*[3]	Pyrogenes	Pyrogenes strain Salinem	Pyrogenes
R5	578	Khon Kaen	*R. norvegicus*	Pyrogenes	Pyrogenes strain Salinem	Pyrogenes
R6	783	Nakorn Phanom	*R. losea*[4]	Australis, Bangkok	ND	Australis
R7	809	Nakorn Phanom	*B. indica*	Australis, Bangkok	Australis	Australis
R8	853	Khon Kaen	*R. norvegicus*	Pyrogenes	Pyrogenes strain Salinem	Pyrogenes
R9	865	Buri Ram	*B. indica*	Autumnalis, Bratislava, Djasiman	ND	Autumnalis
R10	941	Khon Kaen	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	Pyrogenes strain Salinem	Pyrogenes
R11	992	Khon Kaen	*R. norvegicus*	Pyrogenes	Pyrogenes strain Salinem	Pyrogenes
R12	1018	Surin	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R13	1020	Surin	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R14	1078	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R15	1083	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R16	1090	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R17	1107	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae

MAT, microscopic agglutination test; CAAT, cross-agglutination-absorption test; MAb coated latex test, monoclonal coated latex test
*ND, Not done; [1]*Bandicota indica*, [2]*Rattus exulans*, [3]*Rattus norvegicus*, [4]*Rattus losea*, [5]*Bandicota savilei*

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Table 7 Rat species and *Leptospira* isolated from field rats in the Northeastern regions of Thailand during 1999-2000 (continued)

No.	Lab Code	Province of isolate	Rat species	Serovar identification		
				MAT	CAAT	MAb coated latex test
R18	1131	Buri Ram	*B. savilei*[5]	ND	ND	ND
R19	1133	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R20	1134	Buri Ram	*R. norvegicus*	Bataviae, Tarassovi	ND	Bataviae
R21	1172	Buri Ram	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R22	1175	Kala Sin	*R. norvegicus*	Pyrogenes, Icterohaemorrhagiae, Sarmin	ND	Pyrogenes
R23	1183	Kala Sin	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R24	1186	Kala Sin	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R25	1209	Kala Sin	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R26	1210	Kala Sin	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R27	1222	Khon Kaen	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R28	1229	Khon Kaen	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R29	1237	Khon Kaen	*R. norvegicus*	Pyrogenes	ND	Pyrogenes
R30	1241	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R31	1244	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R32	1246	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R33	1252	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R34	1264	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes

MAT, microscopic agglutination test; CAAT, cross-agglutination-absorption test; MAb coated latex test, monoclonal coated latex test
*ND, Not done; [1]*Bandicota indica*, [2]*Rattus exulans*, [3]*Rattus norvegicus*, [4]*Rattus losea*, [5]*Bandicota savilei*

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Table 7 Rat species and *Leptospira* isolated from field rats in the Northeastern regions of Thailand during 1999-2000 (continued)

No.	Lab Code	Province of isolate	Rat species	Serovar Identification		
				MAT	**CAAT**	**MAb coated latex test**
R35	1265	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R36	1266	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R37	1277	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R38	1278	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R39	1283	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes
R40	1290	Nakorn Phanom	*R. norvegicus*	Icterohaemorrhagiae, Pyrogenes	ND	Pyrogenes

MAT, microscopic agglutination test; CAAT, cross-agglutination-absorption test; MAb coated latex test, monoclonal coated latex test
*ND, Not done; [1]*Bandicota indica*, [2]*Rattus exulans*, [3]*Rattus norvegicus*, [4]*Rattus losea*, [5]*Bandicota savilei*

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3. Human isolates from patient with leptospirosis

Serovars of three clinical isolates were identified. Serovar of a Pyrogenes strain Salinem identified by MAT at AFRIMS and immunoblotting at the Department of Pathobiology, Faculty of Science, Mahidol University. In addition, this strain was isolated from a leptospirosis patient in Buri Ram province in 1999. The remaining 2 isolates obtained from Loei province in 2003 were characterized in three serovars as Autumnalis, Bratislava and New by immunoblotting at the Department of Pathobiology, Faculty of Science, Mahidol University.

4. Culture conditions

Leptospires were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (**Appendix A)** for up to 7-14 day at 29°C -30°C.

5. Pulsed-field gel electrophoresis (PFGE)

5.1 Preparation of bacterial DNA

Leptospiral cultures in EMJH liquid medium which had optical density of 0.20 at a wavelength of 420 nm were used in the experiment. Twenty milliliters (10^9 cells) of leptospira cultures were pelleted by centrifugation at 10,000 × *g* for 10 minutes at 4°C. Supernatant was discarded and the pellet was washed with TE buffer (100 mM Tris pH 7.5, 100 mM EDTA pH 7.5), centrifuged at 4,000 × *g* for 15 minutes at 24°C and then resuspended in 200 µl of TE buffer. Agarose plug was prepared by mixing an equal volume of the cell suspension with 1.6% certified low melt agarose (Bio-Rad Laboratories, U.S.A.). The mixture was immediately and carefully dispensed into a plug mold. After solidification, the agarose plugs containing leptospires were incubated in 1.5 milliliters of lysis solution (ESP solution: 0.5 M EDTA pH 9.0, 1% sodium-lauroyl-sarcosine, 1 mg/ml of proteinase K) and left overnight at 55°C. Subsequently, lysis solution was removed and the agarose plugs were washed three times in 10 milliliters of Plug Wash Buffer (10.0 mM Tris, 1.0 M EDTA pH 7.5). For each wash, the agarose plugs were incubated in the solution for 20 minutes at 50°C with gentle mixing (about 150 strokes/min) in an incubator shaker. The agarose plugs containing intact *Leptospira* genomic DNA were stored in fresh Plug Wash Buffer at 4°C until used.

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5.2 Restriction endonuclease digestion

Prior to digestion, the agarose plugs containing the intact *Leptospira* genomic DNA were cut into 2-3 mm thick pieces. The plugs were preincubated with 100 µl of 1X *Not*I restriction buffer for 1 hour at room temperature. The buffer was removed and replaced with a fresh mixture containing 30 U of *Not*I restriction enzyme in 1X restriction buffer. The digestion was carried out at 37°C for 8 hours.

5.3 Electrophoresis

The plug slices were placed on the ends of the comb teeth before placing the comb into the comb holder. Then, 1% Pulsed field certified agarose (Bio-Rad Laboratories, USA) was poured into the gel casting platform. Electrophoresis of the prepared samples was performed on a contour-clamped homogeneous electric field CHEF DR III system (Bio-Rad Laboratories, USA) with 2.5 liters of 0.5X TBE running buffer (Tris-borate-EDTA pH 8.0). The condition for running is initial switch time 2.2 seconds; final switch time 35.0 seconds; runing time 22 hours; 120° angle; gradient 6.0 V/cm; temperature 14°C. After electrophoresis, the gel was stained for 1 hour in 250 milliliters of sterile solution containing 1 mg of ethidium bromide and destained in three times of 30 minutes each by using 250 milliliters of sterile distilled water. The gel was photographed with Gel Documentation (SynGene, USA). A standard molecular weight marker (Bio-Rad Laboratories, USA) consisting of concatemers of the λ phage starting at 48.5 kb was used.

6. Cluster analysis of fingerprinting patterns from PFGE

DNA restriction patterns of isolates were analyzed for the genetic relationship by Dice Coefficient (106). Following is Dice Coefficient equation:

Coefficient (b) = nzy / (nx + ny - nxy)

Where nx and ny are the numbers of bands in lane x and lane y, respectively, and nxy is a number of shared bands between the two lanes. The larger number of coefficient reflects the higher number of the matching bands. After the comparison of the electrophoretic bands, cluster analysis was performed. Dendogram was constructed based on the unweighted pair group matching band average (UPGMA) and the similarity value in the matrix, with the aid of BIO-PROFIL software (Vilber Lourmat, Marne-La-Vallec, France).

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7. Typeability, reproducibility and discriminatory power

Typeability, reproducibility and discriminatory power of PFGE were calculated.

Typeability is the percentage of isolates that can be assigned a positive typing marker and can be defined as the ratio between the number of strains producing a banding pattern and the number of strains tested (107). Reproducibility is the percentage of isolates that give the same result on repeated testing (107). Discriminatory power is its ability to distinguish between unrelated strains and was calculated as using the following equation (108). Values between 1.0 and 0 may be obtained. The value of 1.0 denotes that all strains are differentiated into individual types and the value of 0 assigns all strains studied to the same type.

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{s} n_j (n_j - 1)$$

Where N is the total number of strains in the sample population. S is the total number of types described, an nj is the number of strains belonging to the jth type.

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CHAPTER V
RESULTS

1. PFGE analysis for differentiation of leptospiral reference serovars

1.1 Fingerprinting patterns of leptospiral reference serovars given by *Not*I restriction enzyme

Fingerprinting patterns for 27 reference serovar strains generated with restriction enzyme *Not*I resulted in 26 different patterns as designated as PFGE pattern 1-26 **(Figure 3).** Twenty-seven reference serovars gave different fingerprinting patterns with the exception for serovars Copenhageni and Icterohaemorrhagiae. The fingerprinting patterns of these strains were reproducible and showed marked heterogeneity between member serovars. Summarized the results of PFGE typing and strain found in Thailand are shown in **Table 8.** These 26 fingerprinting patterns were described as PFGE patterns 1 (serovar Australis), 2 (serovar Bangkok), 3 (serovar Bratislava), 4 (serovar Autumnalis), 5 (serovar New), 6 (serovar Rachamati), 7 (serovar Ballum), 8 (serovar Bataviae), 9 (serovar Canicola), 10 (serovar Cellidoni), 11 (serovar Djasiman), 12 (serovar Grippotyphosa), 13 (serovar Hebdomadis), 14 (serovar Copenhageni and Icterohaemorrhagiae), 15 (serovar Javanica), 16 (serovar Saigon), 17 (serovar Panama), 18 (serovar Pomona), 19 (serovar Zanoni), 20 (serovar Ranarum), 21 (serovar Sarmin), 22 (serovar Hardjo), 23 (serovar Sejroe), 24 (serovar Wolffi), 25 (serovar Tarassovi) and 26 (serovar Patoc). The discrimination power of these strains by PFGE was 0.99 and the calculation is shown in **Appendix C.**

Serovars Australis, Bangkok, Bratislava, Autumnalis, New, Bataviae, Canicola, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Saigon, Pomona, Ranarum, Sejroe, Wolffi and Tarassovi are the most commonly found in Thailand. In this study, PFGE potentially differentiated of these serovars and each serovars gave different fingerprint patterns as seen in **Figure 3 (lanes 1-5, 8, 9, 13-17, 19, 21, 24-26).** Thus, it makes sure that these serovars were typeability by PFGE.

