Investigation of *Escherichia coli* O157 and *Vibrio parahaemolyticus* spreading across Thai and Malaysian border

Pharanai Sukhumungoon

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Author	Mr. Pharanai Sukhumungoon
Major Program	Microbiology

Major Advisor:

.....

(Assoc. Prof. Dr. Varaporn Vuddhakul)

Examining Committee:

.....

(Assoc. Prof. Prasit Tharavichitkul)

.....

(Assoc. Prof. Dr. Varaporn Vuddhakul)

.....

(Assoc. Prof. Usa Chethanond)

.....

(Dr. Rattanaruji Pomwised)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Microbiology

.....

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์ การศึกษา *Escherichia coli* O157 และ *Vibrio parahaemolyticus* ที่ระบาดข้ามเขตชายแดนไทยและมาเลเซีย
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สาขาวิชา จุลชีววิทยา
ปีการศึกษา 2553

บทคัดย่อ

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 และ *Vibrio parahaemolyticus* เป็นเชื้อก่อโรคสำคัญในอาหาร *E. coli* O157 เป็นสาเหตุของ hemorrhagic colitis และ hemolytic uremic syndrome ส่วน *V. parahaemolyticus* ทำให้กระเพาะและลำไส้อักเสบ ในการศึกษานี้ได้แยกเชื้อ *E. coli* O157:H7 และ *V. parahaemolyticus* จากเนื้อวัวและหอยแครงที่นำเข้าจากประเทศมาเลเซียสู่ประเทศไทย ในการแยกเชื้อโดยเทคนิค immunomagnetic separation และ CHROMagar สามารถแยกเชื้อ *E. coli* O157:H7 จำนวน 14 สายพันธุ์ และ O116:H31 จำนวน 1 สายพันธุ์ จากเนื้อวัว 31 ตัวอย่างที่นำเข้าจากประเทศมาเลเซีย และสามารถยแยกเชื้อ *E. coli* O157:H7 ได้ 6 สายพันธุ์จากเนื้อวัวไทย 36 ตัวอย่าง *E. coli* ทุกสายพันธุ์มีจีน stx_2 ยกเว้น 2 สายพันธุ์ที่ได้จากเนื้อวัวมาเลเซียคือสายพันธุ์ M2 ซึ่งไม่มี stx_1 และ stx_2 และสายพันธุ์ M7 ซึ่งมีทั้ง stx_1 และ stx_2 ทุกสายพันธุ์ถูกนำมาหาปัจจัยที่เกี่ยวข้องกับการก่อโรค เช่น ปัจจัยในการเกาะติดเซลล์ของโฮสต์ (intimin-*eae*) การสร้าง bundle formimg pilli (*bfp*) Locus of enterocyte effacement (*esc*V) และการมี 60-MDa plasmid จากการศึกษาพบว่า *E. coli* O157 ทั้งหมดที่มี stx_2 ถูกจัดเป็น EHEC เนื่องจาก สายพันธุ์เหล่านั้นตรวจพบ *eae*, *esc*V และ 60-MDa plasmid ยกเว้น *E. coli* O116:H31 (M7) ซึ่งไม่พบจีน *eae* ดังนั้น สายพันธุ์นี้ถูกจัดเป็น Shiga toxin-producing *E. coli* (STEC) ส่วนจีน *bfp* ไม่พบใน *E. coli* ทุกสายพันธุ์ที่ตรวจสอบ *E. coli* สายพันธุ์ M2 ซึ่งไม่มีทั้ง stx_1 และ stx_2 ถูกจัดเป็น atypical enteropathogenic *E. coli* เนื่องจากสายพันธุ์นี้ตรวจพบทั้งจีน *eae* และจีน *esc*V ในการตรวจสอบการสร้าง Stx2 โดยวิธี TNP-PCR พบว่า *E. coli* ทุกสายพันธุ์ให้ผลบวกยกเว้นสองสายพันธุ์คือ $stx_1^-stx_2^-$ (M2) และ $stx_1^+stx_2^+$ (M7) แสดงว่า สายพันธุ์ที่ให้ผลบวกเหล่านี้ ไม่สามารถที่จะสร้าง Stx2 ได้ และได้ทำการทดสอบยืนยันว่าสายพันธุ์เหล่านี้ไม่สามารถสร้าง Stx2 อีกครั้งโดยวิธี reverse passive latex agglutination (RPLA) การทดสอบการดื้อต่อยาปฏิชีวนะ 12 ชนิด พบว่า *E. coli* O157 1 สายพันธุ์จากมาเลเซีย (M14) และ *E. coli O157* 1 สายพันธุ์จากไทย (T3) ให้แบบแผนการดื้อต่อยาปฏิชีวนะเหมือนกัน ความสัมพันธ์ระหว่าง

E. coli ที่แยกจากมาเลเซียและไทยจำนวน 21 สายพันธุ์ถูกวิเคราะห์โดยเทคนิค IS-printing และ Pulsed-field gel electrophoresis (PFGE) วิธี IS-printing สามารถจัดกลุ่มความสัมพันธ์ของเชื้อได้เป็น 7 กลุ่ม (กลุ่ม I ถึง VII) โดยเชื้อสายพันธุ์ไทยทั้งหมด 6 สายพันธุ์ (T1-T6) และเชื้อสายพันธุ์มาเลเซียส่วนใหญ่ (ยกเว้นสายพันธุ์ M1 และ M7) ถูกจัดอยู่ในกลุ่มที่ III วิธี PFGE แสดงประสิทธิภาพในการจัดกลุ่มเชื้อได้ดีกว่าวิธี IS-printing พบว่าลายพิมพ์ DNA ที่ได้จากเชื้อสามารถจัดกลุ่มได้ 11 กลุ่ม (กลุ่ม I ถึง XI) เชื้อสายพันธุ์ไทยทั้งหมดถูกจัดอยู่ในกลุ่มที่ IX และ ลายพิมพ์ DNA ของ 2 จาก 6 สายพันธุ์ (T4 และ T5) มีความเหมือนกัน เชื้อ 15 จาก 19 สายพันธุ์ของมาเลเซีย ถูกจัดจำแนกเป็น 7 กลุ่ม (กลุ่มที่ I, II, III, V, IX, X และ XI) ที่น่าสนใจก็คือ เชื้อสายพันธุ์มาเลเซียสองสายพันธุ์คือ M14 และ M15 ถูกจัดอยู่ในกลุ่มเดียวกับเชื้อสายพันธุ์ไทย (กลุ่มที่ IX) จากข้อมูลนี้ บ่งบอกว่า เชื้อ *E. coli* O157:H7 สายพันธุ์จากไทยและมาเลเซียมีความใกล้ชิดกัน ดังนั้น มีความเป็นไปได้ว่าเชื้อ *E. coli* O157:H7 จากประเทศมาเลเซียเข้าสู่ประเทศไทยผ่านทางเนื้อวัวนำเข้า ในการศึกษานี้ยังได้ตรวจหาเชื้อสายพันธุ์ที่สามารถสร้าง Stx ในปริมาณที่สูงโดยทำการตรวจสอบเนื้อวัวจากตลาดหลายแห่งในเมืองหาดใหญ่ พบเชื้อ Shiga toxin-producing *E. coli* (PSU 5023) ซึ่งสามารถสร้าง Stx1 ในปริมาณที่สูง (titer= 1: 2,048) และพบว่า การที่เชื้อผลิต toxin ได้สูงนั้นเป็นผลมาจากเชื้อมียีน Q_{933}

ในการศึกษานี้ได้ทำการตรวจหา *V. parahaemolyticus* pandemic strain ในตัวอย่างหอยที่นำเข้ามาจากประเทศมาเลเซียโดยใช้เทคนิค immunomagnetic separation และ CHROMagar vibrio แอนติซีรั่มต่อ K6 และ K25 ได้นำมาใช้ในการแยกเชื้อ ในการทดลองนี้ได้ตรวจสอบตัวอย่างหอยจำนวน 24 ตัวอย่าง คือ หอยแครงไทย หอยแครงมาเลเซีย หอยแมลงภู่ไทย และหอยตลับไทย สามารถแยกเชื้อ *V. parahaemolyticus* ที่ให้ผลบวกต่อยีน *tdh*, GS-PCR และ 16 kb insertion sequence ซึ่งเป็น marker ของ pandemic strain ได้จำนวน 15 สายพันธุ์ จากจำนวนเชื้อ *V. parahaemolyticus* ทั้งหมด 42 สายพันธุ์ โดยเป็นเชื้อจากหอยแครงจากไทย 3 สายพันธุ์ หอยแครงจากมาเลเซีย 1 สายพันธุ์ หอยแมลงภู่ไทย 8 สายพันธุ์ และ หอยตลับไทย 3 สายพันธุ์ การวิเคราะห์ลายพิมพ์ DNA ของเชื้อโดยวิธี AP-PCR พบว่าทั้ง 8 สายพันธุ์ของเชื้อ *V. parahaemolyticus* pandemic strain ที่แยกได้จากหอยแมลงภู่มีต้นกำเนิดเดียวกัน แต่ *V. parahaemolyticus* pandemic strains ที่แยกได้จากหอยแครงไทยจำนวน 2 สายพันธุ์ (PSU 4063/1-2) และจากหอยตลับไทยจำนวน 3 สายพันธุ์ (PSU 4070/1-3) มาจากต้นกำเนิดที่ต่างกัน ที่น่าสนใจก็คือ ลายพิมพ์ DNA ของ 1 สายพันธุ์ที่แยกได้จากหอยแครงของไทย (PSU 4063/2) นั้น มีลายพิมพ์ DNA ที่เหมือนกับสายพันธุ์ที่แยกได้จากมาเลเซีย (PSU 4067) จากการวิเคราะห์ลายพิมพ์ DNA โดยวิธี PFGE เพื่อยืนยันความสัมพันธ์ของเชื้อ *V. parahaemolyticus* pandemic strains ที่แยกได้จากสิ่งแวดล้อมและจากตัวอย่างผู้ป่วยจากประเทศไทยและมาเลเซีย (ระหว่างปี ค.ศ. 1998 ถึง 2008) รวมถึง

