รายงานการวิจัยฉบับสมบูรณ์

ทุนอุดหนุนการวิจัยเงินงบประมาณแผ่นดิน ประจำปี 2548

เรื่อง

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

Study of homology of *mce* and *invA* genes among different serovars of *Leptospira interrogans* and their *in vivo* expression as a step of vaccine development for leptospirosis

โดย

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กิตติกรรมประกาศ

ขอขอบคุณ ผู้ช่วยวิจัย เจ้าหน้าที่ภาควิชาจุลชีววิทยา เจ้าหน้าที่ของ Center of Excellence, Viral Hepatitis Research Unit ภาควิชากุมารเวชศาสตร์ และเจ้าหน้าที่ฝ่ายวิจัย คณะแพทยศาสตร์ที่เกี่ยวข้องทุกท่านที่ช่วยให้การดำเนินงานวิจัยนี้สำเร็จลุล่วงไปด้วยดี

งานวิจัยนี้ได้รับทุนอุดหนุนการวิจัยจากเงินงบประมาณแผ่นดิน ประจำปีงบประมาณ 2548 งานวิจัยนี้เป็นส่วนหนึ่งของการศึกษาโรคเลปโตสไปโรซิสเพื่อให้มีความรู้เกี่ยวกับเชื้อก่อโรค และพยาธิกำเนิดของโรคมากยิ่งขึ้น อันจะนำไปสู่การพัฒนาการวินิจฉัยและวัคซีนป้องกันโรค ต่อไปในอนาคต

บทคัดย่อ

โรคเลปโตสไปโรซิสหรือโรคฉี่หนูเป็นโรคที่เป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทย พยาธิกำเนิดของโรคนี้ยังไม่ทราบแน่ชัด ปัจจุบันลำดับเบสทั้งหมดของสารพันธุกรรมของเชื้อ *Leptospira interrogans* เสร็จสมบูรณ์แล้วทำให้มีการพบยีนก่อโรคของเชื้อเพิ่มขึ้น ซึ่งงานวิจัยนี้สนใจยีน 2 ยีนที่มีความคล้าย (homologue) กับยีนที่มีหน้าที่เกี่ยวข้องกับการบุกรุกของเชื้อก่อโรคอื่น ได้แก่ invasionA (*invA*) ของเชื้อ *Rickettsia prowazekii* และยีน mammalian cell entry (*mce*) gene ของเชื้อ *Mycobacterium tuberculosis* ผู้วิจัยตั้งสมมติฐานว่าถ้ายีนทั้งสองเป็นยีนก่อโรคจึงควรพบยีนนี้ในเชื้อ *Leptospira interrogans* ใน serovars ที่ก่อโรคในคน ผลการศึกษาโดยใช้วิธี polymerase chain reaction (PCR) ซึ่งอาศัย primers ที่ออกแบบให้จับกับบริเวณที่มีการอนุรักษ์ลำดับเบสหรือลำดับกรดอะมิโนพบว่าจากเชื้อชนิดก่อโรคทั้งหมดที่ใช้ในการศึกษา จำนวน 10 serovars มีการตรวจพบยีน *invA* homologue จำนวน 7 serovars และตรวจพบยีน *mce* homologue จำนวน 8 serovars โดยไม่พบยีนทั้งสองในเชื้อชนิดไม่ก่อโรค (serovar Patoc) เมื่อทำการหาลำดับเบสทั้งหมดของยีนที่พบใน serovars ต่างๆและนำมาเปรียบเทียบลำดับเบสและลำดับกรดอะมิโนพบว่ายีน *invA* homologue ของเชื้อที่พบ 7 serovars มีความเหมือนกันทั้งลำดับเบสและลำดับกรดอะมิโนมากกว่า 99 เปอร์เซ็นต์ ส่วนยีน *mce* homologue ของเชื้อที่พบ 8 serovars มีความเหมือนกันของลำดับเบสประมาณ 90 ถึง 100 เปอร์เซ็นต์ซึ่งเมื่อเปรียบเทียบลำดับกรดอะมิโนพบว่ามีความเหมือนกันมากกว่า 98 เปอร์เซ็นต์ แต่การศึกษาการแสดงออกของยีนทั้งสองในร่างกาย (*in vivo*) ไม่สามารถตรวจพบด้วยการตรวจหาแอนติบอดีที่จำเพาะต่อโปรตีนในซีรั่มของผู้ป่วยโรคเลปโตสไปโรซิสโดยใช้โปรตีนที่สร้างขึ้นใน *E.coli* (recombinant proteins) การศึกษาขั้นต่อไปคือผู้วิจัยจะตรวจหาการแสดงออกของยีนทั้งสองในร่างกายโดยการศึกษาในระดับ transcription ในสัตว์ทดลอง

Abstract

Leptospirosis is one of major health problems in Thailand. Pathogenesis of leptospirosis is not well understood. 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. We proposed that these gene homologues should be conserved in pathogenic serovars of *Leptospira*. Ten different pathogenic serovars and one non-pathogenic serovar (serovar Patoc) of leptospires were used in this study to determine the presence and the conservation of each gene in various serovars of pathogenic *Leptospira*. Polymerase chain reaction (PCR) 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 found 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. Their expression *in vivo* was determined by Western blot and immunodetection of purified recombinant proteins of each gene with sera of patients infected with leptospirosis. However, neither recombinant protein was able to bind to patients'sera. Further experiment is to detect gene expression at the transcription level in animal model.

