สัญญาเลขที่ MRG4780083

รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาความเหมือนกันของยีน *mce* และ *invA* ระหว่างเชื้อ *Leptospira interrogans* serovars ต่างๆ และการแสดงออกของยีน *in vivo*

Study of homology of *mce* and *invA* genes among different serovars of *Leptospira interrogans* and their *in vivo* expression

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สังกัด

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

Project code: MRG4780083

Project title: Study of homology of *mce* and *invA* genes among different serovars of *Leptospira interrogans* and their *in vivo* expression

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Leptospirosis is a worldwide zoonotic disease. Pathogenesis of leptospirosis is not well understood. Identification of virulence factors of pathogenic *Leptospira* and their roles in pathogenesis is crucial. Based on available whole-genome sequences of *Leptospira*, two presumptive virulence genes which are homologous to the invasionA (*invA*) gene of *Rickettsia prowazekii* and the mammalian cell entry (*mce*) gene of *Mycobacterium tuberculosis*, have been identified. The function of these genes may involve in the attachment and invasion of eukaryotic cells. We proposed that these gene homologues should be conserved in pathogenic serovars of *Leptospira*. Thus, our objective of the study is to determine the presence and the conservation of each gene in various serovars of pathogenic *Leptospira*. Ten different pathogenic serovars and one non-pathogenic serovar (serovar Patoc) of leptospires were used in this study. Polymerase chain reaction using primers designed to bind to the conserved regions of each gene were performed. The amplified PCR products of the *invA* gene homologue were obtained in seven pathogenic serovars whereas eight pathogenic serovars contained the homologue of mce gene. Neither gene homologue was amplified in the non-pathogenic serovar. The nucleotide sequences of the *invA* gene homologue of seven serovars were greater than 99% identity. The *mce* gene homologues of eight serovars were approximately 90 to 100% nucleotide

identity resulting in more than 98% amino acid identity. Therefore, the gene homologues of *invA* and *mce* are found to be conserved in most pathogenic serovars of *Leptospira* with high-degree similarity at nucleotide and amino acid levels. These two homologues were absent in the non-pathogenic serovar. However, convalescent sera of patients with leptospirosis could not detect recombinant proteins of InvA and Mce. *In vivo* expression of these genes requires further investigations.

Keywords: *Leptospira, mce, invA*

บทคัดย่อ

รหัสโครงการ: MRG4780083

ชื่อโครงการ: การศึกษาความเหมือนกันของยีน *mce* และ *invA* ระหว่างเชื้อ *Leptospira interrogans* serovars ต่างๆ และการแสดงออกของยีน *in vivo*

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ระยะเวลาโครงการ: 2 ปี

โรคเลปโตสไปโรซิสเป็นโรคติดต่อจากสัตว์สู่คนที่พบได้ทั่วโลก พยาธิกำเนิดของโรคยังไม่ทราบแน่ชัด การค้นหาปัจจัยก่อโรคของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคและกลไกก่อโรคจึงมีความสำคัญอย่างยิ่ง อาศัยความรู้จากลำดับเบสของทั้งจีโนมของเชื้อเลปโตสไปราพบว่ามียีนที่อาจถอดรหัสเป็นปัจจัยก่อโรคได้เนื่องจากมีความคล้ายคลึงกับปัจจัยก่อโรคที่มีรายงานในเชื้อแบคทีเรียชนิดอื่น ได้แก่ invasionA (*invA*) ของเชื้อริคเก็ตเซีย และ mammalian cell entry (*mce*) ของเชื้อมัยโคแบคทีเรีย ซึ่งยีนทั้งสองมีรายงานว่าเกี่ยวข้องกับการเกาะจับและบุกรุกเซลล์เจ้าบ้าน คณะผู้วิจัยจึงทำการศึกษาหายีนดังกล่าวในเชื้อเลปโตสไปรา เชื้อสายพันธุ์ก่อโรคเทียบกับสายพันธุ์ไม่ก่อโรค โดยใช้เชื้อสายพันธุ์ก่อโรคจำนวน 10 ซีโรวาร์ และสายพันธุ์ไม่ก่อโรค 1 ซีโรวาร์ การตรวจหายีนอาศัยวิธี polymerase chain reaction ซึ่งใช้ primers ที่ออกแบบให้จับกับส่วนที่อนุรักษ์ของยีน พบว่า 7 และ 8 ใน 10 ของเชื้อสายพันธุ์ก่อโรคพบว่ามียีน *invA* และ *mce* ตามลำดับ และไม่พบยีนดังกล่าวในสายพันธุ์ไม่ก่อโรค ผลการหาลำดับเบสของยีนพบว่า ยีน *invA* ที่พบใน 7 ซีโรวาร์ มีความเหมือนกันมากกว่าร้อยละ 99 ส่วนลำดับเบสของยีน *mce* ที่พบใน 8 ซีโรวาร์ มีความเหมือนกันร้อยละ 90 ถึง 100 ซึ่งคิดเป็นความเหมือนกันมากกว่าร้อยละ 98 ในระดับกรดอะมิโน ดังนั้นยีน invA และ mce จึงเป็นยีนที่มีความอนุรักษ์ในเชื้อเลปโตสไปราสายพันธุ์ก่อโรค และไม่พบในเชื้อสายพันธุ์ไม่ก่อโรค อย่างไรก็ตามซีรั่มของผู้ป่วยที่เป็นโรคเลปโตสไปโรซิสไม่จับกับ

recombinant protein InvA และ Mce การแสดงออกของยีนทั้งสองในระดับ *in vivo* จำเป็นต้องมีการศึกษาเพิ่มเติมต่อไปในอนาคต

คำหลัก: เชื้อเลปโตสไปรา ยีน *mce* ยีน *invA*

Introduction

Leptospirosis, caused by the spirochete *Leptospira interrogans*, is a zoonotic disease of a global health concern [1]. It became one of major health problems in Thailand since its outbreak in 1996 [2]. Pathogenesis of leptospirosis is not well elucidated. After leptospires move through skin or mucosal abrasions, the spirochete spreads hematogenously to multiple target organs resulting in systemic infection [1, 3, 4, 5]. Invasion into host tissues is crucial for pathogens to establish infection. Although *Leptospira* is an extracellular pathogen, several studies demonstrated the spirochete inside cells in various organs [6-7]. In addition, leptospires were shown to be able to adhere and penetrate host cells in experimentally infected animals and various cell lines [8-12]. This property was demonstrated to be correlated with its virulence. Leptospiral genes and proteins that mediate invasion of host cells have not been characterized. Based on available whole-genome sequences of *Leptospira*, two presumptive virulence genes that are associated with cell invasion, the invasion A (*invA*) gene of *Rickettsia prowazekii* [13] and the mammalian cell entry (*mce*) gene of *Mycobacterium tuberculosis* [14], were identified [15].