V. parahaemolyticus pandemic strain ที่แยกได้จากฮ่องกง (ค.ศ. 2005) และประเทศสหรัฐอเมริกา (ค.ศ. 1997 และ 2003) ได้ถูกนำเปรียบเทียบด้วย พบว่าลายพิมพ์ DNA ของ *V. parahaemolyticus* ทั้งหมด 23 สายพันธุ์ เมื่อใช้เอนไซม์ตัดจำเพาะ *Not* I และ *Sfi* I สามารถจัดกลุ่มได้เป็น 6 กลุ่ม (กลุ่ม I-VI) เชื้อ *V. parahaemolyticus* pandemic strains สายพันธุ์จากผู้ป่วยและสิ่งแวดล้อมที่แยกได้จากประเทศไทยและมาเลเซียระหว่างปี ค.ศ. 1998 ถึง 2008 ทั้งหมดถูกจัดอยู่ในกลุ่มที่ IV ที่น่าสนใจก็คือ ในกลุ่มนี้มีเชื้อ 4 สายพันธุ์ที่แยกได้ในปี ค.ศ. 2001 และ 2008 ให้ลายพิมพ์ DNA เหมือนกัน (PSU 2050 จากผู้ป่วยประเทศไทย PSU 4063/2 จากหอยแครงไทย PSU 474 จากหอยแมลงภู่ไทย และ PSU 4067 จากหอยแครงประเทศมาเลเซีย) จากข้อมูลนี้แสดงว่า เชื้อทั้ง 4 สายพันธุ์นี้มาจากต้นกำเนิดเดียวกัน จากการศึกษานี้สรุปว่า มีการปนเปื้อนเชื้อ *E. coli* O157 และ *V. parahaemolyticus* pandemic strain ในเนื้อวัวและหอยแครงนำเข้าจากประเทศมาเลเซีย ซึ่งเป็นอันตรายต่อผู้บริโภค ถึงแม้ว่าจากการศึกษานี้พบว่า *E. coli* O157 ที่แยกได้ไม่สามารถสร้าง toxin ได้ในหลอดทดลอง

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Author	Mr. Pharanai Sukhumungoon
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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and *Vibrio parahaemolyticus* are important food-borne pathogens. *E. coli* O157 is a causative agent of hemorrhagic colitis and hemolytic uremic syndrome and *V. parahaemolyticus* causes gastroenteritis. In this study, *E. coli* O157:H7 and *V. parahaemolyticus* were investigated in beef and bloody clam imported from Malaysia to Thailand. Using immunomagnetic separation technique and CHROMagar, 14 strains of *E. coli* O157:H7 and one strain of *E. coli* O116:H31 (M7) were isolated from 31 Malaysian beef samples whereas 6 strains of *E. coli* O157:H7 were isolated from 36 Thai beef samples. All strains of *E. coli* contained only stx_2 gene except two strains obtained from Malaysian beef which one lacked of stx_1 and stx_2 (M2) and another contained both stx_1 and stx_2 (M7). All of them were characterized by determining some virulence factors such as an adherence factor gene (intimin-*eae* gene), bundle forming pilli gene (*bfp* gene), Locus of enterocyte effacement detection (*esc*V gene) and the presence of 60-MDa plasmid. The results revealed that all stx_2^+ gene *E. coli* O157 were classified as EHEC because they possessed *eae*, *esc*V and 60-MDa plasmid except *E. coli* O116:H31 (M7) which lacked of *eae* gene. Therefore, this strain was classified as Shiga toxin-producing *E. coli* (STEC). *bfp* gene could not be detected in all examined *E. coli* strains. *E. coli* strain M2 which lacked of both stx_1 and stx_2 was classified as an atypical Enteropathogenic *E. coli* because this strian harbored *eae* and *esc*V genes. TNP-PCR was performed to detect Stx2 production; all of 21 tested strains were positive for TNP-PCR except two strains of stx_1^- stx_2^- (M2) and stx_1^+ stx_2^+ (M7). This indicated that they were unable to produce Stx2 and it was confirmed again by reverse passive latex agglutination (RPLA). Drug susceptibility to

12 antibiotics revealed that EHEC of one Malaysian strain (M14) and one Thai strain (T3) exhibited the same antibiotic resistance patterns. Relationship among all 21 isolates of *E. coli* from Malaysian and Thai beef were determined by IS-printing and Pulse-field gel electrophoresis (PFGE) techniques. Using IS-printing, the DNA profiles of all isolates could be classified into seven groups. All six Thai strains (T1-T6) and most of the Malaysian strains (except M1 and M7 strains) were classified in the same group (group III). DNA profile analysis using PFGE showed higher resolution over IS-printing technique because it generated DNA profiles of all isolates into 11 groups designated as group I to XI. All Thai strains (T1 - T6) were in group IX, and the DNA profiles of two of the six strains (T4 and T5) were identical. Fifteen out of 19 Malaysian strains were classified into seven groups (groups I, II, III, V, IX, X and XI). Interestingly, two Malaysian strains, M14 and M15, were in the same group of Thai strains (group IX). The results indicated the close relationship among Thai and Malaysian *E. coli* O157:H7 isolated from beef in this study. Thus, it is possible that *E. coli* O157:H7 might be transferred from Malaysia to Thailand through beef trade. In this study, further examination of Thai local beef from variety markets in Hat Yai revealed Shiga toxin 1-producing *E. coli* (designated as PSU 5023) which produced high concentration of Stx1 (titer= 1: 2,048). The high toxin production might result from the presence of Q_{933} gene in this strain.

In this study, pandemic strains of *V. parahaemolyticus* were investigated in molluscan shelfish imported from Malaysia to Thailand using immunomagnetic separation technique and CHROMagar vibrio. Antisera against two important pandemic serotypes (K6 and K25) were employed. A total of 24 molluscan shellfish samples including bloody clam (Thai and Malaysian bloody clam), green mussel (Thai), and oriental hard clam (Thai), were examined. It was found that 15 out of 42 *V. parahaemolyticus* obtained from those mollusks were positive for *tdh*, GS-PCR and 16 kb inserted sequence which are the markers of pandemic strains. Three, one, eight and three *V. parahaemolyticus* isolates were obtained from Thai bloody clam, Malaysian bloody clam, Thai mussel and Thai hard clam, respectively. DNA profile analysis using AP-PCR indicated that all 8 isolates of pandemic *V. parahaemolyticus* obtained from a green mussel sample (PSU4056/1-8) were the same clone and 2 and 3

isolates of pandemic *V. parahaemolyticus* obtained from a Thai bloody clam (PSU 4063/1-2) and an oriental hard clam (PSU 4070/1-3), respectively, were originated from two different clones. Interestingly, DNA profiles of one isolate obtained from Thai bloody clam (PSU4063/2) were identical to DNA profile of one Malaysian isolate (PSU 4067). PFGE was performed to confirm correlation between some pandemic strains of *V. parahaemolyticus* from clinical and environmental Thai and Malaysian samples (isolates from 1998-2008). Some pandemic strains of *V. parahaemolyticus* from Hong Kong (2005) and U.S.A (1997 and 2003) were included for comparison. A total of 23 *V. parahaemolyticus* strains were analyzed using *Not* I and *Sfi* I restriction enzymes. DNA profiles were classified into 6 groups (group I to VI). All Thai and Malaysian *V. parahaemolyticus* pandemic strains isolated from clinical and environmental samples between 1998 and 2008 were in group IV. Interestingly, within this group DNA profiles of four isolates obtained in 2001 and 2008, one Thai clinical isolate (PSU 2050) and two Thai environmental isolates (PSU 4063/2 and PSU 474) and one Malaysian isolate (PSU 4067), were identical. This indicated that they were derived from the same origin. The results could be concluded that *E. coli* O157:H7 and pandemic strain of *V. parahaemolyticus* were contaminated in imported beef and bloody clam from Malaysia to Thailand, respectively. These food-borne pathogens were harmful although Stx production could not be demonstrated *in vitro*.