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A restriction enzyme digestion of these 27 reference serovars DNA with *Not*I produced at least 8 to more than 17 bands with sizes ranging from less than 48.5 kb to more than 485.0 kb. No common band was found among these 27 strains. Moreover, member of serovars in the same serogroup showed distincts fingerprinting patterns. Serogroup Australis (serovars Australis, Bangkok and Bratislava) produced PFGE patterns 1, 2 and 3, respectively. Serogroup Autumnalis (serovars Autumnalis, New and Rachamati) produced PFGE patterns 4, 5 and 6, respectively. Serogroup Sejroe (serovars Hardjo, Sejroe and Wolffi) produced PFGE patterns 22, 23 and 24, respectively.

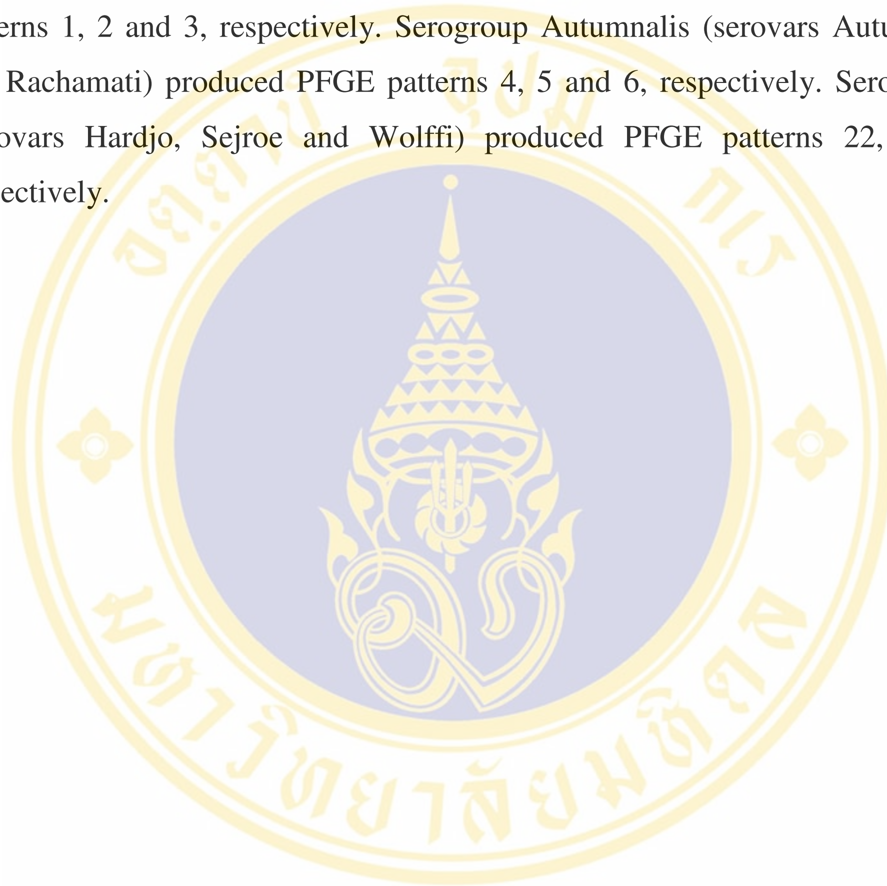

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Figure 3 PFGE fingerprinting patterns of *Not*I digested chromosomal DNA of 27 reference *Leptospira* serovars

Lane M: lambda marker
Lane 1: Australis
Lane 2: Bangkok
Lane 3: Bratislava
Lane 4: Autumnalis
Lane 5: New
Lane 6: Rachamati
Lane 7: Ballum
Lane 8: Bataviae
Lane 9: Canicola
Lane 10: Cellidoni
Lane 11: Djasiman
Lane 12: Grippotyphosa
Lane 13: Hebdomadis
Lane 14: Copenhageni
Lane 15: Icterohaemorrhagiae
Lane 16: Javanica
Lane 17: Saigon
Lane 18: Panama
Lane 19: Pomona
Lane 20: Zanoni
Lane 21: Ranarum
Lane 22: Sarmin
Lane 23: Hardjo
Lane 24: Sejroe
Lane 25: Wolffi
Lane 26: Tarassovi
Lane 27: Patoc

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Table 8 Summary of PFGE pattern of *Leptospira* reference strains digested with *Not*I restriction enzyme

No.	Serovar	PFGE pattern	Serovar findings in Thailand
1	Australis	1	+
2	Bangkok	2	+
3	Bratislava	3	+
4	Autumnalis	4	+
5	New	5	+
6	Rachamati	6	-
7	Ballum	7	-
8	Bataviae	8	+
9	Canicola	9	+
10	Cellidoni	10	-
11	Djasiman	11	-
12	Grippotyphosa	12	-
13	Hebdomadis	13	+
14	Copenhegeni	14	+
15	Icterohaemorrhagiae		+
16	Javanica	15	+
17	Saigon	16	+
18	Panama	17	-
19	Pomona	18	+
20	Zanoni	19	-
21	Ranarum	20	+
22	Sarmin	21	-
23	Hardjo	22	-
24	Sejroe	23	+
25	Wolffi	24	+
26	Tarassovi	25	+
27	Patoc	26	-

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1.2 Fingerprinting patterns of serovars Copenhageni and Icterohaemorrhagiae given by *FspA*I and *Sda*I restriction enzyme

Fingerprinting patterns after digestion with the enzyme *FspA*I and *Sda*I confirmed that both of serovars Copenhageni and Icterohaemorrhagiae could not be differentiated from each others **(Figure 4).** Restriction digestions of chromosomal DNA of the two serovars with enzymes *Not*I, *FspA*I and *Sda*I, produced 10 bands with sizes range from 62 kb to 380 kb, more than 13 bands with sizes range from less than 48.5 kb to 412.5 kb and more than 15 bands with sizes range from less than 48.5 kb to 436.5 kb, respectively. These results demonstrated similarities between the serovars Copenhageni and Icterohaemorrhagiae identified by using serotyping and genotyping.

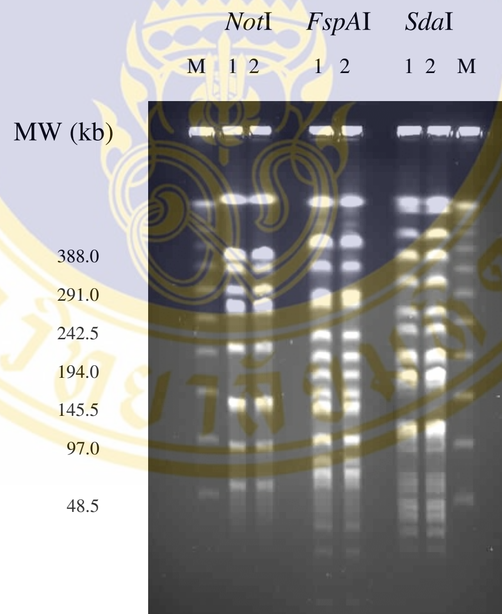

Figure 4 PFGE fingerprinting patterns of *Not*I, *FspA*I and *Sda*I digested chromosomal DNA of serovars Copenhageni and Icterohaemorrhagiae. Lane M, lambda marker; lanes 1 and 2, serovars Copenhageni and Icterohaemorrhagiae

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1.3 Cluster analysis of PFGE fingerprinting patterns of leptospiral reference serovars

PFGE patterns of the reference serovars, except for serovars Sarmin, Sejroe and Tarassovi due to their fingerprints with smear bands, were displayed as a dendogram with the aid of BIO-PROFIL software (**Figure 5**). Clustering of these reference strains showed three distinct clusters without relatedness to each others. Cluster I containing serovars Autumnalis, Australis, Bratislava, New, Bangkok, Patoc and Saigon. The members of cluster II were serovars Copenhageni, Icterohaemorrhagiae, Ranarum, Zanoni, Canicola, Bataviae, Grippotyphosa, Pomona, Panama, Wolffi, Hardjo, Djasiman and Rachamati. Serovars Ballum, Hebdomadis, Javanica and Cellidoni were grouped in the cluster III. Clearly, serovars Copenhageni and Icterohaemorrhagiae were grouped in the same cluster and showed 100% similarity at 95% confidentiality. In addition, each strain had a unique individual clone except the same clone for serovars Copenhageni and Icterohaemorrhagiae.

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Percentage similarity

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Autumnalis
Australis
Bratislava
New
Bangkok
Patoc
Saigon
Copenhageni
Icterohaemorrhagiae
Ranarum
Zanoni
Canicola
Bataviae
Grippotyphosa
Pomona
Panama
Wolffi
Hardjo
Djasiman
Rachamati
Ballum
Hebdomadis
Javanica
Cellidoni

cluster I

cluster II

cluster III

Figure 5 Dendogram of leptospiral reference serovars by BIO-PROFIL software

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2. DNA restriction analysis of leptospires isolated from field rats by PFGE

Forty rat isolates which were serotypicaly typed were further differentiated by PFGE. If two isolates had 100% of band matching to each others or only one band difference, these isolates were proposed to classify in the same group. Thus, PFGE pattern of these isolates were classified as 5 major patterns, patterns I, II, III, IV and V, based on their differences in banding patterns (**Figure 6**). Rat isolates no. R3-R5, R8, R10, R13 and R21-R40 as serovar Pyrogenes and the fingerprinting pattern of these isolates was designated as pattern I. Among isolates of pattern I, there subdivided as patterns Ia and Ib. Members of pattern Ia were rat isolate no. R3-R5, R10, R13, R21-R24 whereas R8 was only one member of pattern Ib. Pattern Ib had one missing band at 304 kb fragment different from the others pattern (**Fig. 6A, lane 4**). Members of pattern I gave unmatched pattern from all of reference strains. Rat isolates no. R11, R12, R14-R17, R19 and R20 as serovar Bataviae and possessed PFGE pattern II. Interestingly, all of these isolates of pattern II gave similar pattern with reference serovar Bataviae as shown in **Figure 7.** The results confirmed that these rat isolates were serovar Bataviae. Rat isolates no. R1, R2, R9 and R18 as serovar Autumnalis which displayed as pattern III. Pattern III was subdivided as patterns IIIa and IIIb. Rat isolate no. R1, R9 and R18 were grouped in patterns IIIa and R2 was placed in patterns IIIb. Patterns IIIb had one additional band at 157.5 kb fragment different from pattern IIIa (**Fig. 6 C, lane 2**). In addition, pattern III did not match to reference serovar Autumnalis strain Akiyami A used in this study (**Figure 8 (A)**) and had different nine or ten banding. The remaining two rat isolates no. R6 and R7 as serovar Australis presented patterns IV and V, respectively. These two isolates of pattern IV and pattern V gave different pattern from reference serovar Australis strain Ballico used in this study (**Figure 8, (B)**) and had approximately ten bands different from their reference pattern.