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คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

invA	invasionA gene
InvA	protein product of *invA*
mce	mammalian cell entry
Mce	protein product of *mce*
^{o}C	degree Celsius
min	minute
h	hour
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
bp	base pair
M	molar
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IPTG	isopropyl β-D-thiogalactoside
Ni-NTA	nickel-nitriolotriacetic acid
BCIP	5-bromo-4-chloro-3-indoxyl-phosphate
NBT	nitroblue tetrazolium

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* and to compare their sequence similarity. In addition, the expression of gene products *in vivo* will be determined. The finding will support the role of these genes as virulence factors.

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°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing EMJH basal medium (Difco&BBL, Sparks, MD, USA) 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.

Human serum samples

Human serum samples used in this study were collected from patients with clinically suspected of leptospirosis, and then confirmed by four-fold rising titers of the microscopic agglutination test (MAT). Pooled sera were derived from sera of five patients with a MAT titer of at least 1: 400.

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°C until used or -20°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. All primers were purchased from GIBCO Invitrogen Corporation (Grand Island, NY, USA). 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 *inA* 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 (°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, Madison, WI, USA). 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, USA). 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, USA) before they were directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) 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 (Applied Biosystems). Nucleotide sequences of each gene homologue from different serovars were compared by the ClustalX program.

Cloning of *invA* and *mce* gene homologues into *E.coli*

PCR was used to amplify the full-length sequence of leptospiral *invA* and *mce* genes. For amplification of *invA* gene, the forward primer, FinvA (GCGCGGATCCGAATGGACAAACCCTAC), and the reverse primer, RinvA (GCCGAATTCCATTACGATCTATTTCC), contained a *Bam*HI restriction site (underlined) and an *Eco*RI restriction site (underlined), respectively. The forward primer, Fmce (GCGCGGATCCGATTGAATATGAATTCGTTACGT) containing a *Bam*HI restriction site (underlined) and the reverse primer, Rmce (GCGCCATGGTTAAAAAGCACTTAAGGCAG) containing a *Nco*I restriction site (underlined), were used to amplify the *mce* gene. Genomic DNA purified from serovar Lai was used as a DNA template. DNA polymerase with proofreading activity,

DyNAzyme EXT™ (Finnzymes), was used in the PCR reaction. The amplification conditions were the same as previously described above except the annealing temperature was 60°C. The amplified products were purified, digested with the corresponding endonucleases (Fermentas, Ontario, Canada) and ligated into pre-digested pRSET C expression plasmid (GIBCO Invitrogen) with T4 DNA ligase (Promega). The vector provided 6His-tag at the N-terminus of recombinant proteins. The resulting constructs, pRSET C-*invA* and pRSET C-*mce*, were separately transformed into *E.coli* BL21(DE3)pLysS (Novagen, Merck Biosciences, Darmstadt, Germany). The transformants were selected on LB agar supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, MO, USA) and 35 μg/ml chloramphenicol (Sigma). The nucleotide sequence of the insert was confirmed by automated sequencer (Applied Biosystems) using primers in the region of T7 promotor and T7 transcription terminator of the vector.

Expression and purification of 6His-InvA and 6His-Mce recombinant proteins

The clone with correct insert was grown in LB broth to an OD_{600} of 0.4-0.6 before induction of protein expression with isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM at 37°C for 4 hours. Alternatively, the clone was grown in the Overnight Express Instant TB Medium (Novagen) at 37°C overnight. The recombinant protein was purified using nickel-nitriolotriacetic acid (Ni-NTA) resin column (His-Bind Purification Kit, Novagen) according to the supplier's instruction. Briefly, cells were harvested by centrifugation and resuspended in BugBuster protein extraction reagent (Novagen) containing protease inhibitor cocktail set III (Calbiochem, Merck Biosciences, Darmstadt, Germany). The soluble and insoluble fractions were separated by centrifugation at 10,000Xg for 10 min. The recombinant protein was found to be mostly in the insoluble fraction. Therefore, purification was performed under denaturing conditions with Binding, Wash, and Elution buffers containing 6 M urea. The insoluble fraction was solubilized in Binding buffer including 6 M urea and centrifuged at 16,000Xg for 30 min to remove insoluble material. The supernatant was filtered through a 0.45-μm membrane prior to loading to pre-charged and equilibrated His-Bind Resin column. The bound protein was eluted with Elute buffer containing 1 M imidazole and 6

M urea. The eluate was collected and then centrifuged with Amicon Ultra-15 5,000 MWCO Centrifugal Filter Unit (Millipore, Carrigtwohill, County Cork, Ireland) to concentrate proteins and to replace the Elute buffer with phosphate buffered saline (PBS) pH 7.4. The proteins were kept at -20°C until use or -80°C for long-term storage.