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LIST OF ABBREVISTIONS AND SYMBOLS

aEPEC	=	Atypical Enteropathogenic *Escherichia coli*
A/E	=	Attaching and Effacing
AP-PCR	=	Arbitraliry Primed-polymerase chain reaction
APW	=	Alkaline peptone water
BHI	=	Brain Heart Infusion
bp	=	base pair (s)
CFU	=	Colony forming unit
CA-YE	=	Casamino Acid-Yeast Extract
CHEF	=	Contour-clamped homogeneous electric field
Da	=	Dalton (s)
dNTPs	=	Deoxyribonucleotide triphosphate
EHEC	=	Enterohaemorrhagic *Escherichia coli*
EPEC	=	Enteropathogenic *Escherichia coli*
Gb_3	=	Globotriaosylceramide
GS-PCR	=	Group-specific polymerase chain reaction
h	=	Hour (s)
HBECs	=	Human Brain Endothelial cell (s)
HC	=	Hemorrhagic colitis
HUS	=	Hemolytic Uremic Syndrome
IL	=	Interleukin
IMS	=	Immunomagnetic Separation
IS	=	Insertion sequence (s)
kb	=	Kilobase (s)
kDa	=	Kilodalton (s)
KP	=	Kanagawa phenomenon
LB	=	Luria-Bertani
LEE	=	Locus of Enterocyte Effacement
MDa	=	Megadalton (s)
MHA	=	Meuller Hinton agar
MHB	=	Meuller Hinton broth

LIST OF ABBREVISTIONS AND SYMBOLS (continued)

min	=	Minute (s)
OMP	=	Outer membrane protein
ORF	=	Open reading frame
PAIs	=	Pathogenicity islands
PFGE	=	Pulsed-field gel electrophoresis
RPLA	=	Reverse Passive Latex Agglutination
RTX	=	Repeats in Toxin
s	=	Second (s)
SF	=	Sorbitol Fermenting
SMAC	=	Sorbitol-McConkey agar
STEC	=	Shiga toxin-producing *Escherichia coli*
Stx	=	Shiga toxin (s)
TCBS	=	Thiosulfate-citrate-bile salt-sucrose
TDH	=	Thermostable direct hemolysin
Tir	=	Translocation of intimin receptor
TNP-PCR	=	Toxin-Non-Producing polymerase chain reaction
TNF	=	Tumor Necrosis Factor
TRH	=	Thermostable direct hemolysis-related hemolysin
TSB	=	Tryptic soy broth
TTSS	=	Type three secretion system
VBNC	=	Viable but not culturable
µM	=	Micromolar (s)
µl	=	Microliter (s)

CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

Thailand is located in Southeast Asia and shares the borders with many countries such as Myanmar, Laos, Cambodia and Malaysia. Population movements, international trades and services have been exchanged across these international borders. Many diseases have also been transferred through these lines via contamination of microorganisms by humans and either exported or imported products. Food-borne pathogens are the second rank in causing disease in this area (Minami et al. 2010). Transportation of these organisms across international borders is not clearly understood. However, it was expected that imported food from neighboring countries may be one of an important vehicle (Radu et al. 1998).

Enterohemorrhagic *Escherichia coli* (EHEC) is an important food-borne pathogen that causes gastroenteritis including hemorrhagic colitis and hemolytic uremic syndrome which are life-threatening especially in children. (Karmali et al., 1983; Riley et al., 1983**)**. The diseases were first reported in patients infected with *E. coli* O157:H7, the major serotype of EHEC (Riley et al., 1983). Important EHEC virulence factors include Shiga toxin 1 and 2 (Stx1 and Stx2), encoded by *stx1* and *stx2*, respectively and the outer membrane protein, intimin, encoded by the *eae* gene (McKee *et al*., 1995; Schmidt *et al*., 1995; Nataro & Kaper, 1998). Domestic ruminants, mainly cattle, are the principal reservoir of this pathogen (Beutin et al., 1993; Vuddhakul et al., 2000**)** and infections have been mostly associated with consumption of beef.

The southern part of Thailand has a common border with Malaysia and some foods are exported from Malaysia to Thailand through this border. Beef is popular in this area because of Muslim population. To gain insight into the microbiological safety of food products routinely traded across Thai border, beef exported from Malaysia to southern Thailand should be examined for *E. coli* O157. In

addition, characterization of bacterial isolates will obtain the potential of the organisms in causing disease.

Along with imported beef, some seafood such as shellfish are also imported from Malaysia to Thailand through the southern border. One of the important seafood-borne pathogens is *Vibrio parahaemolyticus* which is a causative agent of human gastroenteritis after consumption of seafood.

V. parahaemolyticus is a halophilic bacterium which is widely distributed in marine and estuarine environments. Most clinical isolates of *V. parahaemolyticus* produce a major virulence factor, known as the thermostable direct hemolysin (TDH) which is encoded by *tdh* gene (Takeda *et al*., 1983). The TDH is responsible for β-hemolytic activity, called the Kanagawa phenomenon (KP), displayed on special blood agar (Wagatsuma agar). Another virulence factor, the TDH-related hemolysin (TRH), which is generally associated with some KP-negative strains of *V. parahaemolyticus*, is also involved in food poisoning outbreaks (Honda et al., 1987; Kelly and Stroh. 1989).

Since 1996, an increased incidence of gastroenteritis due to *V. parahaemolyticus* O3:K6 has been reported in many parts of the world including Asia (Chowdhury *et al*., 2004; Matsumoto *et al*., 2000), North America (Center for disease control and prevention 1998), South America (González-Escalona *et al*., 2005), and Africa (Ansaruzzaman et al., 2005). These O3:K6 strains carry *tdh* gene but not *trh* gene and recently other clinical strains that belongs to this O3:K6 clone but different in serotypes (such as O1:K25, O4:K68 etc.) have been reported (Bhuiyan et al., 2002; Chowdhury et al., 2000; Matsumoto et al., 2000). These strains are considered to be a pandemic strains.

Since shellfish especially mollusk are filter feeder and have been reported to harbor *V. parahaemolyticus* (Vuddhakul et al., 2006). Screening exported mollusk such as bloody clam from Malaysia to Thailand for pandemic *V. parahaemolyticus* is needed for food safety reasons. In this study, bloody clam will be investigated for *V. parahaemolyticus* and those isolates obtained will be characterized and compared to isolates obtained from Thai clinical samples to confirm whether there are any potential pathogens transferred across Thai border through food

trade. Information obtained from this study will be useful for development program for microbiological safety in food imported to Thailand.

LITERATURE REVIEWS

Escherichia coli is a facultative anaerobic bacterium that is predominantly detected in intestinal tract of many animals including human. *E. coli* is one of an important normal microbiota that colonizes infant gastrointestinal tract within hours after birth. Unless abnormal conditions such as immunosuppression, *E. coli* cannot cause infection. However, some strains of *E. coli* are pathogenic; these strains can cause urinary tract infection, sepsis/meningitis and enteric/diarrheal diseases (Nataro and Kaper. 1998). Diarrhaegenic *E. coli* can be classified into 5 groups following criteria of enterotoxin production (ETEC and EAEC), invasion ability (EIEC), and/or intimate adherence property (EPEC and EHEC).

Enterotoxigenic *E. coli* (ETEC)

ETEC is defined as the *E. coli* strains that produce two types of enterotoxin, either heat labile (LT) or heat stable (ST) enterotoxin. Structure and function of LT toxin is closely related to cholera enterotoxin (CT) (Sixma et al. 1993). Two major serogroups of LT, LT-I and LT-II, have been reported. LT-I is detected in *E. coli* strains that are pathogenic to both human and animal whereas LT-II is mostly found in *E. coli* isolated from animal (Nataro and Kaper et al. 1998). ST is small, monomeric toxin that contains multiple cysteine residues, which disulfide bonds account for the heat stability. There are 2 unrelated classes of ST (STa and STb) that differ in structure and mechanism of action. The ST has been reported in some gram-negative bacteria including *Yersinia enterocolitica* and *Vibrio cholerae* non-O1 (Nataro and Kaper. 1998). ETEC colonizes the surface of small bowel and its enterotoxins cause secretory diarrhea.

Enteropathogenic *E. coli* (EPEC)

EPEC is a causative agent of infantile diarrhea in developing countries. The hallmark of infection by EPEC is the attaching and effacing (A/E) lesion. This lesion is characterized by effacement of microvilli and intimate adherence between

the bacterium and the epithelial cell membrane. The organism also can polymerize filamentous-actin (F-actin) and accumulation of actin generates pedestal-like structure of host cell membrane that facilitates bacterium to sit on it. This appearance is unique characteristic of EPEC. Many factors involved in pathogenicity of EPEC. For instance, EAF plasmid and bundle-forming pilus (BFP) play an important role in adherence to epithelial cells, 35-kb pathogenicity island called Locus of enterocyte effacement (LEE) which composes of genes encode for a type III-secretion system and bacterial adhesion called intimin.

Enteroaggregative *E. coli* (EAggEC or EAEC))

EAEC strains are currently defined as *E. coli* strains that do not secrete enterotoxins LT or ST and adhere to HEp-2 cells in an aggregative adherence (AA) pattern. In animal model, infection with EAEC caused hypersecretion of mucus and volunteers that fed with EAEC developed diarrhea with predominantly mucoid (Nataro et al. 1995). The role of excess mucus production in EAEC pathogenesis is unclear but may associate with diarrheagenicity and persistent colonization of bacteria. Production of mucus biofilm is also related with to cytotoxin. Some EAEC strains possess bundle-forming fimbrial structure designated as aggregative adherence fimbriae I (AAF/I) (Nataro et al. 1992). AAF/I mediates adherence to HEp-2 and hemagglutination of human erythrocyte. Genes encoded for AAF/I are organized in two separated gene cluster on 60MDa plasmid (Nataro et al. 1993; Nataro et al. 1994; Savarino et al. 1994). However, it was found that a minority of EAEC strains produce AAF/I. Therefore, it has been suggested that AA may be caused by other factors than the AAF such as outer membrane protein.

Enteroinvasive *E. coli* (EIEC)

This type of *E. coli* possesses biochemically, genetically and pathogenetically characteristics related to *Shigella* spp. Both organisms have been demonstrated to invade colonic epithelium and elaborate one or more enterotoxins that cause diarrhea. Genes involved in epithelial invasion are carried on a 120-MDa

plasmid in *Shigella sonnei* and a 140-MDa plasmid in other *Shigella* species and EIEC (Baudry et al. 1987; Sasakawa et al. 1992; Savarino et al. 1991). This invasion-related plasmid is called pInv. The genes responsible for type III-secretion system (TTSS) in EIEC are *mxi* and *spa* which are located on plasmid whereas TTSS genes of EPEC and EHEC are located on chromosomal DNA.