Our study aimed to identify these two gene homologues in various pathogenic serovars of *Leptospira*. In addition, the DNA sequences of the gene homologues were compared to determine sequence similarity.

Material and Methods

Bacterial strains

Ten different pathogenic serovars (as shown in table 1) and one non-pathogenic serovar (patoc) of leptospires were used in this study. All leptospiral strains were kindly provided by Reference Collection of the Department of Medical Sciences, National Institute of Health, Ministry of Public Health of Thailand.

Table 1. Pathogenic *Leptospira* used in the study

Serogroups	Serovars	Strains
1. Australis	1. Bratislava 1. Bangkok	Jez Bratislava BD92
2. Sejroe	3. Sejroe	M 84
3. Louisiana	4. Saigon	L 79

4. Autumnalis	5. Autumnalis 6. Rachmati	Akiyami A Rachmat
5. Pyrogenes	7. Pyrogenes	Salinem
6. Icterohaemorrhagiae	8. Icterohaemorrhagiae	RGA
7. Bataviae	9. Bataviae	Swart
8. Javanica	10. Javanica	Veldrat Bat. 46

Culture media and growth conditions

All serovars of *Leptospira* were grown at 30 ^{o}C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing EMJH basal medium (Difco&BBL, Sparks, MD) enriched with 10% bovine serum albumin (Sigma, St. Louis, MO). The spirochetes were checked for contamination and then were subcultured every 5 to 7 days.

DNA preparation of leptospiral culture

Phenol-chloroform method was used to extract genomic DNA from stationary-phase leptospiral culture as previously described [16]. The final concentration of DNA was determined by measuring absorbance with spectrophotometer at wavelength of 260 nm. The DNA preparations were stored at 4 ^{o}C until used or -20 ^{o}C for long term use.

Design of PCR primers

Known DNA and amino acid sequences of *invA* gene of *Rickettsia prowazekii*, *mce* gene of *Mycobacterium tuberculosis* and their homologues in *Leptospira* serovar lai and serovar copenhageni were obtained from the GenBank nucleotide sequence database via the National Center for Biotechnology Information BLAST network service. The ClustalX program was used to align *invA* and *mce* gene sequences with their corresponding homologues. Primers were designed using Primer3 web software to be conserved regions at DNA or amino acid sequence level and were used to determine the presence of the homologues in different serovars of *Leptospira*. In addition, primers located at the upstream and downstream regions of the *invA* or *mce* genes were designed for PCR and consequent DNA sequencing. The primers were purchased from GIBCO Invitrogen Corporation (Grand Island, NY). The primer names, their sequences, the size of amplicons, and annealing temperature used in the reaction are shown in table 2 and 3.

Table 2. Primers designed for amplification of *invA* gene homologue.

Primer name	Direction of primer	Primer sequences (5'->3')	Amplicon size (base pairs)	Annealing temperature (^{o}C)
FinvA1	F	ACCCTACCGAAAAAATGTCG	357	55
RinvA1	R	AAACGAACCGTTCCAAATTC		
Nudix-F	F	AACTCTCGTGGAGAGGTTTTG	136	55
Nudix-R	R	CGATTCCGACTTCTTCATATAATTC		
Flank invA5'	F	ACGGCGTTTAACGACAAACTA	884	50
Flank invA3'	R	ACTATTTTCGCCAACAGAACC		

F: forward primer, R: reverse primer, bp: base pairs

Table 3. Primers designed for amplification of *mce* gene homologue.

Primer name	Direction of primer	Primer sequences (5'->3')	Amplicon expected size (bp)	Annealing temperature (^{o}C)
Fmce1	F	TCTGGAGAAATGGGAATTGG	167	55
Rmce1	R	TTGAGCTGTTTGGGTTCCAG		
MceRP-5'	F	CACCTTTAGAAACGCGGAAG	119	55
MceRP-3'	R	GAACCTCGGTTCCATTCTCA		
Flank mce5'	F	ATGATCCTCGAAACCAAATCG	951	50
Flank mce3'	R	TAATTCCGGTGCCTATGATGG		

F: forward primer, R: reverse primer, bp: base pairs

PCR conditions and detection of PCR products

PCR was performed using primer sets shown in table 2 and 3. The PCR mixtures included 1x reaction buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 nM of each primer, 0.2 mM of each dNTP, and 1 unit of recombinant *Taq* polymerase in a total volume of 20 µl (Promega Corporation, Madison, WI). Fifty nanograms of DNA were used as template for the amplification reaction. For DNA sequencing reaction, Flank invA5', Flank invA3', Flank mce5', and Flank mce3' were primers used to obtain PCR products. *Taq* polymerase was replaced by a proofreading DNA polymerase, DyNAzyme EXT™ (Finnzymes, Espoo, Findland). The PCR conditions and buffers were adjusted as recommended by the manufacturer.

The PCR conditions were optimized and carried out with a thermal cycler (the GeneAmp PCR System 2400, PerkinElmer Life Sciences, Boston, MA). The initial denaturation at 94 °C for 3 min was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at the temperature shown in table 2 and 3 for 1 min, and extension at 72 °C for 1 min. An additional extension at 72 °C for 7 min was included at the end of the last cycle. Each run included a reaction containing no DNA template (addition of distilled sterile water instead of DNA samples) as a negative control. A positive control used DNA of *Leptospira* serovar lai as a template. In case of negative results, primers conserved for 16S rDNA gene of both pathogenic and non-pathogenic leptospires, 16S 5' (5'-GCGCGTCTTAAACATGCAA-3') and 16S 3' (5'-CGTAGGAGTATGGACCGTGT-3') which gave PCR product of 289 base pairs in size, were used to detect the presence of DNA template. The PCR conditions were the same as previously reported [17].

Five microlitres of each PCR product was electrophoresed in a 1% agarose gel stained with ethidium bromide. The amplified product was visualized as a band of expected size under UV transillumination.