PFGE fingerprinting patterns of 40 leptospires isolated from field rat were compared with all reference serovars patterns as seen in **Table 9.** As described, fingerprint of rat isolates serovar Bataviae exactly matched with pattern of reference serovar Bataviae whereas the others fingerprints were found unmatchable to any reference patterns.

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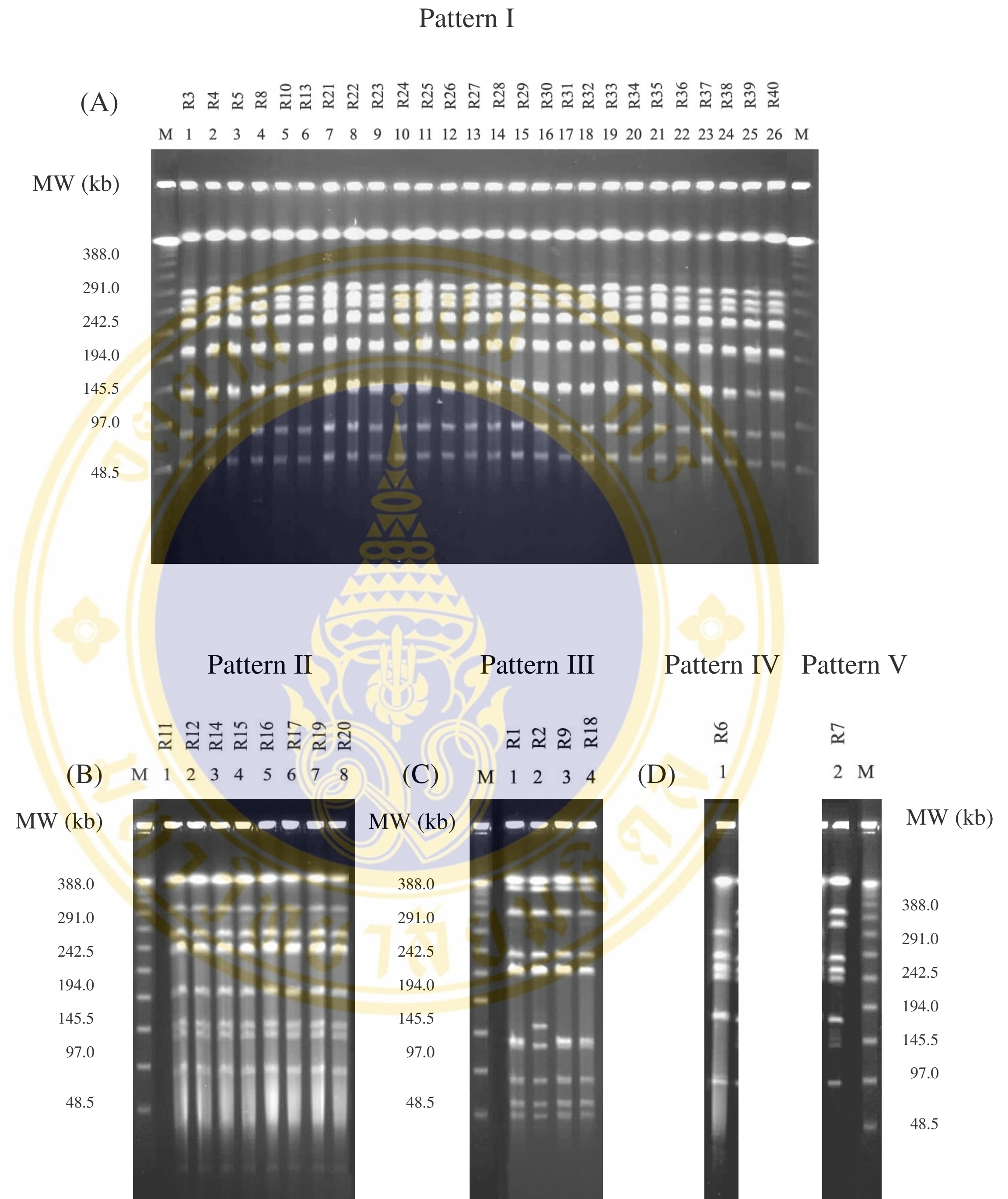

Figure 6 PFGE fingerprinting patterns of *Not*I digested chromosomal DNA of 40 Leptospires isolated from field rats. (A) Lane M, lambda marker; lanes 1-26, no. R3-R5, R8, R10, R13 and R21-R40. (B) Lanes 1-8, no. R11-R12, R14-R17 and R19-R20. (C) Lanes 1-4, no. R1, R2, R9 and R18. (D) Lanes 1 and 2, no. R6 and R7.

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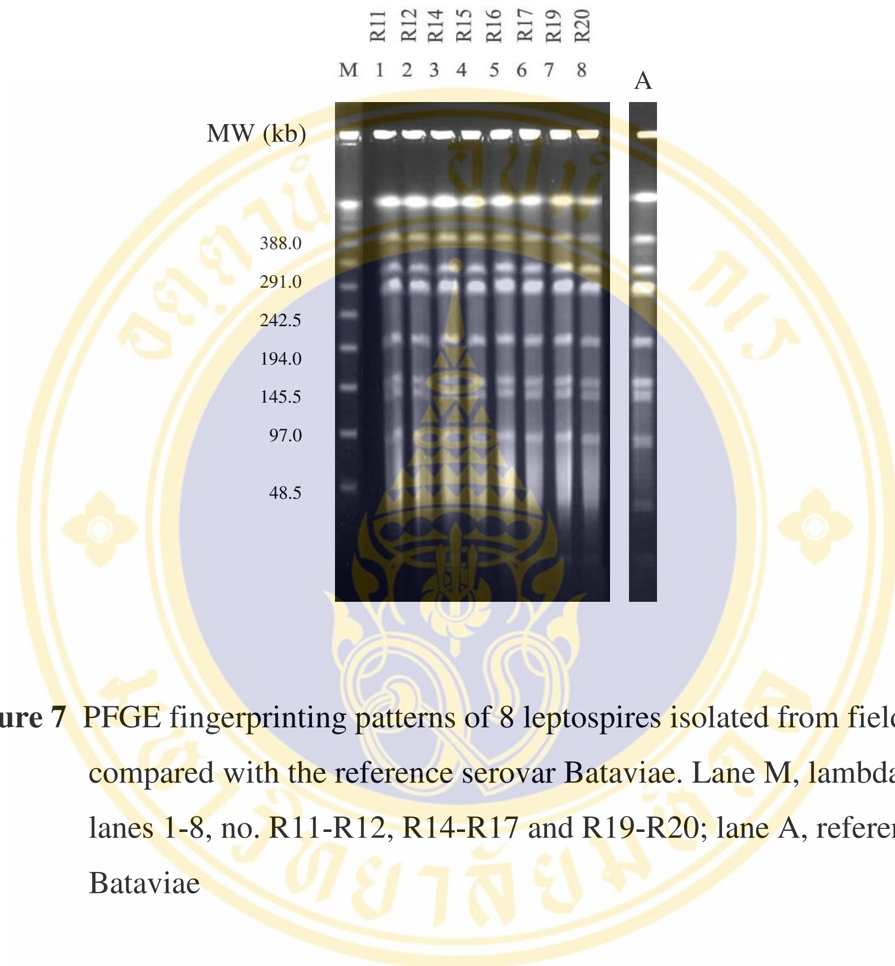

Figure 7 PFGE fingerprinting patterns of 8 leptospires isolated from field rats compared with the reference serovar Bataviae. Lane M, lambda marker; lanes 1-8, no. R11-R12, R14-R17 and R19-R20; lane A, reference serovar Bataviae

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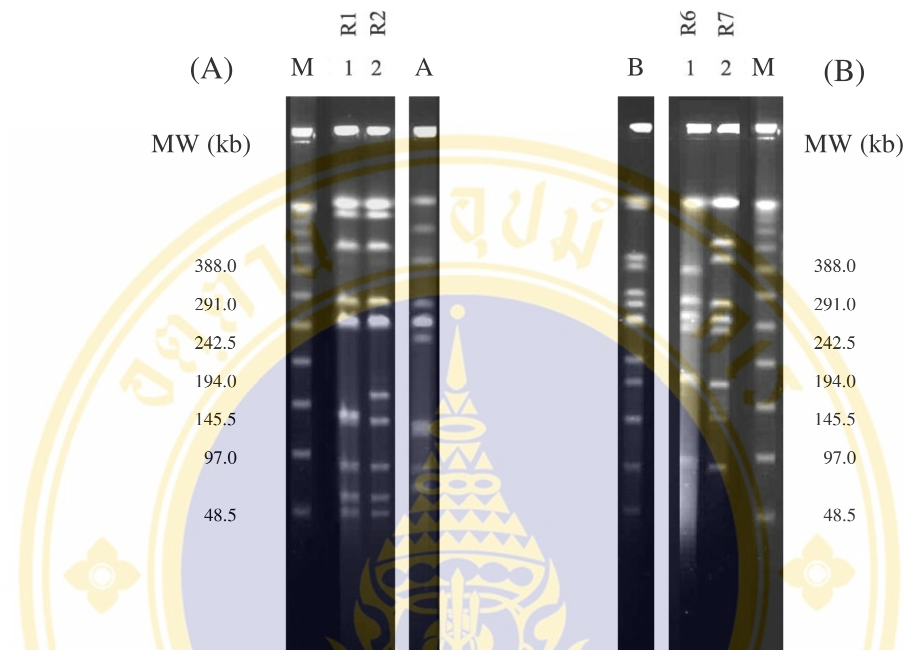

Figure 8 The fingerprinting pattern between rat isolates compared with the Reference serovars Autumnalis and Australis. (A) Lane M, lambda marker; lanes 1-2, rats isolates no. R1 and R2 as representative banding patterns of serovar Autumnalis; lane A, reference serovar Autumnalis. (B) Lanes 1-2, rat isolates no. R6 and R7 as representative banding patterns of serovar Australis; lane B, reference serovar Australis.