Enterohemorrhagic *E. coli* (EHEC)

This type of *E. coli* was first reported in 1983 by Riley and coworker (1983). They observed patients who developed severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea with a little or no fever. This infection was designated as hemorrhagic colitis (HC) and was associated with consumption of undercooked hamburgers at a fast food restaurant chain. In the same time, Karmali and coworkers (1983) reported the association of sporadic cases of hemolytic uremic syndrome (HUS) with cytotoxin-producing *E. coli*. The main virulence factor of EHEC is Verocytotoxin or Shiga toxins (Stx). This toxin is similar to Stx in causing the cytopathic effect on Vero cells. In the 1980s, it was demonstrated that gene encoded for Stx was found on bacteriophage of *E. coli* (O'Brien et al. 1984; Scotland et al. 1983; Smith et al. 1983) and more than 100 different serotypes of *E. coli* can produce Stx (Karmali 1989). However, the most predominant serotype that associated with disease is *E. coli* O157:H7. EHEC can cause A/E lesion on epithelial cells, and possesses a ca. 60-MDa plasmid (Levine et al. 1987; Levine et al. 1984).

Part I *Escherichia coli* O157:H7

Escherichia coli O157:H7 is a bacterial normal microbiota in the intestine of cattle and ruminant. This serotype of *E. coli* is very harmful to human because it possesses an important virulence factor, Shiga toxin (Stx).

1. *E. coli* O157 and its pathogenicity

E. coli serotype O157 was first demonstrated in 1983 (Karmali et al. 1983; Riley et al. 1983). It is an important agent of hemorrhagic colitis (HC) and hemolytic uremic sysdrome (HUS). In hemorrhagic colitis, the bacteria destruct brush border microvili of patients and cause bloody diarrhea (Nataro and Kaper 1998; Griffin et al. 1995). In some patients, the bacteria may penetrate blood circulation and cause septicemia. A few organs especially kidney and brain possess Gb_3 receptor which is the target of Stx. Binding Stx to kidney results in hemolytic uremic syndrome which leads to death (Kaneko et al. 2001). Human brain endothelial cells (HBECs) are normally resistant to Stx. However, in the patients with hemolytic uremic syndrome, HBECs are more sensitive to Stx (Ergonul et al. 2003). Tumor necrosis factor (TNF)-α was demonstrated to increase HBECs sensitivity to Stx, resulting in apoptosis of HBECs (Ergonul et al. 2002). Brain injury is the most frequent cause of mortality among patients with HUS (Ergonul et al. 2002). This inflammatory cytokine including interleukin-1β (IL-1β) has been reported to increase expression of Gb_3 receptor and caused more severity in HUS patients (Jacewicz et al. 1986). Moreover, it was found that enterohemolysin of *E. coli* O157 was capable of inducing IL-1β production (Taneike et al. 2002). Therefore, the presence of enterohemolysin might enhance kidney damage via induction of Stx receptor.

2. Progenitor of *E. coli* O157

It is not clearly understood on the origin of *E. coli* O157:H7. However, there were evidences showing that EHEC O157:H7 was derived from enteropathogenic *E. coli* (EPEC) O55:H7. Four sequential events to develop O157:H7 was proposed (Wick et al. 2005). First, EPEC O55:H7 (A1) might acquire *stx*$_2$-containing bacteriophage and became *stx*$_2^+$ O55:H7 *E. coli* (A2) (Fig. 1). Second, A2 obtained pO157 plasmid and *rfb* region. pO157 plasmid contains genes encoding virulence factors such as *tox*B, *ehx*, *kat*P, *stc*E and *esp*P which enhance pathogenicity of this bacterium. *Rfb* region caused antigenic shift from O55 to O157 (A3). Third, A3 might receive *stx*$_1$-containing bacteriophage and lost flagella (non-motile) (A4) or

lost ability to utilize D-sorbitol (A5). Finally, loss of beta-glucoronidase activity (GUD) was occurred in A5 which led to development of typical *E. coli* O157:H7 (Sakai or EDL933 strains) (A6).

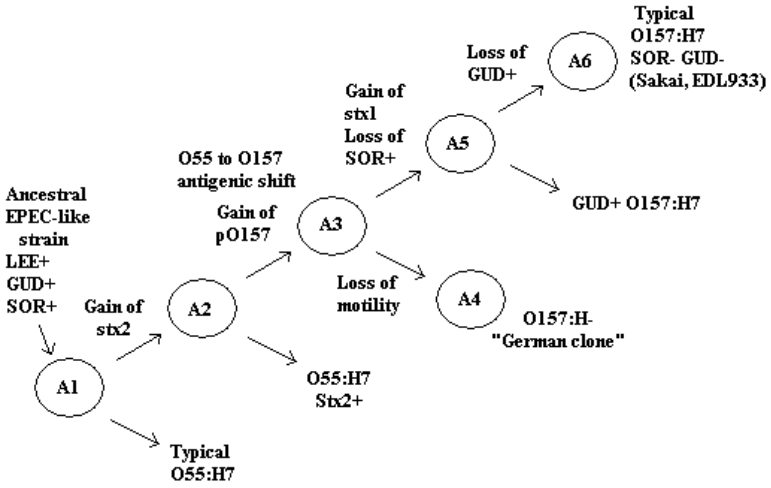

Figure 1 Evolution of *E. coli* O157:H7 from enteropathogenic *E. coli* O55:H7 (From Wick et al. 2005 with slightly modification)

3. Detection of pathogenic *E. coli* O157:H7

Investigation of *E. coli* O157 in clinical or environmental samples is difficult because the number of organism is low and contaminated with other *E. coli* normal microbiota. Therefore, enrichment technique is required to increase the number of *E. coli* O157 and subsequently culture on differential or selective medium. In addition, employing immunomagnetic technique to concentrate *E. coli* O157 has been proposed (Vuddhakul et al. 2000).

3.1 Enrichment medium for *E. coli* O157

Enrichment media for promotion growth of the bacterium are tryptic soy broth (TSB), brain heart infusion broth (BHI), or buffered peptone water. *E. coli* O157 grows simultaneously with other bacteria in the sample. However, subsequently

separation from other bacteria by immunomagnetic separation technique or selective medium can enhance the number of *E. coli* O157.

3.2 Immunomagnetic separation technique (IMS) for *E. coli* O157

Immunomagnetic separation technique plays a significant role in recovery of *E. coli* O157 after enrichments. O157 antibody-coated magnetic bead binds to O-antigenic epitope on cell wall of *E. coli* O157 and form bead-bacteria complex (Fig. 2). Several investigators showed the reliability and sensitivity of IMS in *E. coli* O157 detection. Lejeune and colleagues (2006) demonstrated sensitivity of *E. coli* O157 detection in bovine feces by broth enrichment followed with IMS and direct plating method. The sensitivity of detection was 100 CFU/g of bovine feces. However, if the initial number of *E. coli* O157 was less than 100 CFU/g of feces, the sensitivity of detection was very poor for both direct plating and IMS methods. Chapman and colleagues (1994) demonstrated that use of buffered peptone water followed with IMS was approximately 100-fold more sensitive for detection *E. coli* O157 than direct culture on cifixime rhamnose sorbitol MacConkey (CR-SMAC) agar and cifixime tellurite sorbitol MacConkey (CT-SMAC) agar.

3.3 Media for differentiation of *E. coli* O157

In the past decade, differentiation of *E. coli* O157 is determined by colorless colonies on Sorbitol-MacConkey agar (SMAC) because the bacteria are incapable of utilizing sorbitol (SF). Although most *E. coli* are sorbitol fermenters, around 6% of the isolates will not ferment sorbitol. These atypical strains will appear identical to O157:H7 colonies on SMAC. However, some strains of *E. coli* O157 can ferment sorbitol (Mellmann et al. 2008).

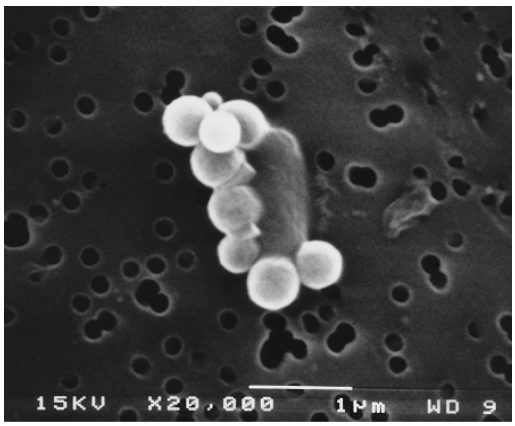

Figure 2 Scanning electron micrograph illustrates magnetic beads binding *E. coli* (From Pyle et al. 1999)

Modification of SMAC has been reported by adding potassium tellurite and cifixime into MacConkey agar to inhibit other bacteria including non-sorbitol fermenting species (Zadik et al. 1993). This agar was suitable for selection growth of Stx-positive *E. coli* O157 and *Shigella sonnei* (Zadik et al. 1993).

CHROMagar O157 is recently developed as a selective and differential medium for *E. coli* O157. The colony of *E. coli* O157 appears as mauve (pink) color on this medium.

Radu and colleagues (2000) demonstrated isolation and differentiation of *E. coli* O157:H7 by using Rainbow agar**.** Most bacteria were suppressed or grew as white to creamed color colonies in this medium whereas non-toxigenic *E. coli* was violet to red, blue, or pink colonies. *E. coli* O157 was detected in black or dark grey colonies.