DNA sequencing of PCR products

PCR products of corrected sized obtained from different serovars were used in the DNA sequencing reaction. The amplified products were purified using a QIAquick PCR purification Kit (QIAGEN, Valencia, CA) before they were directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. DNA sequencing was carried out in both

directions using the same primers as for PCR. DNA sequences were detected on an Applied Biosystems 310 automated sequencer (ABI PRISM, Applied Biosystems, Foster City, CA). Nucleotide sequences of each gene homologue from different serovars were compared by the ClustalX program.

Results

PCR products with correct sizes of the *invA* gene homologue were detected in seven of ten pathogenic serovars used in the study except serovar Saigon, Sejroe, and Javanica (Figure 1). However, PCR amplicons of the *mce* gene homologue were absent in only serovar Sejroe and Javanica (Figure 2). In another word, the *mce* gene homologue was found in one additional serovar, serovar Saigon. All primer sets used in the study gave the same results which are summarized in table 4. In addition, neither gene homologue was found in non-pathogenic serovar Patoc. However, PCR products of 16S rDNA gene were observed in all serovars that gave negative amplification (data not shown). Therefore, the negative amplification of *invA* and *mce* homologues in those serovars was not the result of the absence of DNA template.

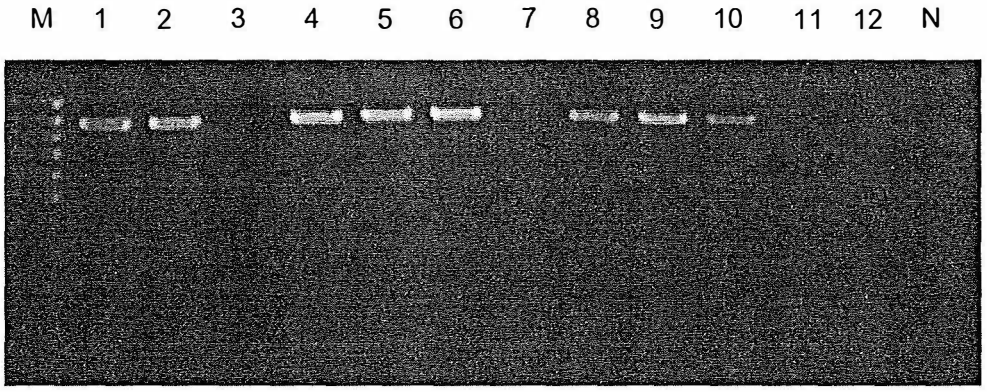


Figure 1. Detection of the *invA* gene homologue in *Leptospira* by PCR using Flank invA5' and Flank invA3' as primers. Lanes: 1, serovar Lai; 2, Bangkok; 3, Javanica; 4, Rachmati; 5, Bratislava; 6, Autumnalis; 7, Saigon; 8, Icterohaemorrhagiae; 9, Pyrogenes; 10, Bataviae; 11, Sejroe; 12; Patoc; M, 100-base pairs (bp) molecular weight marker; N, negative control without DNA