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Table 9 Comparison of PFGE fingerprinting patterns among leptospires isolated from field rats with banding patterns of reference serovars

No.	LAB. Code	Serovar using	Serology[**]
R1	050	NM[*]	Autumnalis
R2	526	NM	Autumnalis
R3	571	NM	Pyrogenes
R4	574	NM	Pyrogenes
R5	578	NM	Pyrogenes
R6	783	NM	Australis
R7	809	NM	Australis
R8	853	NM	Pyrogenes
R9	865	NM	Autumnalis
R10	941	NM	Pyrogenes
R11	992	NM	Pyrogenes
R12	1018	Bataviae	Bataviae
R13	1020	Bataviae	Bataviae
R14	1078	Bataviae	Bataviae
R15	1083	Bataviae	Bataviae
R16	1090	Bataviae	Bataviae
R17	1107	Bataviae	Bataviae
R18	1131	NM	Autumnalis
R19	1133	Bataviae	Bataviae
R20	1134	Bataviae	Bataviae
R21	1172	NM	Pyrogenes
R22	1175	NM	Pyrogenes
R23	1183	NM	Pyrogenes
R24	1186	NM	Pyrogenes
R25	1209	NM	Pyrogenes
R26	1210	NM	Pyrogenes
R27	1222	NM	Pyrogenes
R28	1229	NM	Pyrogenes
R29	1237	NM	Pyrogenes
R30	1241	NM	Pyrogenes
R31	1244	NM	Pyrogenes
R32	1246	NM	Pyrogenes
R33	1252	NM	Pyrogenes
R34	1264	NM	Pyrogenes
R35	1265	NM	Pyrogenes
R36	1266	NM	Pyrogenes
R37	1277	NM	Pyrogenes
R38	1278	NM	Pyrogenes
R39	1283	NM	Pyrogenes
R40	1290	NM	Pyrogenes

[*]NM, Not match to reference serovars used in this study, [**]Detected by MAT, CAAT and MAb Coated Latex Test

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3. Confirmation and characterization of serovar Pyrogenes among leptospires isolated from rats by PFGE

Since the unmatchable patterns among leptospires isolates identified as serovar Pyrogenes by serotyping methods was pattern I, serovar Pyrogenes was additionally included in experiment. To confirm these results, rat isolates no. R3 and R8 which as a representative of pattern I and serovar Pyrogenes strain Salinem isolated from human in Buri Ram province since 1999 were selected for PFGE analysis. The results are shown in **Figure 9.** The fingerprinting patterns of 2 rat isolates were similar to serovar Pyrogenes strain Salinem with slightly difference. As described, the pattern from rat isolate no. R3 showed the presence of a 53 kb fragment and the absence of a 168 kb fragment. Rat isolate no. R8 gave a similar banding pattern as that of rat isolate no. R3 and more missing band of a 304 kb fragment. However, These 2 rat isolates were confirmed as serovar Pyrogenes with the same clonality. In addition, all of these strains were only isolated from rat genus *Rattus* which 25 isolates obtained from *R. norvegicus* and 1 isolate obtained from *R. exulans.*

PFGE fingerprinting patterns of 40 leptospires isolated from field rat were compared with all reference serovars patterns, including the pattern of serovar Pyrogenes strain Salinem. The results were summarized in **Table 10.** As described, fingerprint of rat isolates serovars Bataviae and Pyrogenes matched with reference serovar Bataviae and serovar Pyrogenes strain Salinem patterns, respectively. However, unmatchable patterns of serovars Autumnalis and Australis were not yet confirmed with the other strain of these serovars.

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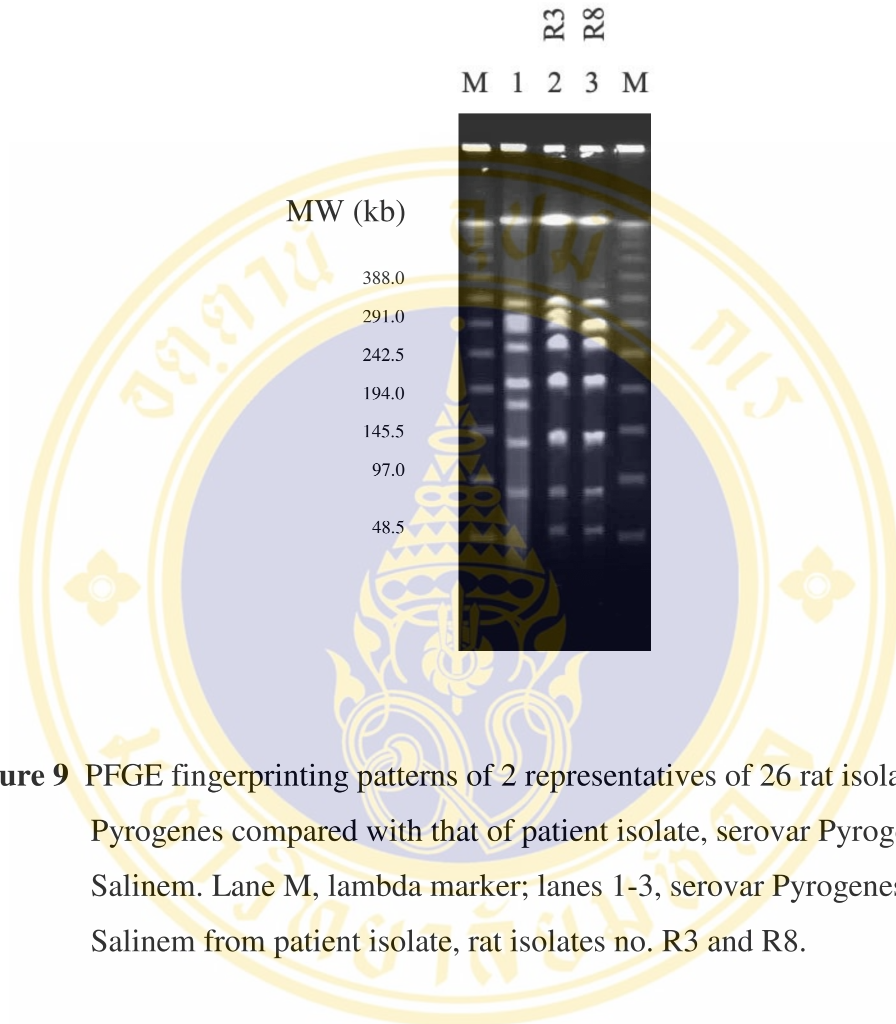

Figure 9 PFGE fingerprinting patterns of 2 representatives of 26 rat isolates serovar Pyrogenes compared with that of patient isolate, serovar Pyrogenes strain Salinem. Lane M, lambda marker; lanes 1-3, serovar Pyrogenes strain Salinem from patient isolate, rat isolates no. R3 and R8.

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Table 10 Comparison of PFGE banding patterns among rats isolates with patterns of reference serovars, included Pyrogenes strain Salinem

No.	LAB. Code	Serovar using	Serology**
R1	050	NM*	Autumnalis
R2	526	NM	Autumnalis
R3	571	Pyrogenes	Pyrogenes
R4	574	Pyrogenes	Pyrogenes
R5	578	Pyrogenes	Pyrogenes
R6	783	NM	Australis
R7	809	NM	Australis
R8	853	Pyrogenes	Pyrogenes
R9	865	NM	Autumnalis
R10	941	Pyrogenes	Pyrogenes
R11	992	Pyrogenes	Pyrogenes
R12	1018	Bataviae	Bataviae
R13	1020	Bataviae	Bataviae
R14	1078	Bataviae	Bataviae
R15	1083	Bataviae	Bataviae
R16	1090	Bataviae	Bataviae
R17	1107	Bataviae	Bataviae
R18	1131	NM	Autumnalis
R19	1133	Bataviae	Bataviae
R20	1134	Bataviae	Bataviae
R21	1172	Pyrogenes	Pyrogenes
R22	1175	Pyrogenes	Pyrogenes
R23	1183	Pyrogenes	Pyrogenes
R24	1186	Pyrogenes	Pyrogenes
R25	1209	Pyrogenes	Pyrogenes
R26	1210	Pyrogenes	Pyrogenes
R27	1222	Pyrogenes	Pyrogenes
R28	1229	Pyrogenes	Pyrogenes
R29	1237	Pyrogenes	Pyrogenes
R30	1241	Pyrogenes	Pyrogenes
R31	1244	Pyrogenes	Pyrogenes
R32	1246	Pyrogenes	Pyrogenes
R33	1252	Pyrogenes	Pyrogenes
R34	1264	Pyrogenes	Pyrogenes
R35	1265	Pyrogenes	Pyrogenes
R36	1266	Pyrogenes	Pyrogenes
R37	1277	Pyrogenes	Pyrogenes
R38	1278	Pyrogenes	Pyrogenes
R39	1283	Pyrogenes	Pyrogenes
R40	1290	Pyrogenes	Pyrogenes

*NM, Not match to reference serovars used in this study, **Detected by MAT, CAAT and MAb Coated Latex Test

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4. Confirmation and characterization of serovar Autumnalis among leptospires isolated from rats by PFGE

Two human isolates from Loei province in 2003 were identified as serovars Autumnalis, Bratislava and New by immunoblotting method. These isolates were further differentiated by PFGE. Interestingly, their fingerprinting patterns were similar to that of serovar Autumnalis among field rat isolated from Buri Ram (**Figure 10**). These results confirmed that the 2 human isolates were of serovar Autumnalis.