4. Bacterial virulence factors

4.1 Shiga toxins

4.1.1 General information of Stx

Shiga toxin (Stx) or Verocytotoxin is the major virulence factor of EHEC. It was firstly reported in *Shigella* spp. in 1903 and was formally named as

VT1 (Conradi et al. 1903; Neisser and Shiga 1903). This toxin causes cytopathic effect on Vero cells. In 1977, Konowalchuk and colleagues demonstrated that certain strains of *E. coli* could produce Stx. Therefore, this toxin was previously called Shiga-like toxin. However, the terms Shiga-like toxin, and Verocytotoxin have been changed to be Shiga toxin currently to avoid confusing.

Two types of Shiga toxin, Stx1 and Stx2 encoded by stx_1 and stx_2 genes, respectively, have been reported in EHEC. A single EHEC strain may express either Stx1 or Stx2, or both toxins. Stx1 is identical to Shiga toxin from *S. dysenteriae* I. At least 5 genetic variations of Stx1 and 11 variations of Stx2 have been described in the Stx family (Scheutz et al. 2001)

4.1.2 Structure

Shiga toxins comprise 2 subunits, A and B subunits. A subunit is 32 KDa protein composed of 28 KDa A_1-subunit and 4 KDa A_2-subunit. A_1-subunit has *N*-glycosidase activity. This enzyme is capable of eliminating one alanine residue from 28s ribosomal RNA, resulting in suppression of protein synthesis which leads to cell death. A_2-subunit functions as a bridge to link A_1 subunit and B-subunit together. B-subunit is a 35 KDa pentamer, composed of five identical 7 KDa proteins raveled together (Fig. 3). Shiga toxin was first reported to affect Vero cells by binding B subunit to globotriaosyl ceramide (Gb_3) receptor located on plasma membrane of the host (de Sablet et al. 2008). Subsequently, toxin is engulfed into cytoplasm by endocytosis. The prototypical Stx1 and Stx2 toxins have 55 and 57% in nucleotide sequence identity in the A and B subunits, respectively (Jackson et al. 1987).

4.1.3 Subtypes of Stx

Stx2 has more variation in nucleotide sequence than Stx1. Sequence variation within Stx2 also generates many subtypes such as Stx2c, Stx2d, Stx2e, Stx2f.

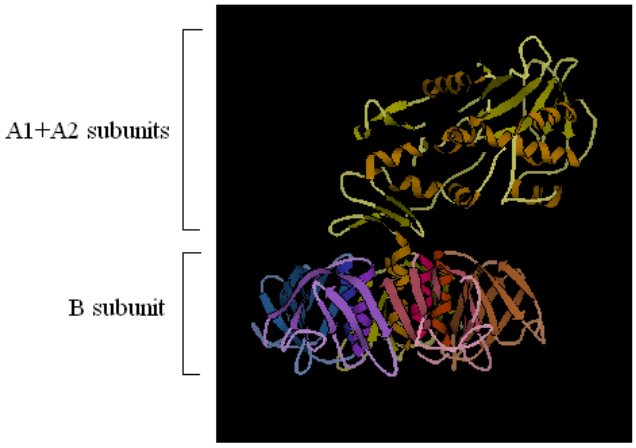

Figure 3 Three-dimentional structure of Shiga toxin illustrated in ribbon diagram (http://commons.wikimedia.org/wiki/File:Shiga_Toxin_Stx1_1DM0.png)

Prototype Stx2 has high potency to cause cell lysis, but various subtypes of Stx2 have been associated with clinical diseases (Marques et al. 1987). Stx2e caused pig edema disease in swine but only caused rash in human. In addition, it was found that this subtype induced cytotoxicity to Vero cells but not HeLa cell (Marques et al. 1987). *E. coli* O91:H21 and O91 nonmotile producing Stx2d were highly virulent to streptomycin-treated mouse whereas O91:H21 strains that produced Stx2 was not (Teel et al. 2002).

4.1.4 Transmission of *stx* gene

Stx is encoded by *stx* gene. Both stx_1 and stx_2 genes are found on lysogenic bacteriophages (Plunkett et al. 1999). Transfer of these genes among *E. coli* via bacteriophages has been documented (Allison et al. 2007; Herold et al. 2004; Schmidt et al. 1999). Several animal models were evaluated to demonstrate the transfer of *stx*-converting bacteriophage *in vivo* such as mice (Acheson *et al*., 1998), sheep (Cornick et al. 2006), and insect (Petridis et al. 2006).

Horizontal transfer of *stx* results in the presence or absence of virulence gene in *E. coli* and converts pathogenicity of bacteria. In addition, loss of *stx* will change the bacterium that classified as EHEC (stx^+ eae^+) to be atypical EPEC (stx^- and eae^+). Bielaszewska and colleagues (2007) demonstrated that EHEC

O26:H11 was isolated from stool of patient in the early stage of illness, after that they could isolate atypical EPEC (aEPEC) O26:H11 from the same patient. Pulsed-field gel electrophoresis revealed that both EHEC and aEPEC O26:H11 isolates possessed similar DNA fingerprinting. Moreover, aEPEC O26:H11 strain could be lysogenized by *stx*-encoding phage which was isolated from EHEC O26 strain.

Five bacteriophage insertion sites were reported (Ruth Serra-Moreno et al., 2007). They are *wrb*A gene encoding a NADH: quinolone oxidoreductase (Patridge et al., 2006), *yeh*V gene encodes a transcriptional regulator (Yokoyama et al. 2000), *sbc*B encodes exonuclease (Ohnishi et al. 2002), Z2577 encodes oxidoreductase (Koch et al., 2003), and *yec*E gene which its function is unknown (Recktenwald et al. 2002). *yeh*V gene is the preference integration site for *stx*1 phage while *yec*E, *wrb*A, and *sbc*B genes are integration sites for *stx*2 phages.

In the stx_2 phage genome, stx_2 gene is located downstream of the *Q* gene (Fig. 4). Expression of stx_2 is controlled by promoter p_{stx2}. However, a promoter of bacteriophage (P_R') also plays an important role in high level stx_2 transcription (Plunkett et al. 1999; Wagner et al. 2001). Q protein is an antiterminator that is required for continuation of transcription through t_R. This Q protein promotes RNA polymerase to readthrough transcription terminator t_R that activates P_R', resulting in stx_2 expression.

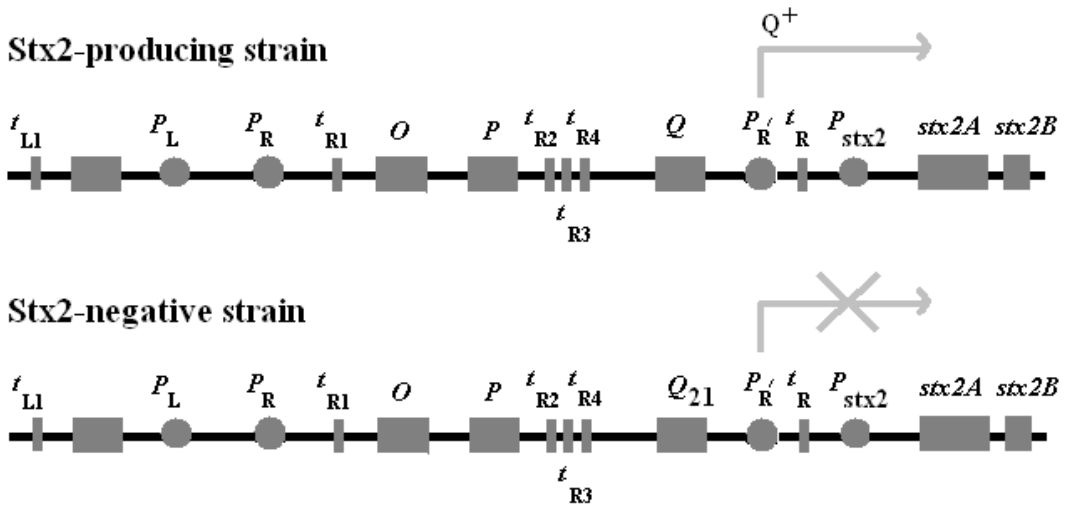

Figure 4 Diagram of stx_2 gene and its regulators (Modified from Wagner et al. 2002)

4.1.5 Expression of *stx*

Koitabashi and colleagues (2006) demonstrated that some strains of *E. coli* O157:H7/H$^-$ from Asia such as Thailand and Japan could not express stx_2 or produced Stx2 in low level. Those strains were named Stx2-negative strains. The low expression of stx_2 caused by the presence of *Q* gene which was different from *Q* gene previously reported in 933W phage which showed high antitermination activity. This new detected *Q* gene was similar to Φ21- bacteriophage *Q* gene and showed weak "Q_{21}" antitermination activity, resulting in low production of Stx2 (Fig. 4). Lejeune and colleagues (2004) demonstrated that *Q* gene of bacteriophage 933W can be used as a marker for identification virulence strains of *E. coli* O157:H7.

The expression of stx_2 in Stx2-negative strains might be increased when they obtained the compatible *Q* gene from bacteriophage. Koitabashi and colleagues (2006) demonstrated increasing of Stx2 titer (16-fold) in Thai-12 strain (Stx2-negative) after cloning of *Q* gene (from EDL933 strain- Stx2-positive) into this strain.

E. coli O157:H7 retain their ability to produce Stx1 and Stx2 when they enter viable but non culturable (VBNC) state. *E. coli* O157:H7 were forced to enter VBNC state by incubation in river water, PBS buffer, and deionized water, followed by incubation at 4°C or 25°C. Then, Stx production was evaluated by Real-time PCR (Liu et al. 2010). It was found that *E. coli* O157:H7 in VBNC state could produce Stx. This indicates that *E. coli* O157:H7 in drinking water and river water are capable of causing disease.