Gel electrophoresis and immunoblot analysis

Leptospira total cell lysate, soluble and insoluble fractions, and recombinant proteins purified from Ni-NTA resin column were fractionated on a 15% SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Amersham, Piscataway, NJ, USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA). The blot was incubated with a blocking buffer (5% skim milk in PBST containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 1 h followed by a primary antibody, mouse anti-His monoclonal antibody (Amersham) at a 1:3,000 dilution or pooled patient sera (1:100 dilution in blocking buffer). The blot was washed with PBST and incubated with 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (KPL,Gaithersburg, MD, USA) or alkaline phosphatase-labeled goat anti-human IgG (KPL) at a 1:3,000 dilution for 1 h. The blot was washed with PBST before addition of substrate reagents, BCIP/NBT phosphatase substrate (1-component) solution (KPL) for chromogenic development.

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.

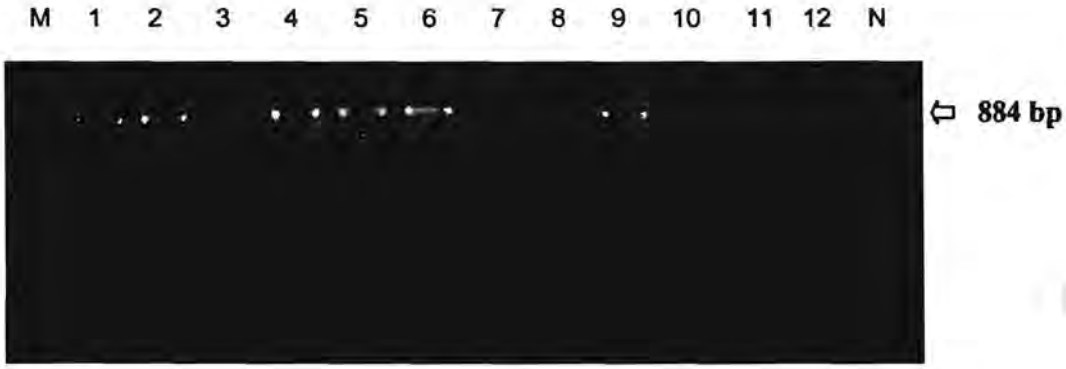


Figure 1. Detection of the *invA* gene homologue in *Leptospira* by PCR using Flank invA5' and Flank invA3' as primers. Lanes: 1, serovar Lai; 2, Bangkok; 3, Javanica; 4, Rachmati; 5, Bratislava; 6, Autumnalis; 7, Saigon; 8, Icterohaemorrhagiae; 9, Pyrogenes; 10, Bataviae; 11, Sejroe; 12; Patoc; M, 100-base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