PFGE fingerprinting patterns of 40 leptospires isolated from field rat were compared with all reference serovars patterns, including the patterns of serovar Pyrogenes strain Salinem and Autumnalis from humans isolates **(Table 11).** As described, fingerprint of rat isolates serovars Bataviae, Pyrogenes and Autumnalis matched with reference serovars Bataviae, Pyrogenes strain Salinem and Autumnalis from humans isolates patterns, respectively. However, unmatchable patterns of serovars Australis were not yet confirmed with the other strain of this serovars.

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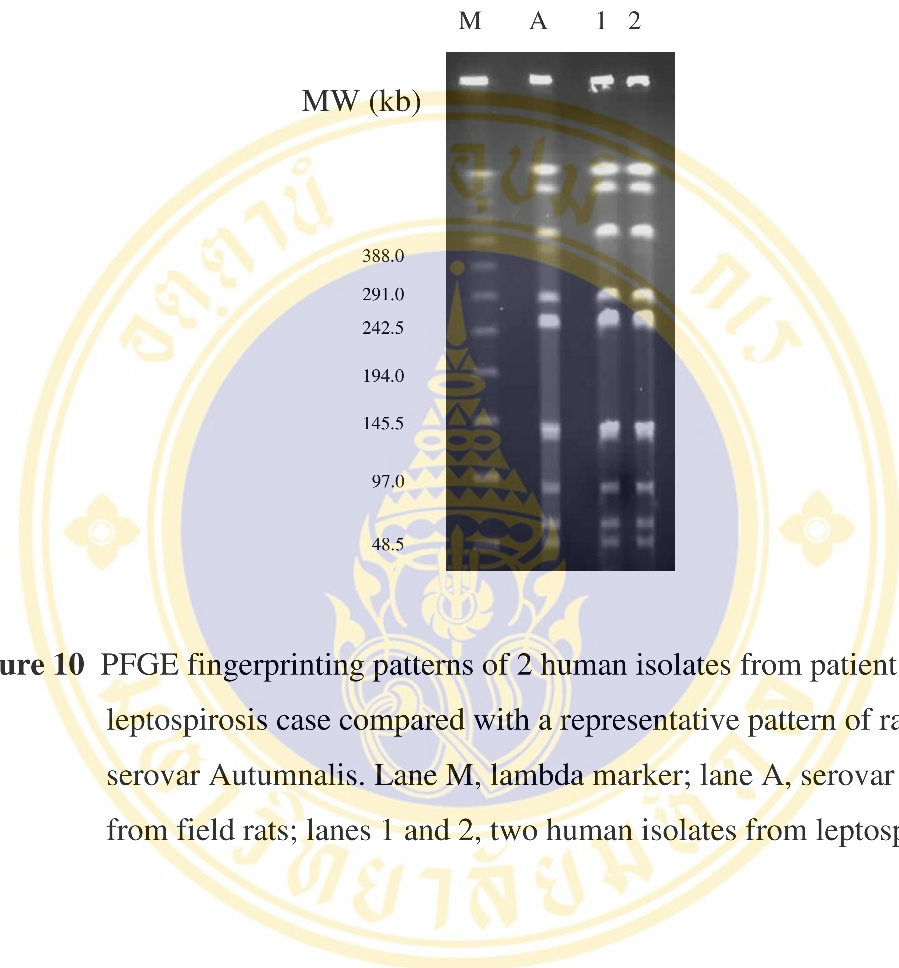

Figure 10 PFGE fingerprinting patterns of 2 human isolates from patient leptospirosis case compared with a representative pattern of rat isolates serovar Autumnalis. Lane M, lambda marker; lane A, serovar Autumnalis from field rats; lanes 1 and 2, two human isolates from leptospirosis cases

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Table 11 Comparison of PFGE banding patterns among rats isolates with patterns of reference serovars, included Pyrogenes strain Salinem and Autumnalis from human isolates

No.	LAB. Code	Serovar using	Serology**
R1	050	Autumnalis	Autumnalis
R2	526	Autumnalis	Autumnalis
R3	571	Pyrogenes	Pyrogenes
R4	574	Pyrogenes	Pyrogenes
R5	578	Pyrogenes	Pyrogenes
R6	783	NM*	Australis
R7	809	NM	Australis
R8	853	Pyrogenes	Pyrogenes
R9	865	Autumnalis	Autumnalis
R10	941	Pyrogenes	Pyrogenes
R11	992	Pyrogenes	Pyrogenes
R12	1018	Bataviae	Bataviae
R13	1020	Bataviae	Bataviae
R14	1078	Bataviae	Bataviae
R15	1083	Bataviae	Bataviae
R16	1090	Bataviae	Bataviae
R17	1107	Bataviae	Bataviae
R18	1131	Autumnalis	Autumnalis
R19	1133	Bataviae	Bataviae
R20	1134	Bataviae	Bataviae
R21	1172	Pyrogenes	Pyrogenes
R22	1175	Pyrogenes	Pyrogenes
R23	1183	Pyrogenes	Pyrogenes
R24	1186	Pyrogenes	Pyrogenes
R25	1209	Pyrogenes	Pyrogenes
R26	1210	Pyrogenes	Pyrogenes
R27	1222	Pyrogenes	Pyrogenes
R28	1229	Pyrogenes	Pyrogenes
R29	1237	Pyrogenes	Pyrogenes
R30	1241	Pyrogenes	Pyrogenes
R31	1244	Pyrogenes	Pyrogenes
R32	1246	Pyrogenes	Pyrogenes
R33	1252	Pyrogenes	Pyrogenes
R34	1264	Pyrogenes	Pyrogenes
R35	1265	Pyrogenes	Pyrogenes
R36	1266	Pyrogenes	Pyrogenes
R37	1277	Pyrogenes	Pyrogenes
R38	1278	Pyrogenes	Pyrogenes
R39	1283	Pyrogenes	Pyrogenes
R40	1290	Pyrogenes	Pyrogenes

*NM, Not match to reference serovars used in this study, **Detected by MAT, CAAT and MAb Coated Latex Test

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5. Comparison of PFGE and ribotyping results

There was 100% concordance between the results of PFGE compared with ribotyping reported by Miss Kamonnaree Chotinantakul's thesis as shown in **Table 12.** It revealed that 2 out of 40 rat isolates (no. R6 and R7) were serovar Australis but not strain Ballico. Four rat isolates (no. R1, R2, R9 and R18) were serovar Autumnalis but not strain Akiyami A. Eight rat isolates (no. R11, R12, R14-R17, R19 and R20) were serovar Bataviae. Twenty-six rat isolates (no. R3-R5, R8, R10, R13 and R21-R40) were serovar Pyrogenes similar to strain Salinem. In addition, rat isolates serovars Autumnalis, Bataviae and Pyrogenes were presented each single clone among 4, 8 and 26 rat isolates, respectively. While, rat isolates serovar Australis were shown as two distinct clones among 2 rat isolates.

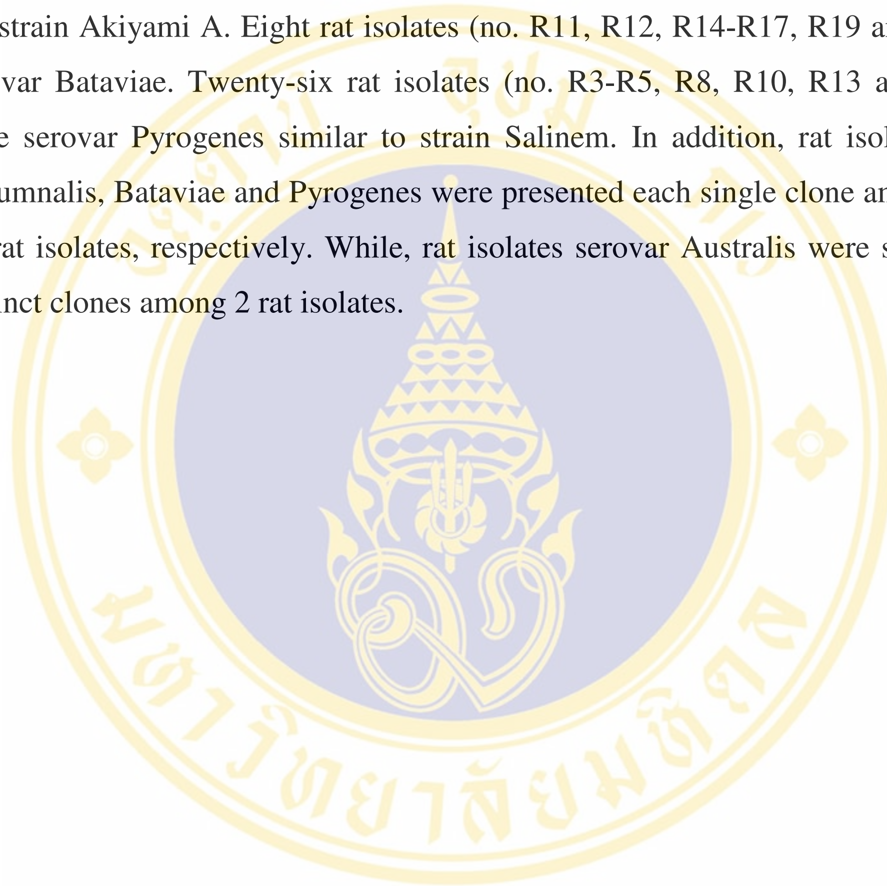

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Table 12 Comparison of genotyping results analyzed by PFGE and ribotyping (27)