4.1.6 Techniques to detect Stx

Since Stx is a major virulence factor of EHEC. Stx production can be used to characterize EHEC or *E. coli* O157 strains. This cytotoxin can be illustrated using Vero cells (Konowalchuk et al. 1977). Antigen-antibody-based on ELISA technique such as Ridascreen® test (R-biopharm, Darmstadt, Germany) is also useful to determine Stx production (Werber et al. 2002). In addition, PCR technique has been developed to investigate *stx* gene (Karch et al. 1989; Paton et al. 1993). PCR

and ELISA technique were found to be sensitive equally (Bonardi et a*l*. 2000) and were more rapid and easier than using Vero cells (Bettelheim and Beutin. 2003).

PROSPECT®(Lenexa, KS, USA) is another technique to determine Shiga toxin using ELISA microplate assay (Bettelheim, unpublished data). However, Reverse Passive Latex Agglutination (RPLA) has been developed to determine Stx (Beutin et al. 2002; Chart et al. 2001). Either Stx1 or Stx2 is evaluated using specific Stx1 or Stx2 antibody-coated latex particles react with supernatant from enrichment bacterial culture such as Casamino acid-Yeast extract (CA-YE). Bacterial colonies on agar plate (Brain Heart Infusion agar) can be evaluated by suspension them in 0.85% NaCl supplemented with polymyxin before assay.

4.2 Intimin

Intimin is an outer membrane protein encoded by *eae* gene located in pathogenicity island called Locus of enterocyte effacement (LEE). This gene has been detected in both EPEC and EHEC including *Citrobacter rodentium* and *Hafnia alvei* (Natoro and Kaper 1998). In the early stage of pathogenicity, intimin assists bacteria to attach to cell membrane of host cell by binding to a 90 kDa translocation of intimin receptor (Tir) (Hartland et al. 1999; Sinclair et al. 2006), leading to intimate attachment to epithelial cells and causes destruction to brush border microvilli called attaching and effacing (A/E) lesion (Frankel et al. 1998). Host cells do not have the receptor for intimin protein but it was demonstrated that EHEC O157:H7 cleverily inserted intimin (Tir) into host cell membrane using type III secretion system (Sinclair *et al*., 2006). After that EHEC O157:H7 used its intimin to bind to the Tir receptor and generated pathogenicity. Recently, interaction of intimin to host cell without Tir has been demonstrated (Hartland et al. 1999).

4.3 60MDa plasmid

E. coli O157 contains highly conserved plasmid called 60MDa plasmid or pO157. This plasmid is a non-conjugative plasmid with size ranging from 92 to 104 kb. The pO157 plasmid has been shown to contain many types of mobile genetic

element such as insertion sequence, transposons and prophages. Completed sequences of pO157 revealed 100 open reading frames (ORFs) (Burland *et al*. 1998). Several virulence genes have been characterized in this plasmid, for example, enterohemolysin (*ehxA*), a catalase-peroxidase (*katP*), a serine protease (*espP*), a putative adhesion (*toxB*), a zinc metalloprotease (*stcE*). This indicates that EHEC might obtain pO157 from different origins.

Identification of pO157 is an important criterion in characterization EHEC. Several techniques have been established to investigate pO157 such as Southern blot hybridization and PCR. Fratamico and colleagues (Fratamico et al. 1995) performed multiplex PCR to detect Stx, intimin, and 60MDa plasmid.

4.3.7 Enterohemolysin

Genes encoding for enterohemolysin are located on pO157 (Bauer *et al*., 1996; Schmidt et al., 1995). These genes comprise *ehxC*, *ehxA*, *ehxB*, and *ehxD* which are more than 60% homologous to the alpha-hemolysin gene of *E. coli* (Schmidt et al. 1996). Difference G+C% of these genes from surrounding region indicates that *E. coli* might obtain them from other origins (Lim et al. 2010).

Enterohemolysin, like alpha-hemolysin, is a pore-forming RTX (repeats in toxin) cytolysin that causes sheep erythrocyte lysis (Bauer et al. 1996). Six subtypes of enterohemolysis (A-F) have been reported (Cookson et al. 2007). Subtype A and C are significantly associated with the strains containing *stx* gene and *eae* respectively.

4.3.8 ToxB protein

ToxB is a putative adherent protein which is encoded by gene located in 9.5 kb DNA fragment of pO157 plasmid. Truncated ToxB assisted production and /or secretion of EspB, one of the type III secretion proteins detected in EHEC O157:H7 (Tatsuno et al. 2001). Therefore, ToxB involved in adherence of bacteria through type III secreted proteins.

4.4 Type III secretion systems (TTSSs)

Type III secretion systems have been found in many bacteria and the systems involve in delivering virulence proteins across bacterial membrane into cytoplasm of host cells (Ghosh et al. 2004). In EHEC, translocation of intimin receptor (Tir) to host cells was manipulated through TTSSs (Garmendia et al. 2004). TTSSs reported in *E. coli* O157 also play an important role in attachment to host epithelial cells. EspA, one of proteins in TTSSs, attached host cell through chevron-like fimbriae (Daniell et al. 2003).

4.5 Other virulence factors

4.5.1 EspFu

EspFu (*E. coli* secreted protein F in prophage U, also known as Tir-cytoplasm coupling protein, TccP) is a protein that disrupts phagocytosis which is a mechanism of host cells to destroy invaded bacteria (Campellone et al. 2010). In addition, this protein also involves in movement of bacteria over the surface of epithelial cell (Shaner et al. 2005), resistance to flow-mediated detachment during diarrhea (Campellone et al. 2010), translocation of other factors (Mills et al. 2008), intracellular pressure and cell-to-cell spreading. EspFu has been reported in all EHEC and some strains of EPEC such as EPEC O55:H7 which is proposed to be the progenitor of EHEC O157:H7.

5. Epidemiology of *E. coli* O157:H7

5.1 Transmission

Transmission of *E. coli* O157:H7 to humans is mostly monitored through food especially beef consumption. *E. coli* O157:H7 has been found in the intestines of healthy cattle, deer, goat, and sheep. Shedding of this bacteria in animal

feces contaminates environment as well as food. In addition, improper dissection of animal in slaughter house results in meat contamination with this pathogen.

Transmission of *E. coli* O157 via person-to-person has been reported (Carter et al. 1987). In 1985, an outbreak of *E. coli* O157:H7 occurred in nursing home and caused 11 out of 12 people died. Person-to-person transmission was indicated to be the second phase whereas the first phase infection was the consumption of a sandwich contaminated with *E. coli* O157.

Some animals have been reported as potent vectors to transfer *E. coli* O157. This bacterium was reported in apple cider contaminated by fruit fly (Janisiewicz et al. 1999). Examination of wild animals demonstrated Shiga toxin producing *E. coli* (STEC) in Starling bird (*Sturnus vulgaris*) and a Norway rat (*Rattus norregicus*) (Nielsen *et al*., 2004). PFGE revealed that DNA fingerprints of these STEC isolates were identical to DNA fingerprints of the STEC isolated from the cattle. Schmidt and colleagues (2000) isolated STEC strains in feces of feral pigeons. *E. coli* O157 was isolated from 0.21 % of slugs in Aberdeenshire sheep farm, Scotland (Sproton et al. 2006). These slugs are able to transfer *E. coli* O157 from animal feces to salad vegetables.

5.2 Epidemiology

There have been reports on infection caused by *E. coli* O157 worldwide. In the United States, a total of 350 outbreaks from 49 states caused by *E. coli* O157 have been reported between 1982 and 2002 (Rangel et al. 2005). Most of the cases were in Minnesota State and ground beef was the most potent vehicle. An outbreak of *E. coli* O157:H7 in Colorado, the patients consumed ground beef 7 days before illness began. In 2008, seven patients in Colorado were hospitalized because of eating contaminated ground beef and five developed hemolytic uremic syndrome (HUS). DNA profiles of *E. coli* isolates generated by PFGE were posted on PulseNet (the national molecular subtyping network for food-borne disease surveillance) and it was found that DNA profiles of 8 *E. coli* O157 isolates from California, Iowa, Michigan, South Dakota, Washington, and Wyoming were identical.

Infection caused by consumption of deer meat was reported in 2002. Seven year-old boy developed gastroenteritis after consumption of a large quantity of undercooked grilled meat from a white-tailed deer (Rabatsky-Ehr et al. 2002).

In Europe, central Scotland, the largest number of deaths was caused by an outbreak of *E. coli* O157:H7 in 1996 (Dandas et al. 2001). This outbreak originated from cross-contamination of cooked meat and caused infection to elderly people and subsequently the infection spread to many people of all ages.

In Japan, diffuse outbreaks of O157 were frequently reported in Japan but in 1996, the large outbreak of *E. coli* O157 occurred in Sakai city, Osaka prefecture involving more than 6,000 primary school children. An investigation suggested that radish sprouts were the most likely cause (National Institute of Health and Infectious Diseases Control Division, Ministry of Health and Welfare of Japan 1998).

6. DNA fingerprint

Bacteria have their unique DNA fingerprints which can be used for identifying specific strains that involved in Epidemiology. There are many techniques to generate DNA fingerprints of various pathogenic bacteria such as Arbitarily-primed polymerase chain reaction, IS-printing and Pulsed-field gel electrophoresis.