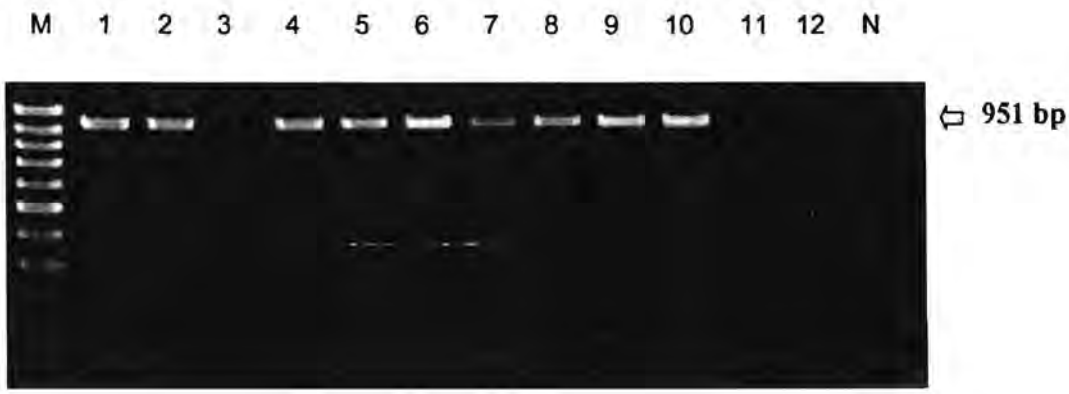
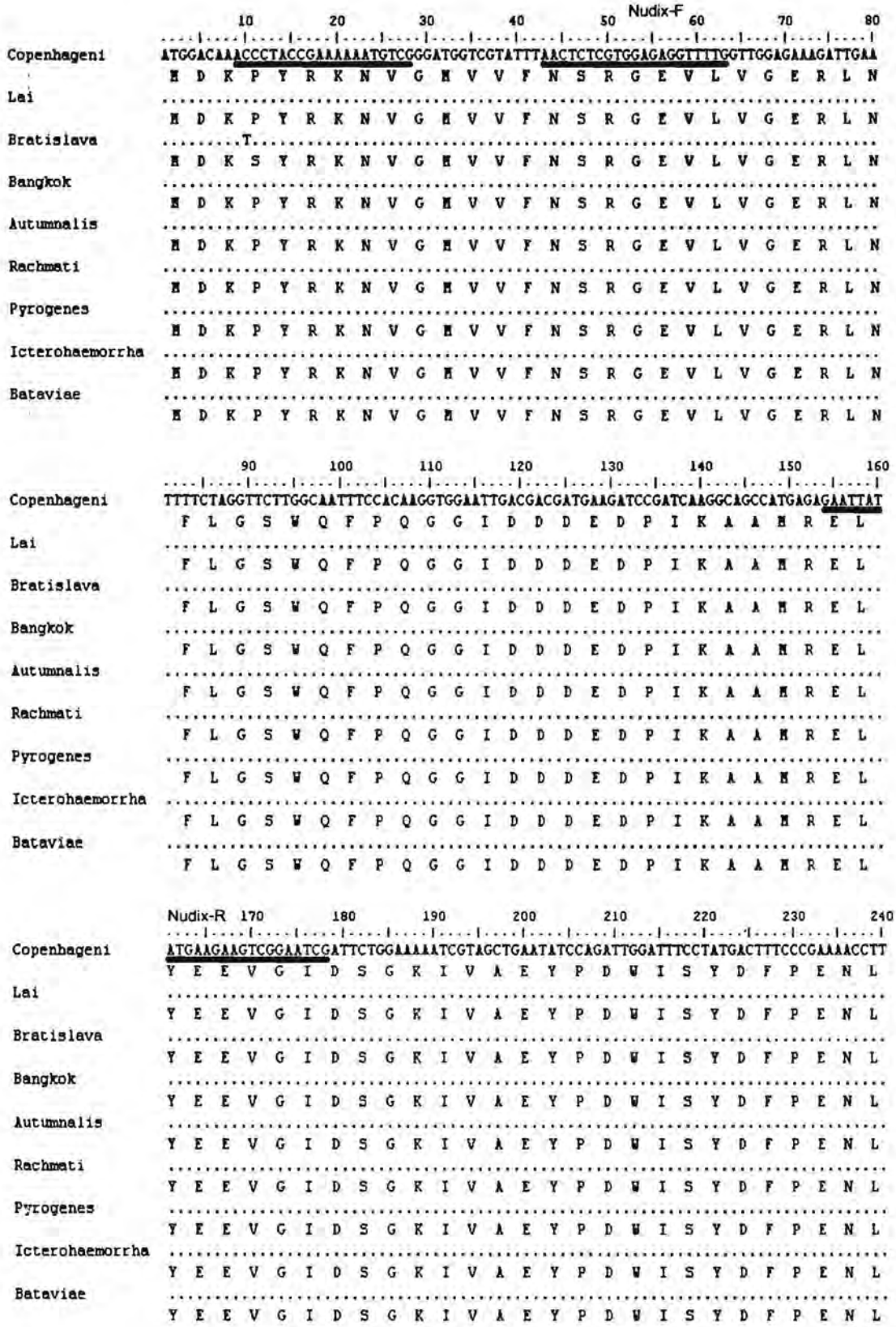


Figure 2. Detection of the *mce* gene homologue in *Leptospira* by PCR using Flank mce5' and Flank mce3' as primers. Lanes: 1, serovar Lai; 2, Bangkok; 3, Javanica; 4, Rachmati; 5, Bratislava; 6, Autumnalis; 7, Saigon; 8, Icterohaemorrhagiae; 9, Pyrogenes; 10, Bataviae; 11, Sejroe; 12; Patoc; M, 100-base 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.