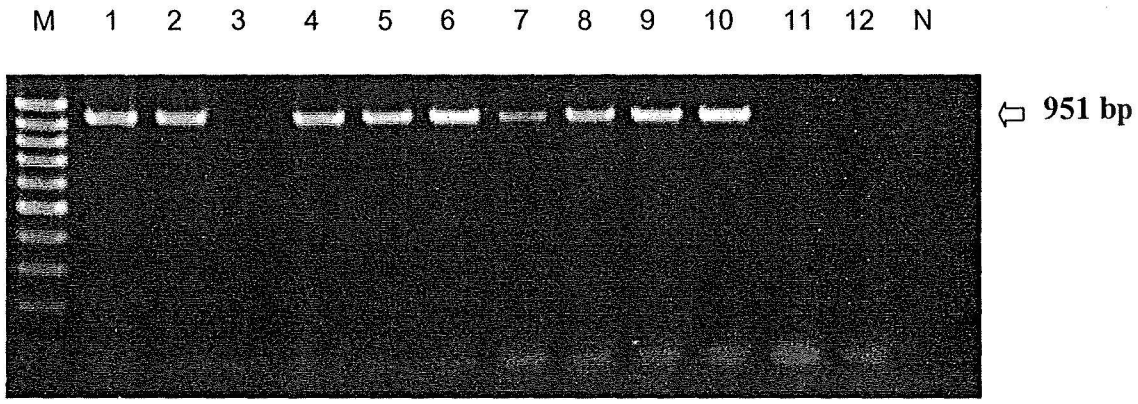


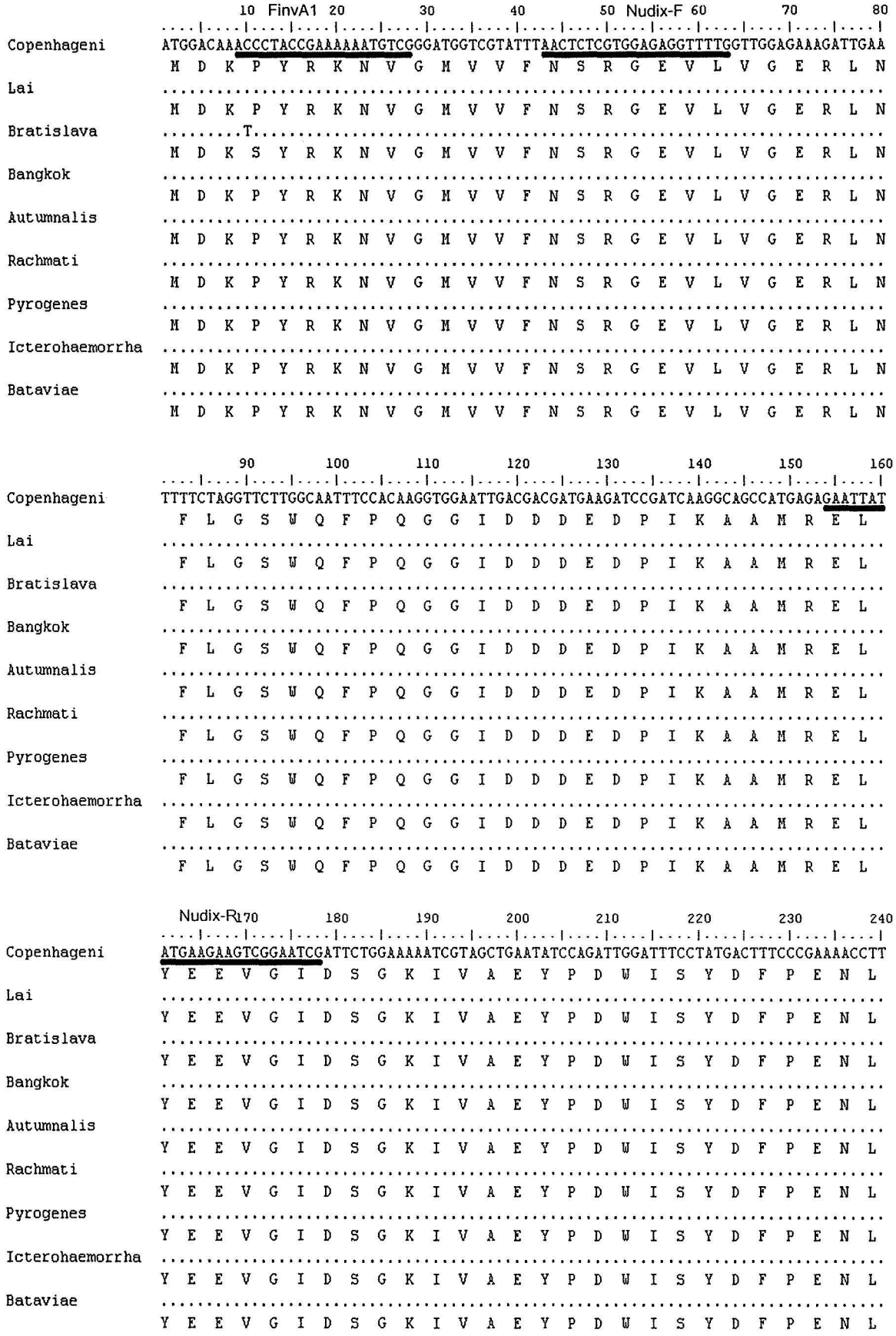
Figure 2. Detection of the *mce* gene homologue in *Leptospira* by PCR using Flank mce5' and Flank mce3' as primers. Lanes: 1, serovar Lai; 2, Bangkok; 3, Javanica; 4, Rachmati; 5, Bratislava; 6, Autumnalis; 7, Saigon; 8, Icterohaemorrhagiae; 9, Pyrogenes; 10, Bataviae; 11, Sejroe; 12; Patoc; M, 100-base pairs (bp) molecular weight marker; N, negative control without DNA

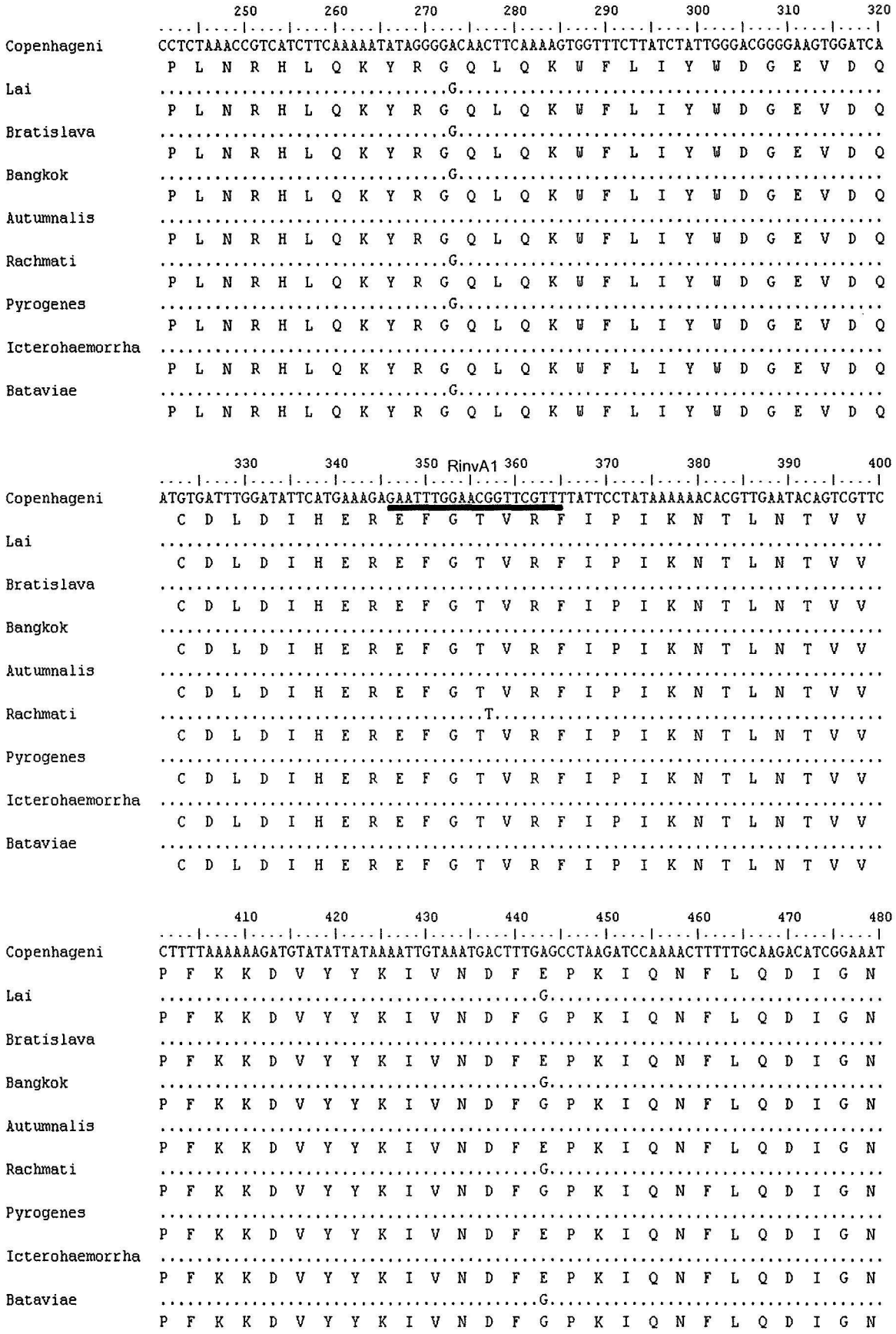
Table 4. Summary of the presence (+) or absence (-) of PCR products of *invA* and *mce* gene homologues in various *Leptospira* serovars.