6.1 Arbitarily-primed polymerase chain reaction (AP-PCR)

AP-PCR or random amplified polymorphic DNA (RAPD) is a technique that determines DNA fingerprints of bacteria in the same species. This technique uses short primer (usually 10 bp in length) that randomly binds and amplifies several regions in bacterial DNA (Fig. 5). Sequence variations in DNA generate different number and size of PCR products that will vary among different strains of bacteria. Therefore, a DNA fingerprint specific to each strain is produced.

6.2 Insertion sequence -printing (IS- printing)

Insertion sequences (IS) are a group of small, mobile genetic elements that widely distributed in the genomes of most bacteria. Their sizes are between hundred and few thousand base pairs and usually code for transposase proteins which allow the IS to move. The coding region in IS is usually flanked by inverted repeats sequences. In *E. coli* O157, it was found that heterogeneity of the strains depends on large-size structural genomic polymorphism (LSSPs) and small-sizes structural genomic polymorphism (SSSPs) which were caused by bacteriophages and IS element respectively (Ooka et al. 2009a). Analysis of 8 isolates of *E. coli* O157:H7 Sakai strains from clinical specimens, genomic polymorphisms ranging from several hundred base pairs to a few thousand base pairs were detected and insertion or deletion of IS*629* and IS*Ec8* were present in this region (Onishi et al. 2002; Ogura et al. 2006). Thus analysis of IS element can be used to identify *E. coli* O157 strains.

Multiplex PCR has been established to amplify many loci of IS*629* of *E. coli* O157:H7 Sakai strain using universal primers OW-L and OW-R (Ooka et al. 2009b). These primers bind to DNA sequence within IS*629* and amplify gene outward of IS*629* (Fig. 6). Meanwhile, primers OS-L and OS-R bind to gene outside IS*629* to amplify upstream and downstream region of IS*629*. This leads to generate combination of amplicons ranging from 0.1kb to 1.0 kb (Ooka et al. 2009b). Therefore, different strains of *E. coli* O157:H7 that possess different gene sequence between IS*629* will generate different DNA profiles.

6.3 Pulsed-field gel electrophoresis (PFGE)

PFGE is a technique used to separate large genomic DNA fragments after digestion with a restriction enzyme (Schwartz and Cantor 1984). The digestion yields several linear segments DNA, if two identical bacterial isolates are compared; the sites at which the restriction enzymes act on the DNA and the length between these sites would be identical and generate identical DNA patterns after electrophoresis in alternating electrical fields (Fig. 7).

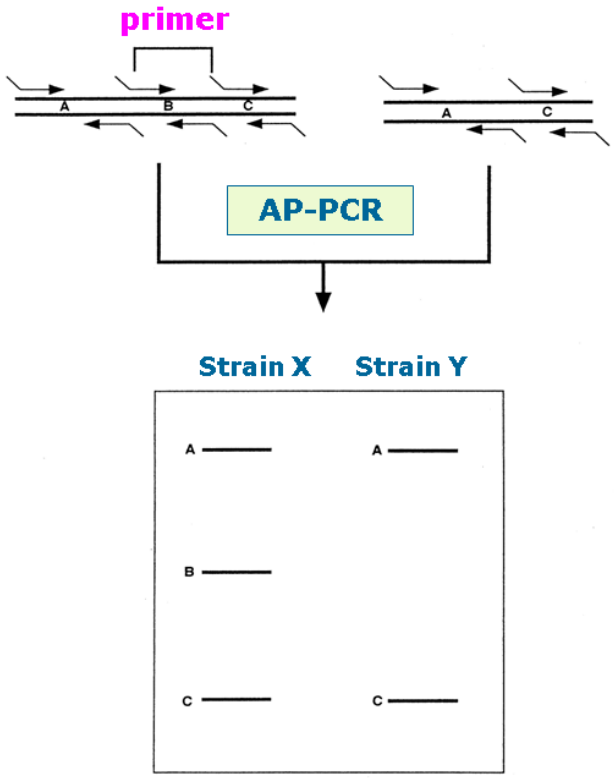

Figure 5 Diagram illustrates AP-PCR analysis. Short primer randomly binds to the genome sequence of bacteria, generating various sizes of amplicons (A, B and C)**.** The amplicon**s** are separated by agarose gel electrophoresis to obtain different DNA patterns of different strains of bacteria.

Conversely, if two isolates are not the same strain, then the sites at which the restriction enzymes act on the DNA and the length between these sites would be different; thus their DNA patterns will be different.

Interpretation of PFGE pattern can be done by naked eyes to determine the causative agent of the outbreak but interpretation of relatedness between strains or between groups of the isolates, should be done by computer program which compares PFGE profile using Dice coefficient and UPGMA (unweighted pair group method using arithmetic averages) algorithm (Kam et al. 2008).

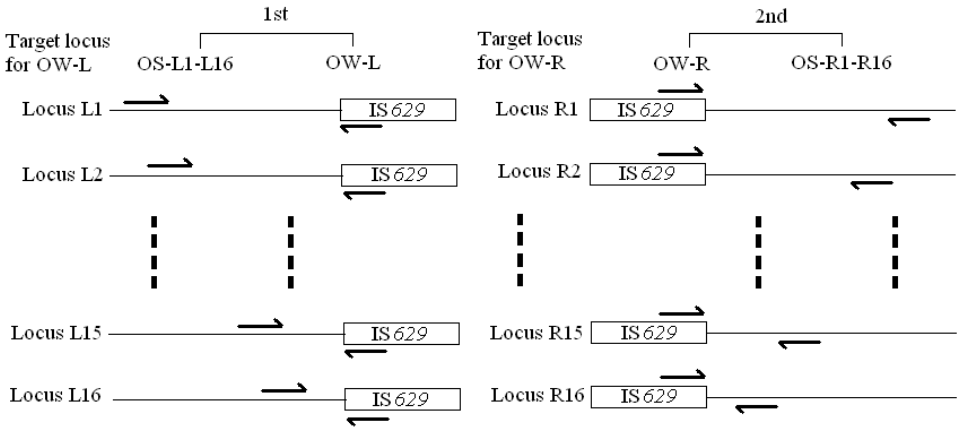

Figure 6 Schematic indication of IS*629* loci and the direction of primer binding in each locus for IS-printing analysis. Sixteen loci of IS*629* were targeted for multiplex PCR. In each locus the upstream (left) and downstream (right) of IS*629* were amplified and various sizes of amplicons in each locus were obtained, displayed as a ladder when applied to agarose gel electrophoresis.

7. Treatment of *E. coli* O157

There has been a controversy of using antibiotics in treatment infection due to *E. coli* O157:H7 (Carter *et al*., 1987). Several reports demonstrated that antibiotics could recover the patients from the disease (Proulx et al. 1992; Takeda et al. 1998). However, some reports showed that some antibiotics exacerbated hemolytic uremic syndrome (HUS). DNA-affecting antibiotics such as Mitomycin C and Ciprofloxacin caused increasing of Stx (Mao *et al*., 1999). Folate metabolism inhibitor such as Trimethoprim-sulfamethoxazol, Norfloxacin, a DNA gyrase inhibitor, also stimulates Stx production (Kimmitt et al. 2000; Matsushiro et al. 1999). However, Rifaximin showed no effect on bacteriolysis which prevents stx gene transfer to other bacteria (Ochoa et al. 2007). Therefore, this antibiotic would be the drug of choice in therapy patients infected with *E. coli* O157:H7.

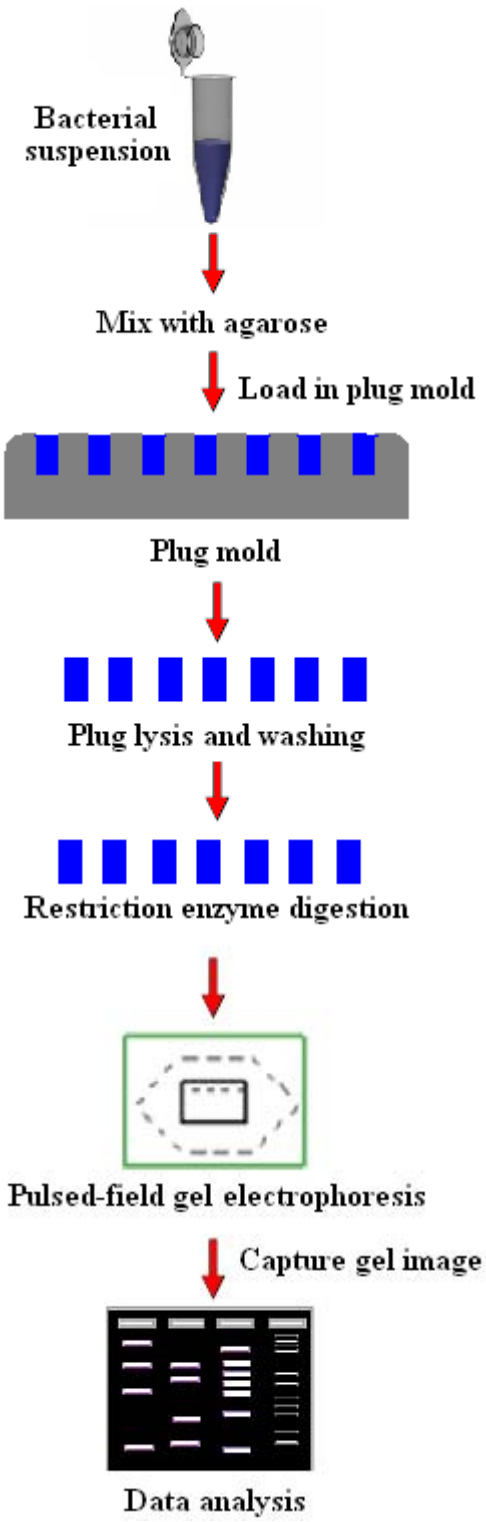

Figure 7 Diagram illustrating pulsed-field gel electrophoresis protocol

Part II *Vibrio parahaemolyticus*

Family *Vibrionaceae*

Bacteria in the genus Vibrio belong to the family *Vibrionaceae*. According to Bergey's taxonomic outline, 2004 (http://dx.doi.org/10.1007/bergeysoutline200310), this family consists of 8 genera: *Vibrio* (57 species), *Photobacterium* (9 species), *Enterovibrio* (1 species), *Allomonas* (1 species), *Catenococcus* (1 species), *Grimontia* (1 species), *Listonella* (2 species), and S*alinivibri*o (2 species). However, phylogenetic analysis of concatenated 16S rRNA, *rec*A, and *rpo*A gene sequences suggested that this family should be split into 4 different families; Vibrionaceae, Photobacteriaceae, Enterovibrionaceae, and Salinivibrionaceae (Thompson et al. 2004). The new family Vibrionaceae comprises only genus *Vibrio* which includes 70 species and 1 biovar (http://www.ciad.mx/caim/VibrioSpecies.html). Some species of bacteria in this genus are pathogenic to humans such as *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*.