Nudix-F
Nudix-R
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Bangkok
Autumnalis
Rachmati
Pyrogenes
Icterohaemorrha
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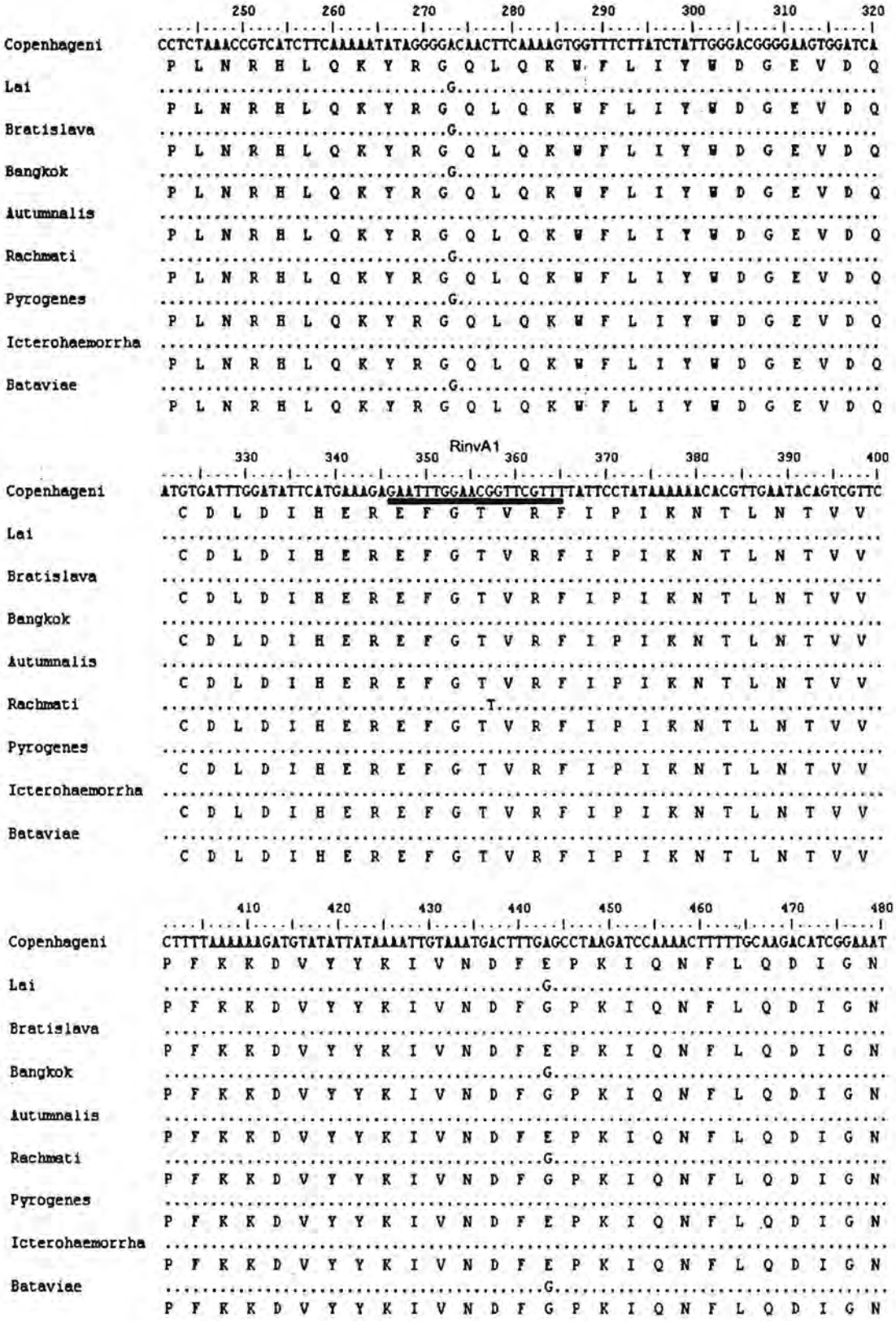
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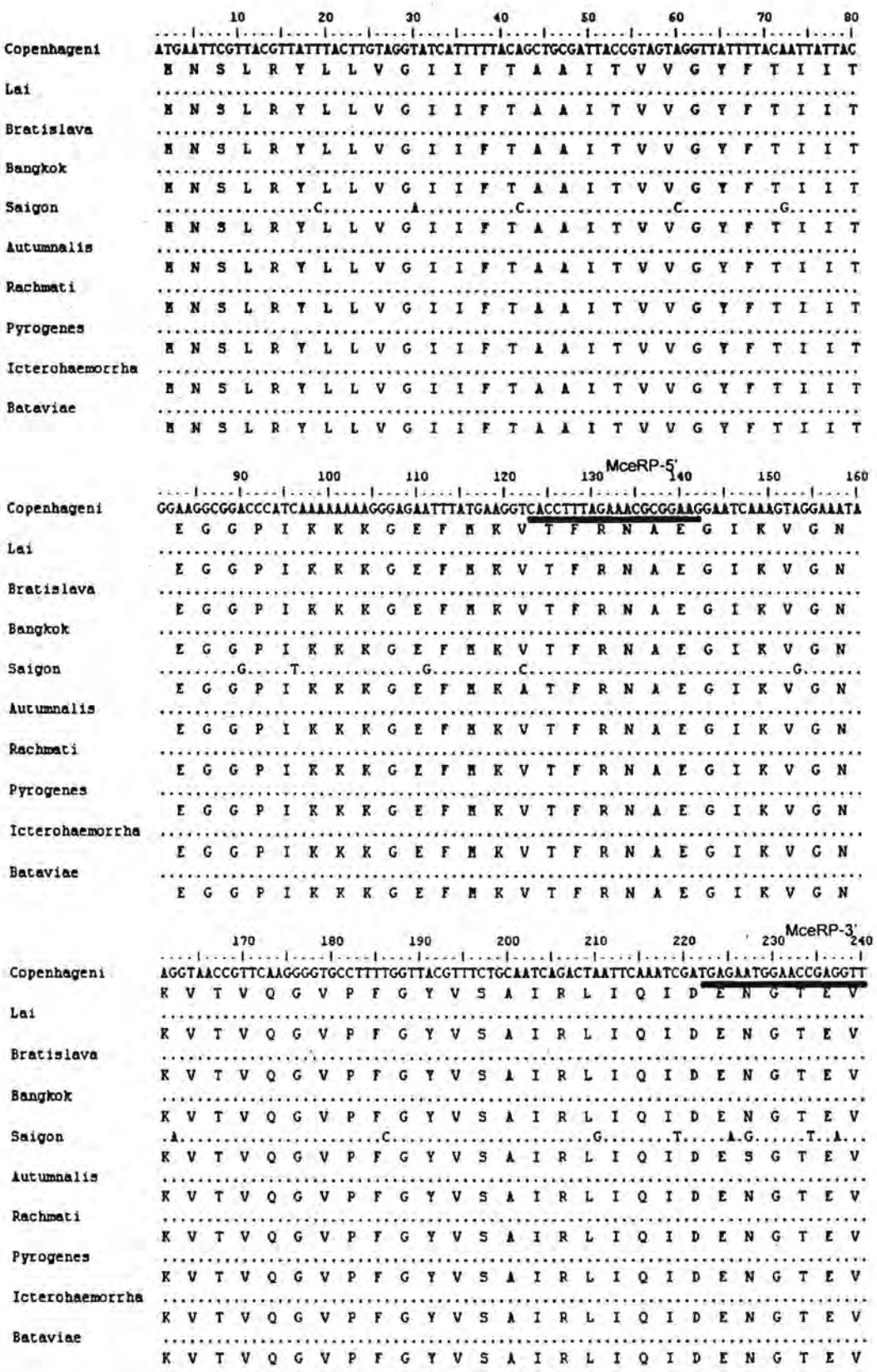
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Lai .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Bratislava .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Bangkok .....G.....
P L N R H L Q K Y R G Q L Q K W F L I Y W D G E V D Q