No.	LAB. Code	Ribotyping	PFGE
R1	050	Autumnalis	Autumnalis*
R2	526	Autumnalis	Autumnalis
R3	571	Pyrogenes	Pyrogenes***
R4	574	Pyrogenes	Pyrogenes
R5	578	Pyrogenes	Pyrogenes
R6	783	Australis	Australis**
R7	809	Australis	Australis
R8	853	Pyrogenes	Pyrogenes
R9	865	Autumnalis	Autumnalis
R10	941	Pyrogenes	Pyrogenes
R11	992	Pyrogenes	Pyrogenes
R12	1018	Bataviae	Bataviae
R13	1020	Bataviae	Bataviae
R14	1078	Bataviae	Bataviae
R15	1083	Bataviae	Bataviae
R16	1090	Bataviae	Bataviae
R17	1107	Bataviae	Bataviae
R18	1131	Autumnalis	Autumnalis
R19	1133	Bataviae	Bataviae
R20	1134	Bataviae	Bataviae
R21	1172	Pyrogenes	Pyrogenes
R22	1175	Pyrogenes	Pyrogenes
R23	1183	Pyrogenes	Pyrogenes
R24	1186	Pyrogenes	Pyrogenes
R25	1209	Pyrogenes	Pyrogenes
R26	1210	Pyrogenes	Pyrogenes
R27	1222	Pyrogenes	Pyrogenes
R28	1229	Pyrogenes	Pyrogenes
R29	1237	Pyrogenes	Pyrogenes
R30	1241	Pyrogenes	Pyrogenes
R31	1244	Pyrogenes	Pyrogenes
R32	1246	Pyrogenes	Pyrogenes
R33	1252	Pyrogenes	Pyrogenes
R34	1264	Pyrogenes	Pyrogenes
R35	1265	Pyrogenes	Pyrogenes
R36	1266	Pyrogenes	Pyrogenes
R37	1277	Pyrogenes	Pyrogenes
R38	1278	Pyrogenes	Pyrogenes
R39	1283	Pyrogenes	Pyrogenes
R40	1290	Pyrogenes	Pyrogenes

*Autumnalis not strain Akiyami A, **Australis not strain Ballico, ***Pyrogenes similar to strain Salinem

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6. Cluster analysis of PFGE fingerprinting patterns of rat isolates and some of leptospiral reference strains

A cluster analysis of the representative fingerprints of rat isolates and the reference serovars Australis, Autumnalis, Bataviae and Pyrogenes (patient isolate) was displayed as a dendogram with the aid of BIO-PROFIL software **(Figure 11).** The dendogram showed 2 major clusters which had 7% similarity at 95% confidentiality. As described, both patterns Ia and Ib of serovar Pyrogenes rat isolates were placed in the same cluster with serovar Pyrogenes, Salinem strain, isolated from a patient at 70% similarity. Pattern II of serovar Bataviae rat isolates producing 100% similarity with reference serovar Bataviae, was grouped in the same cluster at 95% confidentiality. Patterns IV and V of serovar Australis rat isolates were grouped in the same cluster with reference serovar Australis at 37% similarity. Notably, patterns III of serovar Autumnalis rat isolates could not be grouped in the same cluster as reference serovar Autumnalis at 28% similarity. However, patterns III could be grouped as the same cluster with serovar Autumnalis isolated from humans at 100% similarity.

In this study, 40 rat isolates obtained from leptospirosis outbreak in 1999-2000 were selected to investigate of their epidemiology and clonal relationship among them. As seen in **Figure 11**, patterns Ia and Ib of rat isolates serovar Pyrogenes showed the same clonality at 95% similarity. Most serovar Pyrogenes rat isolates were placed in pattern Ia that isolated from *R. norvegicus and R. exulans* in Nakorn Phanom, Khon Kaen, Kala Sin and Buri Ram provinces. The others one isolate of pattern Ib was obtained from *R. norvegicus* in Khon Kaen province. Eight of rat isolates serovar Bataviae showed the same clonality at 100% similarity. Having isolated from *R. norvegicus,* these isolates were found to be distributed in Buri Ram and Surin provinces. Patterns IIIa and IIIb of rat isolates serovar Autumnalis displayed as the same clonality at 95% similarity. In addition, 3 isolates of pattern IIIa were isolated from two of *B. indica* and one of *B. savilei* in Buri Ram province. One isolate of pattern IIIb was found in *B. indica* at Khon Kaen province. The remaining 2 rat isolates of serovar Australis showed two distincts clonality at 76% similarity. One strain of pattern V was isolated from *B. indica* and the others strain of pattern IV was isolated from *R. losea*. Both isolates were obtained from Nakorn Phanom province.

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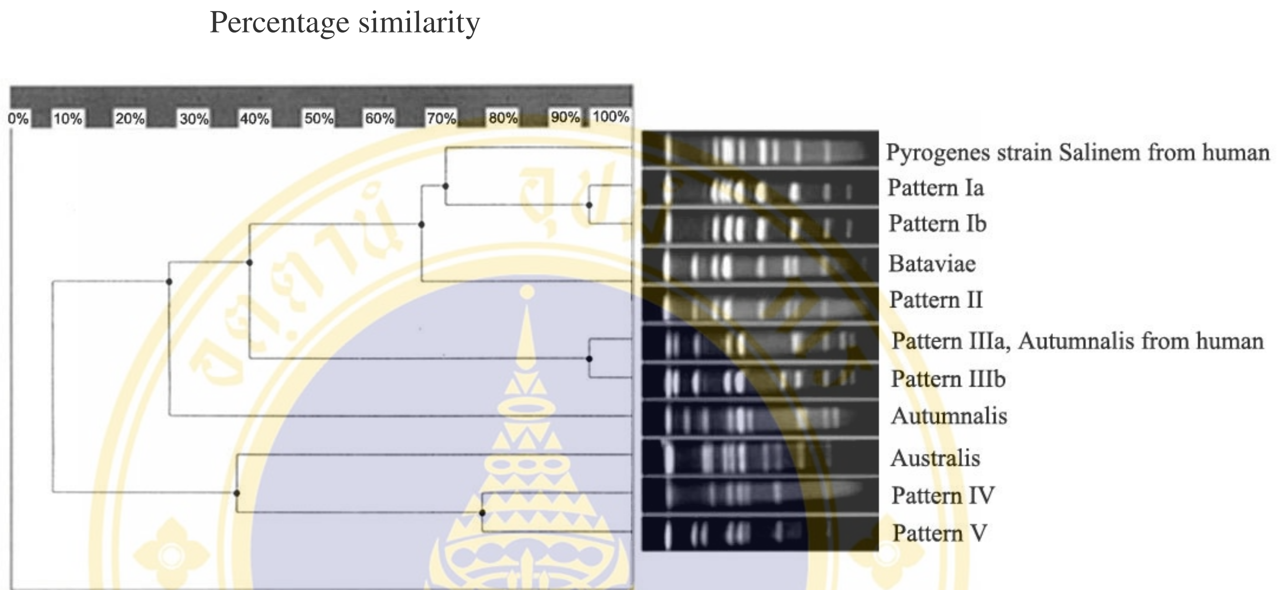

Figure 11 Dendogram of the representative fingerprinting patterns all of rats isolates and the leptospiral reference serovars by BIO-PROFIL software

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CHAPTER VI
DISCUSSION

Identification of serovars is necessary for epidemiological surveillance since some serovars are known to have preferential animal reservoirs, for example, serovar Icterohaemorrhagiae found in rats and serovar Canicola found in dogs (2). Moreover, some serovars are associated with certain clinical forms such as serovar Icterohaemorrhagiae is associated with icterogenic syndrome and serovar Grippotyphosa is associated with benign meningitis syndrome (2). Investigation of serovars not only useful for epidemiological study but also provide a strategy for prevention of the disease.

Microscopic agglutination test is a conventional typing methodology. However, it is laborious and often gives more than one leptospiral serovar results because of cross agglutination. In addition, it is necessary to use of live leptospires in the assay. To overcome such difficulties, genetic methods have recently proved to be valuable for characterization of leptospire strains. In this study, PFGE was performed to characterize reference strains and rat isolates of *Leptospira* during 1999-2000 outbreak. *Not*I was selected for PFGE analysis since it produced approximately 12-13 bands when predicted *in silico* by using genome sequence of *L. interrogans* serovar Lais train 5660 as a template (http://insilico.ehu.es/digest/index.php?mo=Leptospira). In addition, *Not*I has been proven as a useful endonuclease for characterization among leptospires at serovar level reported by previous studies (12, 16).

PFGE successfully differentiated among reference strains. Each of 27 reference serovars possessed a unique profile except serovars Copenhageni and Icterohaemorrhagiae which yield the same pattern. *Not*I fingerprint patterns of these reference strains produced ranging 8 to more than 17 fragments and each pattern different from to each other. The possibility for heterogeneity of banding patterns may result from genetic rearrangment such as translocation, inversions or distribution of mobile genetic elements i.e. insertion sequences, as found scatter in the chromosomal

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DNA of serovars Lai and Copenhageni (40). Notably, serovars from either the same serogroup or the same species might not be placed in the same group as displayed by dendogram. This results confirmed the heterogeneity among member serovars of *Leptospira* previously found by DNA relatedness (22), multiple-locus variable number of tandam repeats (MLVA) assay (19) and PFGE (16). Moreover, these data suggested that most of these strains were not correlated in evolution to each other. Similar to AP-PCR (36), REA (109) and MLVA assay (19), PFGE was not able to discriminate between serovars Copenhageni and Icterohaemorrhagiae. Additionally, these two serovars could not be distinguished by using *Fsp*AI and *Sda*I as seen in this study. These results indicated that seovars Copenhageni and Icterohaemorrhagiae have extremely closely related both serologically and genetically.

PFGE clearly discriminated among leptospires and established a clonal relationship between related strains. However, the appropriate criteria for interpreting PFGE banding patterns remains unsettled. Goering (110) has suggested that more than 3 bands differences should be considered as clonal variants. Similarly, two isolates with 3 or fewer band differences were considered as closely related strain by Tenover et al. (111). In this study, the criteria of interpreting closely related strains as a total of band matching have to 100% or difference only one band, were proposed. Thus, 40 rat isolates were classified into 5 major patterns as patterns I, II, III, IV and V. As described, pattern I for serovar Pyrogenes, patterns II for serovar Bataviae, patterns III for serovar Autumnalis and patterns IV and V for serovar Australis, were observed. These results of PFGE typing that characterized specific serovar reflects their serological method identify clonal specific antigen on leptospires.

Rat isolates of serovar Bataviae and reference serovar Bataviae were found to be identical profile as the same clone at 100% similarity. The findings suggested that conservation of their chromosomal DNA might be occurred within this serovar. However, these results were observed by using *Not*I enzyme and PFGE running condition in this study. Thus, if used other enzyme or changed PFGE running condition, the heterogeneity of this serovar may be observed.

Similarly, the banding pattern of rat isolates serovar Pyrogenes was similar, but not identical, to serovar Pyrogenes strain Salinem from human isolate at 70% similarity. This result revealed that serovar Pyrogenes among field rats and human

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isolate came from different clone. Notably, both human and rat isolates came from the same geographical areas in the northeastern regions of Thailand during the outbreak since 1999 to 2000. This observation confirmed that leptospires harboring in rats as reservoirs and may spread directly to human. However, this possibility requires further investigation with more leptospiral strains isolated from human leptospirosis cases compared to rat isolates during a given outbreak.