Serovars	*invA*	*mce*
1. Bratislava	+	+
2. Bangkok	+	+
3. Sejroe	-	-
4. Saigon	-	+
5. Autumnalis	+	+
6. Rachmati	+	+
7. Pyrogenes	+	+
8. Icterohaemorrhagiae	+	+
9. Bataviae	+	+
10. Javanica	-	-

The primers designed to bind to the flanking upstream and downstream regions of the *invA* (Flank invA5' and Flank invA3') and *mce* (Flank mce5' and Flank mce3')

gene homologues gave the PCR products that were used for the nucleotide sequencing of the whole genes. The results showed that the PCR products are the genes of interest compared to the known sequences in the database. After sequence alignment, the nucleotide sequences of the *invA* gene homologues of all seven serovars were found to be 99 to 100% identity resulting in only 1 or 2 amino acid difference (Figure 3). The DNA sequences of the *mce* gene homologue of the same seven serovars were also 99 to 100% identity. Amplification of the *mce* gene homologue was observed in one additional serovar, serovar Saigon. Notably, the sequence of *mce* gene homologue of serovar Saigon was shown to be the least identical to those of other serovars, approximately 90% nucleotide identity (Figure 4). However, most DNA sequence differences of serovar Saigon conferred the same amino acids as those of other serovars resulting in 98% identity at the amino acid level.


10 FinvA1 20 30 40 50 Nudix-F 60 70 80
Copenhageni ATGGACAAAACCCTACCGAAAAAATGTCGGGATGGTCGTATTTAACTCTCGTGGAGAGGTTTTGGTTGGAGAAAGATTGAA
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Lai
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Bratislava .....T.....
M D K S Y R K N V G M V V F N S R G E V L V G E R L N
Bangkok
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Autumnalis
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Rachmati
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Pyrogenes
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Icterohaemorrha
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
Bataviae
M D K P Y R K N V G M V V F N S R G E V L V G E R L N
90 100 110 120 130 140 150 160
Copenhageni TTTTCTAGGTTCTTGGCAATTTCCACAAGGTGGAATTGACGACGATGAAGATCCGATCAAGGCAGCCATGAGAGAATTAT
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Lai
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Bratislava
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Bangkok
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Autumnalis
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Rachmati
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Pyrogenes
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Icterohaemorrha
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Bataviae
F L G S W Q F P Q G G I D D D E D P I K A A M R E L
Nudix-R170 180 190 200 210 220 230 240
Copenhageni ATGAAGAAGTCGGAATCGATTCTGGAAAAATCGTAGCTGAATATCCAGATTGGATTTCCTATGACTTTCCCGAAAACCTT
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Lai
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Bratislava
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Bangkok
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Autumnalis
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Rachmati
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Pyrogenes
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Icterohaemorrha
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L
Bataviae
Y E E V G I D S G K I V A E Y P D W I S Y D F P E N L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P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Lai .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Bratislava .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Bangkok .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Autumnalis .....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Rachmati .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Pyrogenes .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Icterohaemorrha .....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Bataviae .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
330 340 350 RinvA1 360 370 380 390 400
Copenhageni ATGTGATTTGGATATTCATGAAAGAGAATTTGGAACGGTTCGTTTTATTCCTATAAAAAACACGTTGAATACAGTCGTTC
C D L D I H E R E F G T V R F I P I K N T L N T V V
Lai .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Bratislava .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Bangkok .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Autumnalis .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Rachmati .....T.....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Pyrogenes .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Icterohaemorrha .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
Bataviae .....
C D L D I H E R E F G T V R F I P I K N T L N T V V
410 420 430 440 450 460 470 480
Copenhageni CTTTTAAAAAAAGATGTATATTATAAAATTGTAAATGACTTTGAGCCTAAGATCCAAAACTTTTTGCAAGACATCGGAAAT
P F K K D V Y Y K I V N D F E P K I Q N F L Q D I G N
Lai .....G.....
P F K K D V Y Y K I V N D F G P K I Q N F L Q D I G N
Bratislava .....
P F K K D V Y Y K I V N D F E P K I Q N F L Q D I G N
Bangkok .....G.....
P F K K D V Y Y K I V N D F G P K I Q N F L Q D I G N
Autumnalis .....
P F K K D V Y Y K I V N D F E P K I Q N F L Q D I G N
Rachmati .....G.....
P F K K D V Y Y K I V N D F G P K I Q N F L Q D I G N
Pyrogenes .....
P F K K D V Y Y K I V N D F E P K I Q N F L Q D I G N
Icterohaemorrha .....
P F K K D V Y Y K I V N D F E P K I Q N F L Q D I G N
Bataviae .....G.....
P F K K D V Y Y K I V N D F G P K I Q N F L Q D I G N