Autumnalis .....
P L N R H 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
RinvA1
330 340 350 360 370 380 390 400
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 CTTTTAAAAAAGATGTATATTATAAAATTGTAAATGACTTTGAGCCTAAGATCCAAAACTTTTTGCAAGACATCGGAAAT
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.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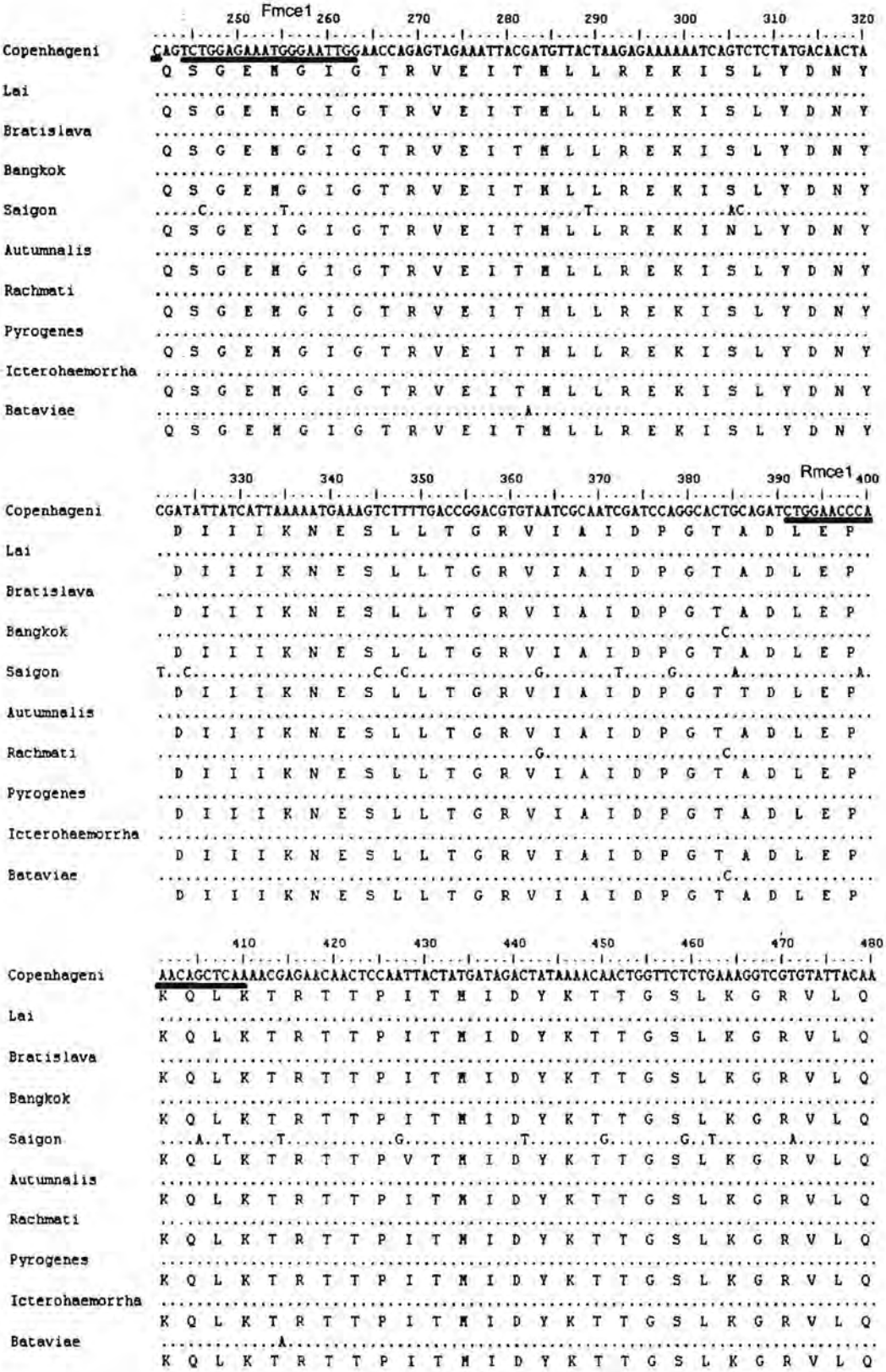
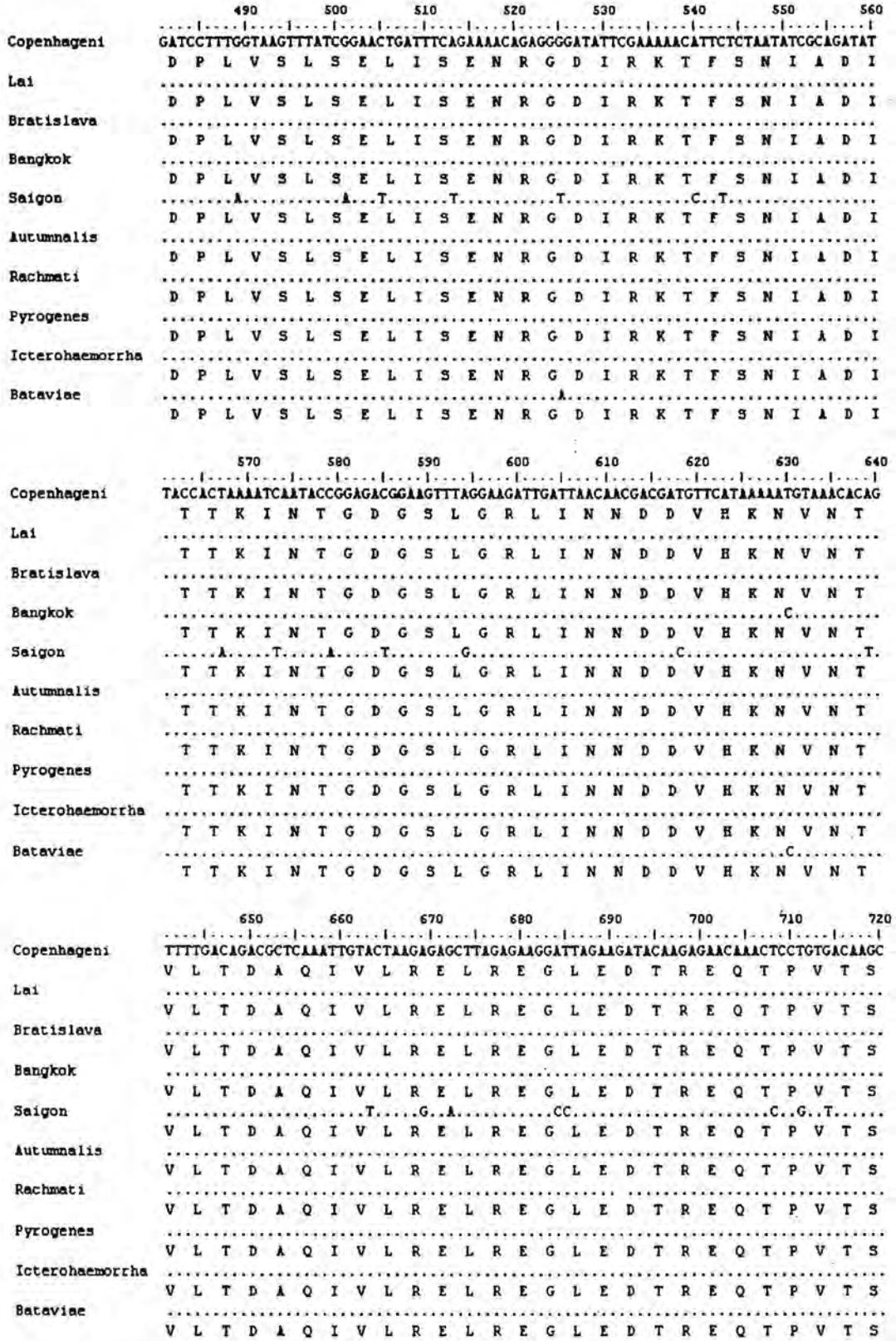
M N S L R Y L L V G I I F T A A I T V V G Y F T I I T
Lai .....
Bratislava .....
Bangkok .....
Saigon .....C.....A.....C.....C.....G.....
Autumnalis .....
Rachmati .....
Pyrogenes .....
Icterohaemorrha .....
Bataviae .....
90 100 110 120 130 140 150 160
MceRP-5'
Copenhageni GGAAGGCGGACCCATCAAAAAAAAGGGAGAATTTATGAAGGTCACCTTTAGAAACGCGGAAGGAATCAAAGTAGGAAATA
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.....
E G G P I K K K G E F M K A T F R N A E G I K V G N
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
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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