Among 26 rat isolates of serovar Pyrogenes, they displayed as two subdivided patterns, patterns Ia and Ib in 25 and 1 isolates, respectively. The majority of pattern Ia was found from rat species *R. norvegicus* which obtained from Buri Ram, Kala Sin, Khon Kaen and Nakorn Phanom provinces. These results demonstrated that serovar Pyrogenes might circulate among wild rats, especially *R. norvegicus* in the northeastern region of Thailand. This result was also supported by previous study demonstrating that the highly prevalence of leptospiral serovars Pyrogenes (36.6%) was found among rodents (53). Additionally, *R. norvegicus* (commensal rat) was the predominant reservoir of serovar Pyrogenes in both high epidemic regions (Kala Sin, and Khon Kaen provinces) and non-epidemic area (Nakorn Phanom province) (53). Kositanont et al. (52) reported the most prevalent antibody to serovar Pyrogenes among wild rats in rural and urban cities in both low and high endemic areas.

Serovars Autumnalis and Australis were not completely supported between their antigenic characterization by serotypic methods and PFGE fingerprinting patterns. The fingerprints for serovars Autumnalis and Australis between rat isolates and their corresponding reference strains were unrelated. As described, rat isolates serovar Autumnalis had distinct clone with reference serovar Autumnalis strain Akiyami A at 28% similarity. Similarly, serovar Australis among rat isolates and reference strain Ballico were found as different clone at 37% similarity. These data suggested that rat isolates serovars Autumnalis and Australis were not strains Akiyami A and Ballico as references in this study, respectively. This suggestion was supported by previously study of Miss Kamolnaree Chotinantakul thesis (27) which found the discordant results between 16S ribotyping of *Eco*RV and *Hind*III digestions of these isolates. Additionally, the finding was supported by the results of serovar Hardjo strain Hardjobovis and Hardjoprajitno, which belong to *L. borgepetersenii* and *L. interrogans*, respectively. Although phenotypic characteristic of both strains were

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similar but genotyping were different, as demostrated by ribotyping, AP-PCR fingerprinting, and mapped restriction site polymorphisms (MRSPs) in *rrs* and *rrl* genes (28).

Rat isolates of serovars Australis (not strain Ballico), Autumnalis (not strain Akiyami A) and Pyrogenes similar to strain Salinem found in this study, should be included in the reference strain battery for mornitoring epidemiological study in Thailand. However, rat isolates serovars Autumnalis and Australis need to be investigated further of their species and strains by others molecular techniques such as DNA-DNA hybridization or phylogenic analysis of 16S rRNA sequence. Notably, fingerprints of serovar Australis among reference strain and 2 rat isolates showed different to each other. This finding revealed that this serovar had high heterogeneity in their chromosomal DNA.

Two human isolates from Loei province in 2003 produced the same banding pattern with 3 rat isolates of serovar Autumnalis. Interestingly, the same clonality was observed among human isolates and field rats isolates. These results suggested that rats serve as reservoirs for transmission of leptospires, serovar Autumnalis, to humans. However, it remained to be investigated further on other leptospire clones of serovar Autumnalis which remained spreading in the northeastern region between 1999 and 2003. Even these 2 human isolates showed immunoreactivity to three serovars, including serovars Autumnalis, Bratislava and New by immunoblotting, their fingerprints showed only one single pattern for serovar Autumnalis. The findings indicated that antigens that recognized by antibodies to this serovar Autumnalis were not clonal specific antigens. Supported with the study of Kusum et al. (112), it was found that inconsistency between culture results and serum assay of patients with leptospirosis in northeastern region of Thailand. All isolates from clinical specimens agglutinated at high titers for serovar Autumnalis and cross-reacted with serovar Djasiman while serum samples from these patients agglutinated at high titers for several serovars such as serovars Autumnalis, Australis and Bratislava.

Geographic distribution of leptospires isolates among field rats are shown in **Figure 12.** Patterns Ia and Ib of serovar Pyrogenes isolates were circulated in several areas as Khon Kaen, Kala Sin, Buri Ram and Nakorn Phanom provinces. Pattern II of serovar Bataviae isolates were found at 2 adjacent provinces as Buri Ram and Surin.

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Rat isolates of serovar Autumnalis (patterns IIIa and IIIb) were observed at Buri Ram and Khon Kaen provinces. Rat isolates of serovar Australis (patterns IV and V) were observed at the same province as Nakorn Phanom.

In the present study, PFGE showed high reproducibility and high degree of discriminatory power to differentiate among reference leptospires serovars. Moreover, this technique showed good agreement with ribotyping to characterize leptospires isolated from field rats. Therefore, PFGE is a useful tool for both characterization and epidemiological study of leptospiral infection.

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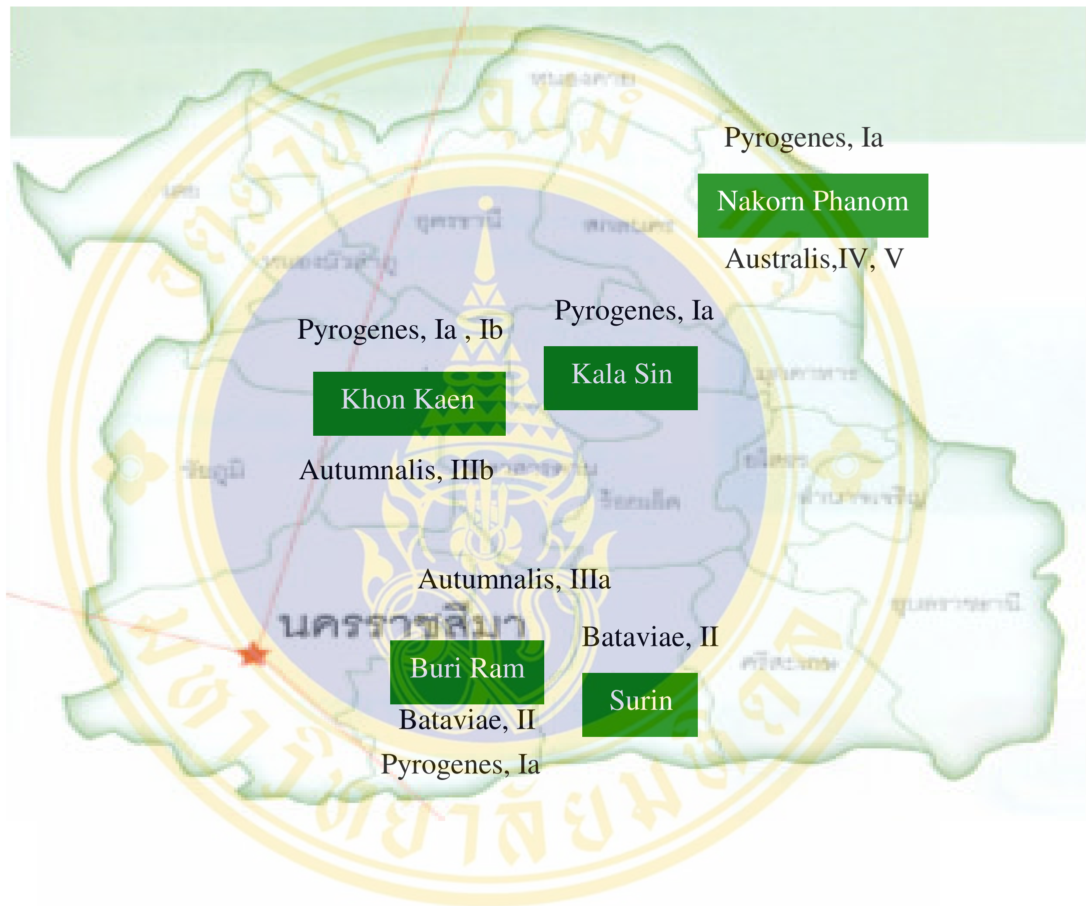

Figure 12 Geographic distribution of leptospires serovars isolated from field rats in the northeastern region of Thailand during 1999-2000

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CHAPTER VII
CONCLUSION

In this study, PFGE used for characterization of leptospires isolates from both reference strains and field rats that captured from the northeastern region of Thailand during the outbreak in 1999-2000.

For 27 reference strains, there were 26 PFGE patterns when using *Not*I digestion. The discriminatory power of these reference strains was 0.99. These results indicated that member serovars of leptospires had high heterogeneity among their chromosomal DNA. Exception for serovars Copenhageni and Icterohaemorrhagiae could not be distinguished from each other when using *Not*I, *FspA*I and *Sda*I restriction enzymes.

For 40 rat isolates, fingerprints of these isolates were grouped as 5 major patterns. Twenty-six isolates of serovar Pyrogenes have 2 patterns as patterns Ia and Ib. Eight isolates of serovar Bataviae have one pattern as pattern II. Four isolates of serovar Autumnalis have 2 patterns as patterns IIIa and IIIb. Two isolates of serovar Australis have 2 patterns as patterns IV and V. Among leptospires isolates of serovars Autumnalis, Bataviae and Pyrogenes were found as one own clone whereas serovar Australis have 2 distinct clones. Patterns of serovars Pyrogenes and Bataviae among rat isolates similar to patterns of serovar Pyrogenes strain Salinem isolated from human and reference serovar Bataviae, respectively. Fingerprints for rat isolates serovars Autumnalis and Australis were those unmatchable to reference serovars Autumnalis strain Akiyami A and Australis strain Ballico, respectively. These results revealed that serovars Autumnalis and Australis among field rats might be not strains Akiyami A and Ballico, respectively.

Interestingly, The same banding pattern of leptospires serovars Pyrogenes, Autumnalis isolated from field rats and human isolates from leptospirosis cases were observed. These results suggested that there was clonal spread of leptospires among wild rats and rats may be the reservoir of leptospires during that outbreak.

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The results of serovar characterization among field rats were compared between PFGE and ribotyping. The results showed 100% concordance between these 2 methods.

Future study should be focused to characterize strains and species of serovars Autumnalis and Australis among field rats in this study. Moreover, it need to be investigate further that rats serve as reservoirs for transmission of leptospires to humans by using more humans and rats isolates during that outbreak.