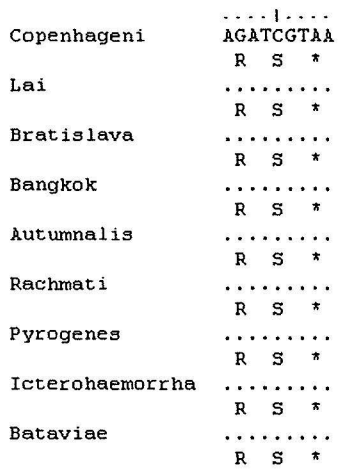
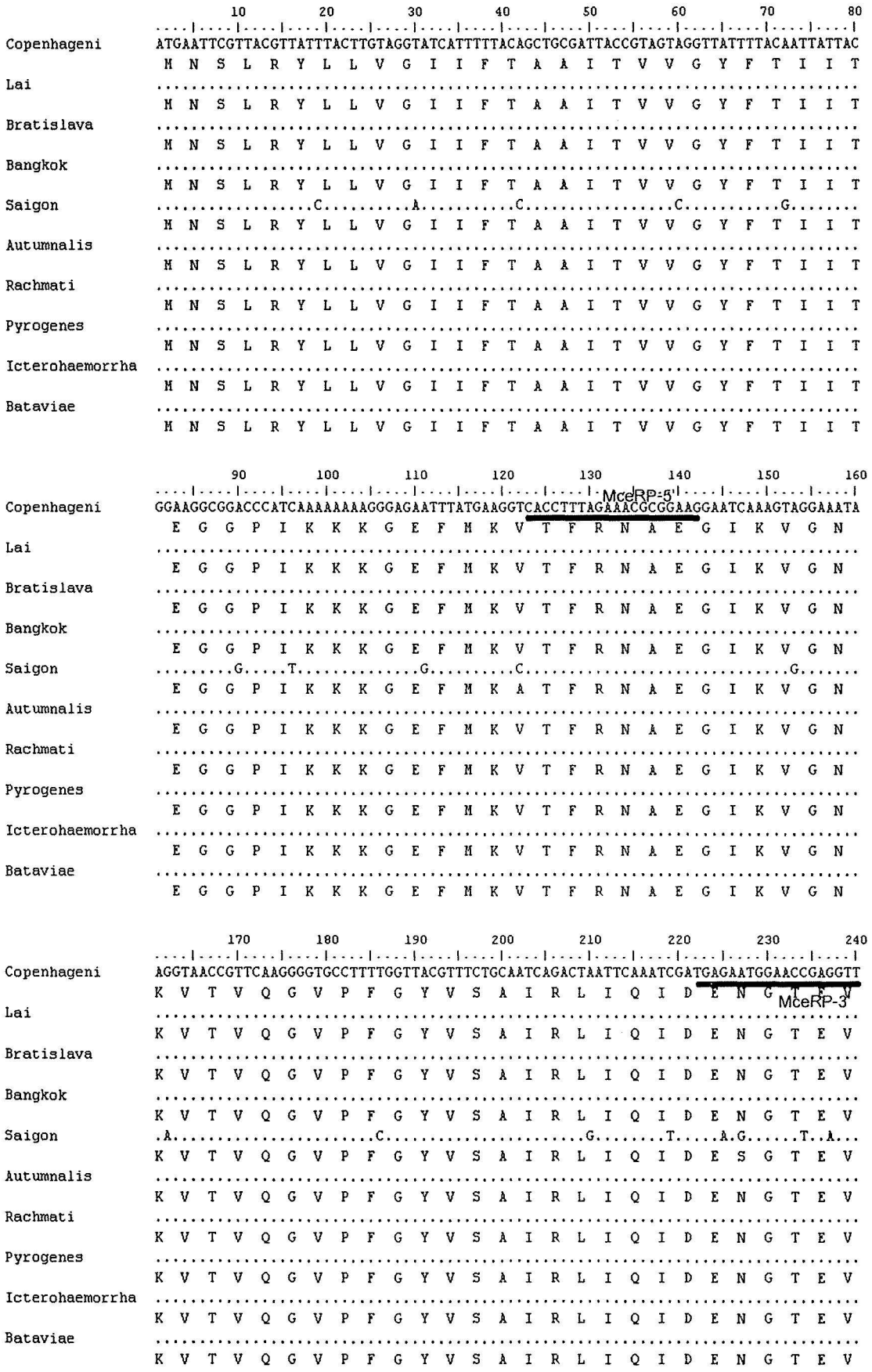
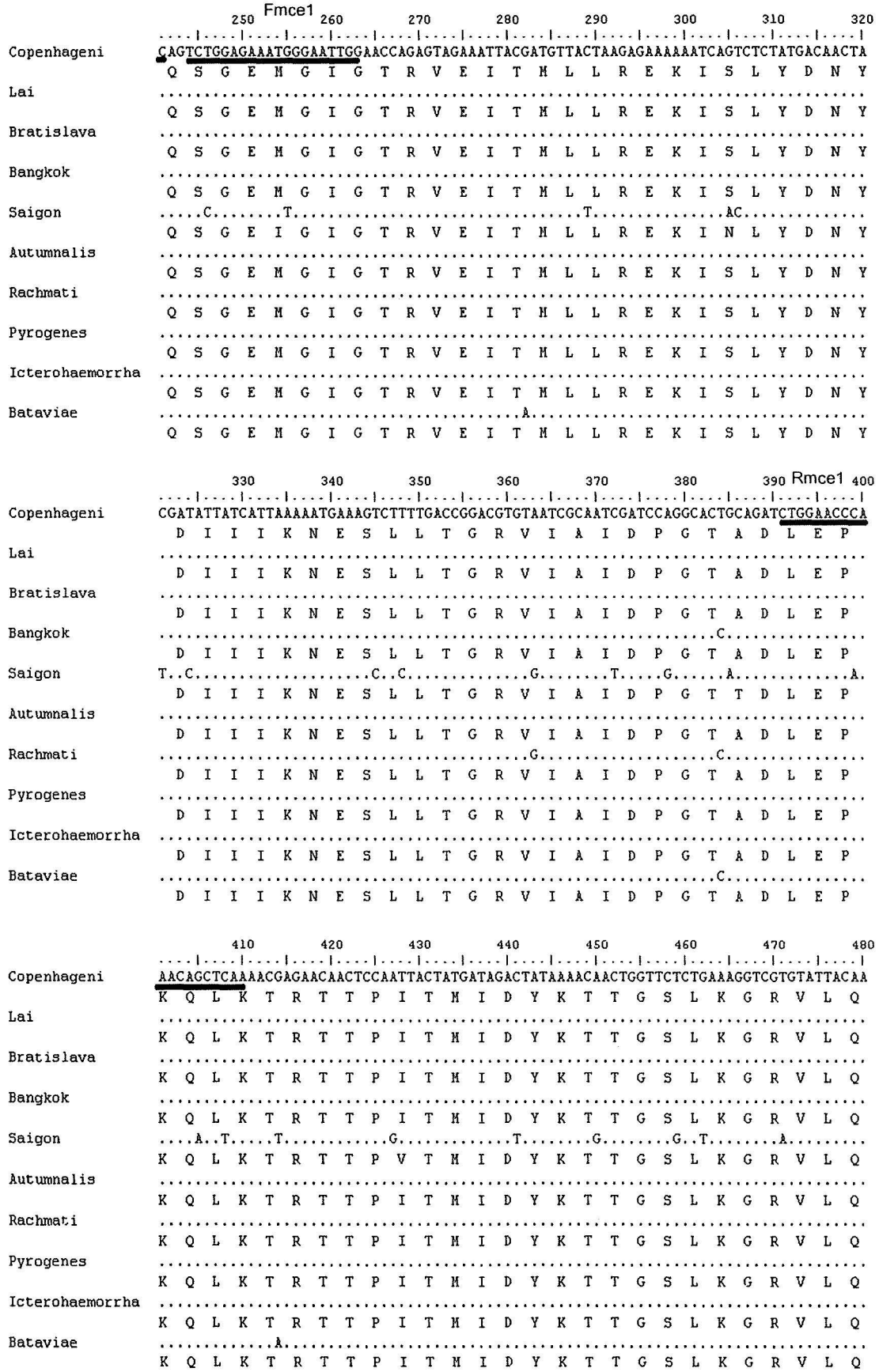
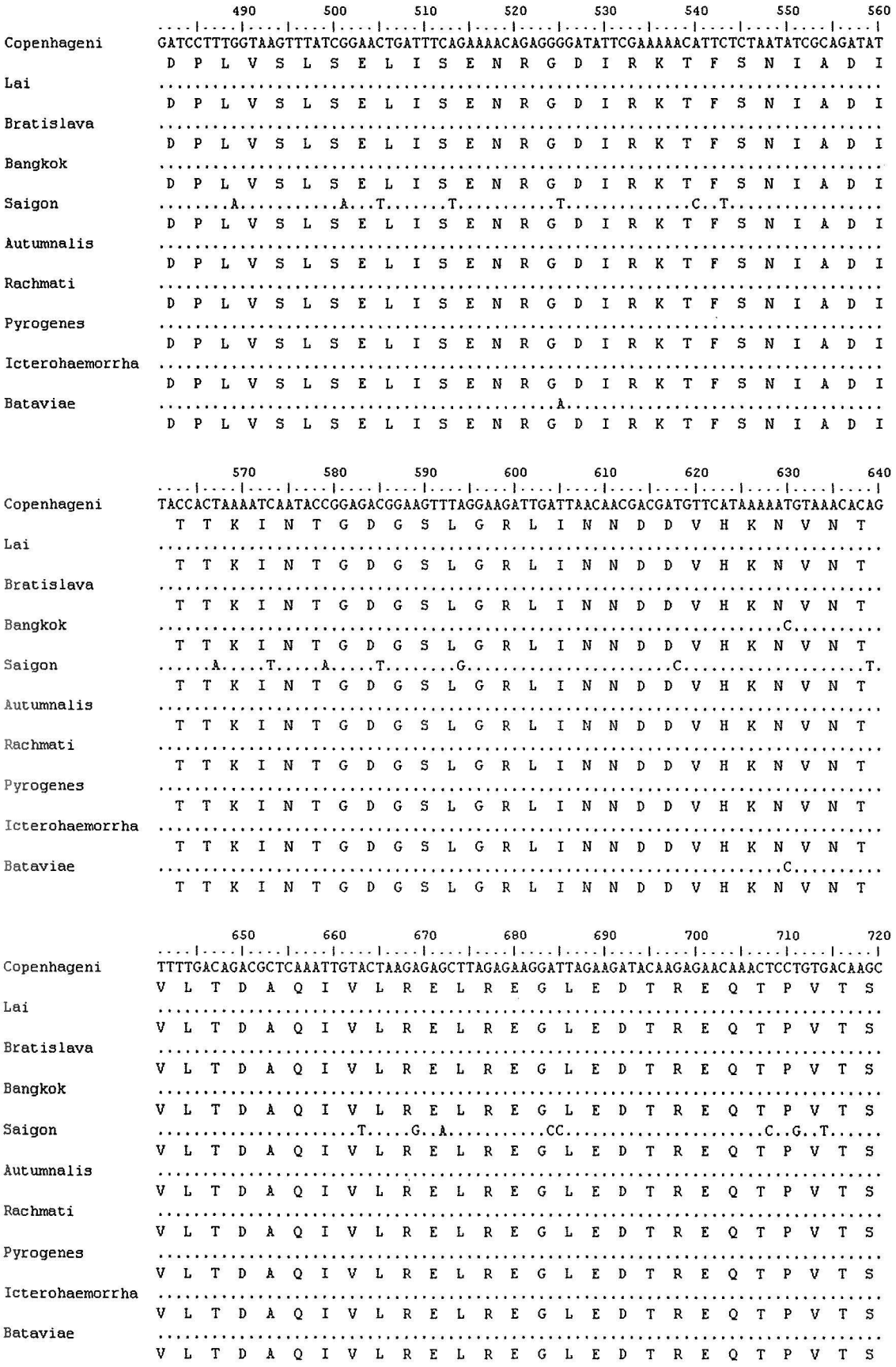