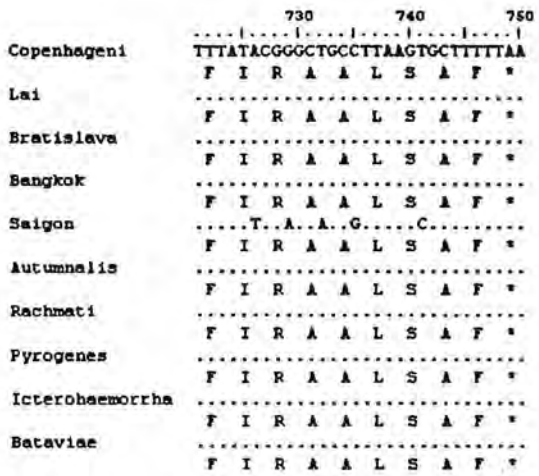

Figure 4. Alignment of DNA sequences (upper row) and their corresponding amino acid sequences (lower row) of *mce* gene homologue of *Leptospira interrogans* serovar Bratislava, Bangkok, Saigon, Autumnalis, Rachmati, Pyrogenes, Icterohaemorrhagiae, and Bataviae compared to known sequences of serovar 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.

To determine expression of *invA* and *mce* genes *in vivo*, recombinant proteins of these genes were produced and used to detect antibodies against these proteins in sera of patients with leptospirosis. The 6His-InvA and 6His-Mce recombinant proteins were found to be in the insoluble fraction or inclusion body (data not shown). Pooled sera from patients with leptospirosis were unable to detect the recombinant proteins although anti-His monoclonal antibody was shown to bind to the recombinant proteins on the blot (Figure 5).

Figure 5. Immunodetection of InvA (A) and Mce (B) recombinant proteins with anti-His monoclonal antibody (lane 1) and pooled sera of patients with leptospirosis (lane 2).

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.

Pooled sera of patients with leptospirosis were unable to detect the 6His-InvA and 6His-Mce recombinant proteins. However, expression of these genes *in vivo* cannot be excluded. The possible explanations for the negative finding of immunodetection are: 1) the InvA and Mce proteins may not be immunogenic. Alternatively, the immunogenicity of the proteins is too poor to induce strong antibody production. Therefore, the antibody against these proteins is overwhelmed by other components of *Leptospira* that are stronger immunogens. 2) the genes may be transiently or temporarily expressed when the proteins are required for infection and then are down regulated. Therefore, antibody response against these proteins may not be strong or only strong enough to be detected at a specific time point of infection. 3) the epitopes on the 6His-InvA and 6His-Mce recombinant proteins are distinct from their native proteins so they cannot be recognized by antibodies present in human sera. However, this assumption may be less likely since pooled sera from patients should contain polyclonal antibodies which hypothetically can recognize several linear and conformational epitopes. Hence, detection of gene transcripts by more sensitive

techniques such as reverse transcription followed by real time PCR should be used to determine the expression of *invA* and *mce* genes *in vivo*.

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. The expression of these two proteins *in vivo* cannot be demonstrated by immunodetection of recombinant proteins with patients' sera.

Our next step is to show the expression of these genes *in vivo* at the transcription level in the infected tissues of animal model. In addition, we plan to determine the role of these genes in tissue invasion. Knowledge obtained from this study provides better insight of pathogenesis of leptospirosis which is crucial for the development of better diagnostic techniques, treatment, and vaccine in the future.

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ประวัตินักวิจัยและคณะ พร้อมหน่วยงานสังกัด

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ผลงานวิจัย

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พ.ศ. 2534 รางวัลผลงานวิจัยดีเด่น เรื่อง การดูแลทารกที่คลอดจากมารดาที่เป็นพาหะไวรัสตับอักเสบ บี ผลการศึกษาระยะยาว 5 ปี จากจุฬาลงกรณ์มหาวิทยาลัย

พ.ศ. 2536 รางวัลผลงานวิจัยดีเยี่ยม เรื่อง ปัญหาและการป้องกันไวรัสตับอักเสบในประเทศไทย จากสภาวิจัยแห่งชาติ

พ.ศ. 2540 รางวัลนักวิทยาศาสตร์ดีเด่น จากมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์ และรางวัลนักวิจัยดีเด่นแห่งชาติ สำนักงานคณะกรรมการวิจัยแห่งชาติ

พ.ศ. 2540-ปัจจุบัน เมธีวิจัยอาวุโส สกว สำนักงานกองทุนสนับสนุนการวิจัย

พ.ศ. 2546 รางวัลมหาวิทยาลัยมหิดล-บี บราวน์ เพื่อการแพทย์และสาธารณสุขไทย

ผลงานวิจัย

ระดับนานาชาติมากกว่า 130 เรื่อง

ระดับประเทศมากกว่า 150 เรื่อง

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