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APPENDIX

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APPENDIX A
Preparation of Reagent and Media

1. Media and reagents for bacterial culture

1.1 EMJH medium was composing of albumin fatty acid supplement and basal medium.

Stock solutions of EMJH medium

Reagents	Grams per 100 ml H_2O	Storage
$CaCl_2$ x $2H_2O$ + $MgCl_2$ x $6H_2O$	1 (each)	-20 ^oC
$ZnSO_4$ x $7H_2O$	0.4	-20 ^oC
$CuSO_4$ x $5H_2O$	0.3	4 ^oC
Vitamin B12	0.02	-20 ^oC
Tween 80	10.0	-20 ^oC
NH_4Cl	25.0	-20 ^oC
Thiamine (Vitamin B1)	0.5	-20 ^oC
Glycerol	10.0	-20 ^oC

Albumin fatty acid suplement

1. Dissolve 2 grams of bovine serum albumin (BSA) in 12 ml sterile glass distilled water by gently stirring on a magnetic stirrer (avoid foaming). It can take several hours depending on the batch of BSA.
2. Add 0.3 ml calcium chloride and magnesium chloride stock solution.
3. Add 0.2 ml zinc sulphate stock solution.
4. Add 0.02 ml copper sulphate stock solution.
5. Add 0.01 g ferrous sulphate and 0.008 g of sodium pyruvate.
6. Add 0.2 ml Vitamin B 12 stock solution.
7. Add 2.5 ml Tween 80 stock solution.
8. Add sterile distilled water up to 20 ml.
9. Adjust pH to 7.4 - 7.6 with 1 N NaOH.

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10. Sterile by filtrate with 0.2 μm pore size filter.

Basal medium

1. Dissolve 0.2 grams of Na_2HPO_4 together with 0.06 grams of KH_2PO_4 and 0.2 grams of NaCl in 20 ml glass distilled water.
2. Add 0.2 ml stock solution ammonium chloride (NH_4Cl)
3. Add 0.2 ml stock solution Thiamin
4. Add 0.2 ml stock solution glycerol
5. Add glass distilled water to a total volume of 40 ml
6. Add 140 ml distilled water
7. Adjust pH to 7.4.
8. Autoclave during 30 minutes at 121°C

Two components of 20 ml albumin fatty acid supplement and 180 ml basal medium were mix to 200 ml of EMJH medium and separate to 5 ml per tube.

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APPENDIX B
Reagents for PFGE

1. Cell suspension TE (Tris-EDTA) buffer (100mM Tris pH 7.5 and 100 mM EDTA pH 7.5)

10 ml of 1M Tris, pH 7.5
20 ml of 0.5 EDTA, pH 7.5
Dilute to 100 ml with sterile distilled water
Filter sterilize, store at room temperature

2. ES buffer (0.5 M EDTA pH 9.0, 1% sodium-lauroyl-sarcorsine)

93.05 gram of EDTA
Add to 359 ml warm distilled water in a beaker with stir bar
While stirring, add NaOH pellets until pH is 8.5, adjust to 9.0 with 10 N NaOH
Add 5 grams of sodium-lauroyl-sarcorsine
Adjust volume to 500 ml
Autoclave for 15 minutes, store at room temperature

3. Plug wash buffer (10.0 mM Tris, 1.0 M EDTA pH 7.5)

10 ml 1M Tris, pH 7.5
2 ml 0.5M EDTA
Dilute to 1L with sterile distilled water
Filter sterilize and store at room temperature

4. 1.6% Certified low melt agarose/SDS agarose mix (plug agarose) (1.6% Certified low melt agarose, 1% SDS)

0.16 grams of Certified low melt agarose
Add 10 ml sterile distilled water
Add 500 ul of 20% SDS and mix well
Store at room temperature

5. 1% Pulsed field certified agarose

1 gram of Pulsed field certified agarose

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Suspend in 100 ml 0.5X TBE

Heat in microwave until crystals fully dissolve

Cool in 50°C water bath for 15 minutes before pouring

6. 10X TBE (Tris-Borate EDTA) (0.9M Tris base, 0.9M Boric acid, 0.02M EDTA pH 8.0)

108 grams Trizma base

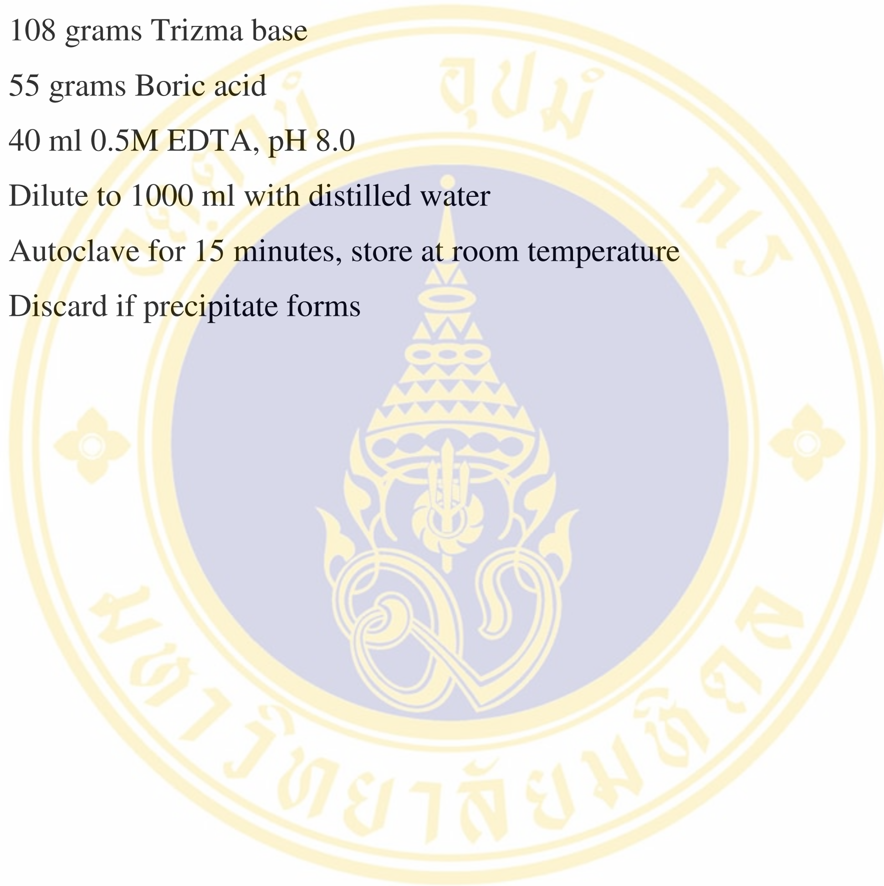
55 grams Boric acid

40 ml 0.5M EDTA, pH 8.0

Dilute to 1000 ml with distilled water

Autoclave for 15 minutes, store at room temperature

Discard if precipitate forms



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APPENDIX C

Calculation of Discriminatory power

Discriminatory power equation :

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{s} n_j (n_j - 1)$$

N is the total number of strains in the sample population = 27 strains

S is the total number of types described = 26 types

nj is the number of strains belonging to the jth type = 1 strain of types 1-13, 15-26 and 2 strains of type 14

As a results :

$$D = 1 - \frac{1}{27(27-1)} \sum_{J=1}^{26} 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1)$$

$$+ 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1)$$

$$+ 1(1-1) + 1(1-1) + 2(2-1) + 1(1-1) + 1(1-1) + 1(1-1)$$

$$+ 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1) + 1(1-1)$$

$$+ 1(1-1) + 1(1-1) + 1(1-1)$$

D = 0.99

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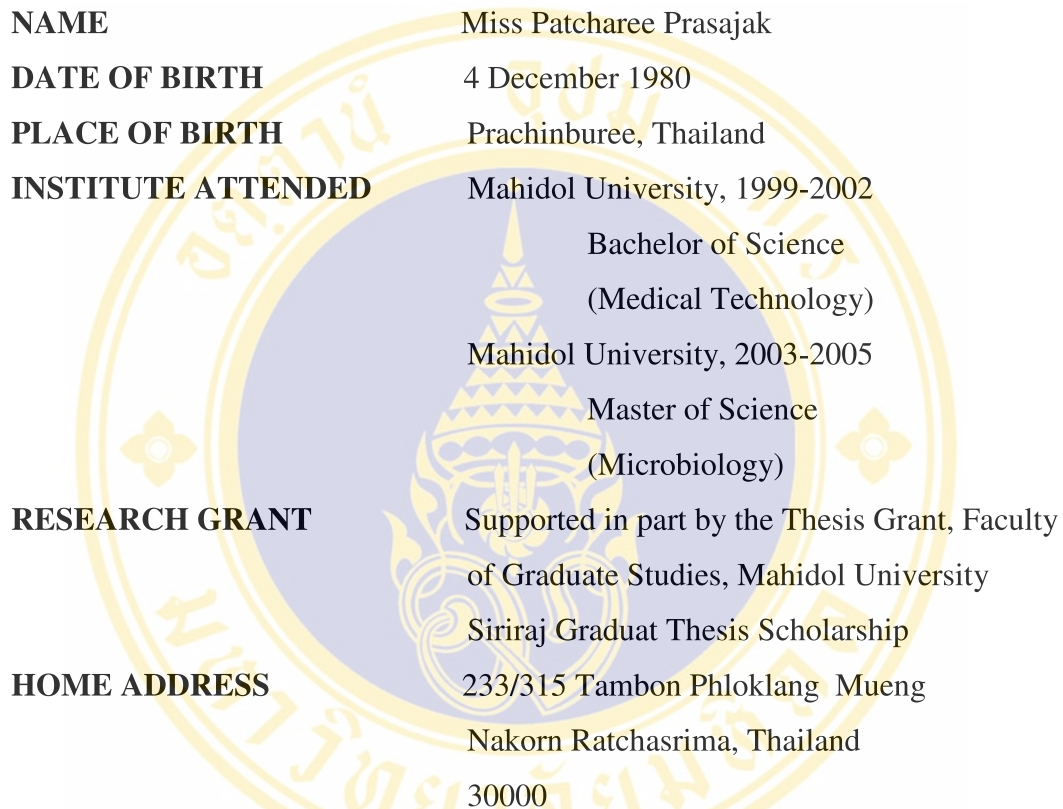
BIOGRAPHY



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