Figure 3. Alignment of DNA sequences (upper row) and their corresponding amino acid sequences (lower row) of *invA* gene homologue of *Leptospira interrogans* serovar Bratislava, Bangkok, Autumnalis, Rachmati, Pyrogenes, Icterohaemorrhagiae, and Bataviae compared to known sequences of serovar Copenhageni and Lai obtained from GenBank. The sequences are shown from start to stop codons. The location of primers was underlined and labeled as indicated.


10 20 30 40 50 60 70 80
Copenhageni ATGAATTCGTTACGTTATTTACTTGTAGGTATCATTTTTACAGCTGCGATTACCGTAGTAGGTTATTTTACAATTATTAC
M N S L R Y L L V G I I F T A A I T V V G Y F T I I T
Lai
Bratislava
Bangkok
Saigon .....C.....A.....C.....C.....G.....
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae
90 100 110 120 130 140 150 160
MceRP-5
Copenhageni GGAAGGCGGACCCATCAAAAAAAAAGGGAGAATTTATGAAGGTCACCTTTAGAAACGCGGAAGGAATCAAAGTAGGAAATA
E G G P I K K K G E F M K V T F R N A E G I K V G N
Lai
Bratislava
Bangkok
Saigon .....G.....T.....G.....C.....G.....
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae
170 180 190 200 210 220 230 240
MceRP-3
Copenhageni AGGTAACCGTTCAAGGGGTGCCTTTTGGTTACGTTTCTGCAATCAGACTAATTCAAATCGATGAGAATGGAACCGAGGTT
K V T V Q G V P F G Y V S A I R L I Q I D E N G T E V
Lai
Bratislava
Bangkok
Saigon .A.....C.....G.....T.....A.G.....T..A...
K V T V Q G V P F G Y V S A I R L I Q I D E S G T E V
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae


Fmce1
Rmce1
Copenhageni
Lai
Bratislava
Bangkok
Saigon
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae


490 500 510 520 530 540 550 560
Copenhageni GATCCTTTGGTAAGTTTATCGGAACTGATTTCAGAAAACAGAGGGGATATTCGAAAAACATTCTCTAATATCGCAGATAT
D P L V S L S E L I S E N R G D I R K T F S N I A D I
Lai
Bratislava
Bangkok
Saigon .....A.....A...T.....T.....T.....C..T.....
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae .....A.....
570 580 590 600 610 620 630 640
Copenhageni TACCACTAAAATCAATACCGGAGACGGAAGTTTAGGAAGATTGATTAACAACGACGATGTTCATAAAAATGTAAACACAG
T T K I N T G D G S L G R L I N N D D V H K N V N T
Lai
Bratislava
Bangkok .....C.....
Saigon .....A.....T.....A.....T.....G.....C.....T.
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae .....C.....
650 660 670 680 690 700 710 720
Copenhageni TTTTGACAGACGCTCAAATTGTACTAAGAGAGCTTAGAGAAGGATTAGAAGATACAAGAGAACAAACTCCTGTGACAAGC
V L T D A Q I V L R E L R E G L E D T R E Q T P V T S
Lai
Bratislava
Bangkok
Saigon .....T.....G..A.....CC.....C..G..T.....
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
Bataviae

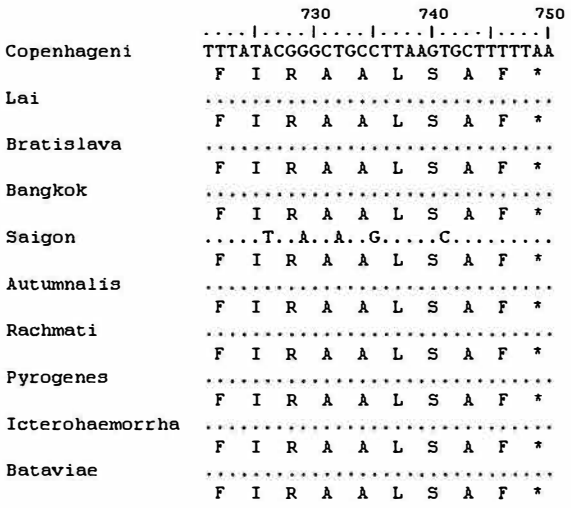


Figure 4. Alignment of DNA sequences (upper row) and their corresponding amino acid sequences (lower row) of *mce* gene homologue of *Leptospira interrogans* serovar Bratislava, Bangkok, Saigon, Autumnalis, Rachmati, Pyrogenes, Icterohaemorrhagiae, and Bataviae compared to known sequences of serovar Copenhageni and Lai obtained from GenBank. The sequences are shown from start to stop codons. The location of primers was underlined and labeled as indicated.

We also made recombinant proteins Mce and InvA in *E. coli* expression system to be antigens to determine expression of these genes *in vivo* using convalescent serum of patients with leptospirosis. However, none of convalescent sera from all 5 patients with leptospirosis did not detect the recombinant proteins on Western blot.

Discussion

The genes that are associated with the ability of *Leptospira* to adhere and penetrate host cells have not been verified. From whole genome sequences in the GenBank, we are interested in two genes of *Leptospira* that are homologues of *invA* gene of *R. prowazekii* [13] and *mce* gene of *M. tuberculosis* [14]. These two genes have been previously reported as virulence genes that are associated with attachment and invasion. The *invA* contains a conserved motif called the Nudix (Nucleotide diphosphates linked to some other moiety, X) box, which is in the Nudix hydrolase family [13]. This protein may play a role in enhancing the intracellular survival of

bacteria during host cell invasion. InvA was observed to be upregulated in the early period of rickettsial infection. Mce was also shown to be expressed during infection and may be associated with the ability of mycobacteria to gain entry into host cells [18]. A purified recombinant Mce protein coated on latex particles was able to promote uptake of the particles into HeLa cells [14]. Knock-out mutants of *mce* genes leaded to attenuation of virulence of *M. tuberculosis* in mice [19]. Hence, these two gene homologues are potential virulence genes of *Leptospira*. We proposed that these two homologues should be found in pathogenic serovars and their sequences should be conserved.

In our study different sets of primers designed based on the conserved DNA and amino acid sequences were used to detect the homologues of *invA* and *mce* genes. Most but not all pathogenic serovars used in the study contains the *invA* and *mce* homologues. The explanation of no amplification of these homologues in some pathogenic serovars is either the homologues are truly absent or the primers used in the study are unable to amplify the existing homologues due to unmatched sequences. Since most obtained sequences are highly conserved, negative amplification using three different sets of primers makes the latter reason to be less likely. However, other methods such as DNA hybridization using a conserved region as a probe may be additionally useful to confirm the results. The DNA and amino acid sequences of either gene seem to be conserved with minor differences in various serovars. Therefore, they should contain the conserved regions important for their functions as virulence factors.

In our study, convalescent sera of patients with leptospirosis did not detect recombinant Mce and InvA on Western blots. However, this result cannot exclude *in vivo* expression of these genes. It is possible that the proteins are not highly immunogenic or expressed at a low level in the hosts.

In conclusion, two presumptive virulence genes, which are homologues of *invA* and *mce* genes, are found to be conserved in most pathogenic serovars of *Leptospira* with high-degree similarity at nucleotide and amino acid levels. The homologues were absent in the non-pathogenic serovar.

To determine whether *invA* and *mce* are virulence genes, virulence of *invA*$^-$ and *mce*$^-$ mutants compared to the wild type needs to be examined in animal models. In addition, *in vivo* expression of these genes should be investigated in target tissues of animal models of leptospirosis by other techniques such as using RT-PCR to detect

gene expression at transcriptional level and using immunohistochemistry or immunofluorescence to detect gene expression at translational level.

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Output จากโครงการวิจัย

1. เสนอผลงานวิจัยในรูปแบบโปสเตอร์ในการประชุม 106th American Society for Microbiology General Meeting ในหัวข้อเรื่อง "Homologues of *invA* and *mce* genes and their conserved sequences among different serovars of *Leptospira interrogans*" ณ เมือง Orlando, Florida ประเทศ สหรัฐอเมริกา ระหว่างวันที่ 19 ถึงวันที่ 27 พฤษภาคม พ.ศ. 2549
2. ผลงานดีพิมพ์ในวารสารวิชาการนานาชาติ อยู่ในระหว่างทำการวิจัยเพิ่มเติมและจะดำเนินการเขียนบทความเพื่อลงตีพิมพ์ ในวารสาร Asian Biomedicine ภายในระยะ 6 เดือน