1. Characteristics of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a halophillic bacterium that has been detected in marine environments worldwide. *V. parahaemolyticus* was first identified in 1950 by Fujino and colleagues as an agent of gastroenteritis outbreak after comsumption of Shirasu (a semidry fish) in Japan. There were 272 people infected and 20 died (Fujino et al. 1953).

V. parahaemolyticus is a gram-negative curved rod, non-spore forming bacterium. For cultivation, this bacterium requires NaCl between 0.5 to 8% but for optimal growth usually requires between 2 and 4% NaCl. The optimal temperature of *V. parahaemolyticus* is between 30 and 35ºC. The upper temperature for growth is 44ºC. The optimal pH ranges are between 7.6 and 8.6. Under the optimal condition, the doubling time of *V. parahaemolyticus* is between 9-13 min (Twedt 1989).

However, in seafood, the doubling time of 12-18 min was demonstrated (Doyle et al. 1997).

2. Virulence factors

2.1 Thermostable-direct hemolysin (TDH)

V. parahaemolyticus has long been recognized as an agent of gastroenteritis associated with the consumption of seafood. Not all strains of *V. parahaemolyticus* are considered to be pathogenic. Only the strains that possess thermostable direct hemolysin (TDH) and able to exhibit β-hemolysis on special blood agar (Wagatsuma agar) called Kanagawa phenomenon (KP) is considered to be pathogenic *V. parahaemolyticus* (Miyamoto et al., 1969; Sakazaki et al. 1968). Almost clinical strains of *V. parahaemolyticus* exhibit KP but only 1-2 % of *V. parahaemolyticus* isolates from environmental are KP-positive (Miyamoto et al., 1969; Sakazaki et al. 1968).

TDH is encoded by *tdh* gene. TDH is a pore-forming toxin and is able to lyse erythrocyte of mouse, rabbit, humans, calf, guinea pig, chicken, sheep, but not horse (Honda et al. 1988). This toxin is stable at 100º C for 10 min. TDH is a tetrameric protein with a molecular weight of 75 KDa, comprising 4 units of approximately 18 KDa each (Hamada et al. 2006). TDH has been reported to exhibit intestinal toxicity, cardiotoxicity, hemolytic activity, and cytolethal activity.

It has been found that the KP-positive isolates usually possess two non-identical copies of the *tdh* gene (designated as tdh_1 and tdh_2). However, weak positive of KP isolates showed only one copy of *tdh* gene and is associated with the tdh_1 gene (Nishibuchi and Kaper 1990; Nishibuchi et al. 1991). In addition, in KP-positive phenotype, more than 90% of total TDH production was caused by the expression of tdh_2 gene (Nishibuchi et al. 1990; Nishibuchi et al. 1991).

V. parahemolyticus toxRS (VP-*toxRS*) regulated the expression of TDH. The expression of tdh_1 and tdh_2 were increased 1.25 and 5.07 fold, respectively, when VP-*toxRS* operon was overexpressed in *E. coli* (Lin et al. 1993).

V. parahaemolyticus tdh was found to be flanked by nucleotide sequence nearly identical to the terminal inverted repeat sequence of IS*102* (Baba et al. 1991; Nishibuchi et al. 1990). This observation suggested the possibility of *tdh* gene to transpose to the chromosome and the plasmid of other *Vibrio* species (Nishibuchi and Kaper 1995).

2.2 Themostable direct hemolysin-related hemolysin (TRH)

Another virulence factor, the TDH-related hemolysin (TRH) has also involved in some food-poisoning outbreaks (Honda et al. 1988). Biological, immunological, and physiochemical characteristics of this hemolysin is similar but not identical to those of TDH (Honda et al. 1988). TRH is encoded by *trh* gene and this gene shares 68.4% and 68.6% identity to the tdh_1 and tdh_2, respectively. Thus, *tdh* and *trh* genes may have evolved from the same ancestor.

TRH is a homodimer protein with a molecular weight of 48 KDa and 2 subunits of 23 KDa (Honda et al. 1988). Two subtypes of *trh*, $trh_1$and trh_2, have been reported (Kishishita et al. 1992). The levels of in vitro expression of trh_1 gene was higher than trh_2 but was lower than tdh_2 of KP-positive strains (Shirai et al. 1990). In addition, the expression of trh_1 was not depended on the VP-ToxRS (Lin et al. 1993). Several studies reported the association of *trh* and urease-positive in clinical isolates of *V. parahaemolyticus* (Abbott et al. 1989; Kaysner et al. 1994; Kelly and Stroh 1989; Suthienkul et al. 1995). Iida et al., (1998) demonstrated that gene encoded for urease (*ure*) was close to the *trh* gene (less than 8.5 kb in distance) of *trh*-positive *V. parahaemolyticus*. Therefore, the urease production can be used as a marker of virulent *V. parahaemolyticus* in clinical diagnosis (Iida et al. 1998; Iida et al. 1997; Okitsu et al. 1997; Suthienkul et al. 1995).

2.3 Thermolabile hemolysin (TLH)

In addition to TDH and TRH, thermolabile hemolysin (TLH) is detected in *V. parahaemolyticus* (Sakurai et al. 1974; Taniguchi et al. 1985). TLH is encoded by *tlh* gene. The G+C content of *tlh* gene is 47.6% which is similar to the

G+C content of *V. parahaemolyticus* genome (45.4%), indicating that TLH may be originally a part of *V. parahaemolyticus* genome (Taniguchi et al. 1986; Taniguchi et al. 1985). TLH is destroyed by heating at 60°C for 10 minutes. Protein of TLH consisted of 398 amino acids with molecular weight of 45.3 KDa. TLH is a lecithin-dependent hemolysin (LDH) which exhibits phospholipase activity designated as phospholipase A_2 or lysophospholipase (Shinoda et al. 1991). TLH is detected in all clinical and environmental *V. parahaemolyticus* isolates. Hence, it can be used as one of the markers to identify *V. parahaemolyticus* (Bej et al. 1999; McCarthy et al. 1999; Sakurai et al. 1974; Taniguchi et al. 1986; Taniguchi et al. 1985).

2.4 Type III secretion system (TTSS)

Over 25 species of Gram-negative bacteria either pathogens or symbionts, possess a special protein export apparatus called a type III secretion systems (TTSSs) (Cornelis 2006). TTTSs are a complex needle like structure that create a channel extending beyond the two bacterial inner and outer membranes and involved in transfering effector proteins from the bacterial cytosol to the host cell. The complete genome of *V. parahaemolyticus* RIMD2210633 revealed the presence of two sets of genes responsible for TTSSs on either chromosome 1 or chromosome 2, designated as TTSS1 and TTSS2, respectively (Makino et al. 2003). TTSS1 is responsible for cytotoxicity whereas TTSS2 is directly involved in the cytotoxicity and enterotoxicity (Kodama et al. 2007; Park et al. 2004). TTSS2 is detected only in *V. parahaemolyticus* possessing the *tdh* gene.

3. Association of *V. parahaemolyticus* in seafood

Most infections caused by *V. parahaemolyticus* have been associated with seafood consumption. In the United States in 1998, 416 persons in 13 States developed gastroenteritis after eating raw oysters harvested from Galveston bay (Daniels et al. 2000). Investigation Blue crab (*Callinectes sapidus*) from Chesapeake Bay, 56 strains of *V. parahaemolyticus* were obtained from 60 processed blue crabs samples (Fishbein et al. 1970).

Sixty two percent of Cockle (*Anadara granosa*) obtained from Kuala Selangor were positive for *V. parahaemolyticus*. PCR analysis revealed 2 and 11 samples were positive for *tdh* and *trh* gene respectively (Bilung et al. 2005).

In Thailand, 230 molluscan shellfish were investigated and 2 and 12 trh^+ and tdh^+ *V. parahaemolyticus* were detected (Vuddhakul et al. 2006).

4. Epidemiology of *V. parahaemolyticus*

V. parahaemolyticus has been reported to cause gastroenteritis worldwide. In the past infections of this bacterium were caused by variety serotypes. However, in 1966 specific serotype O3:K6 of *V. parahaemolyticus* was reported in hospitalized patients in Calcutta, India (Okuda et al. 1997). It was found that this new O3:K6 serotype first appeared and accounted for 50-80% of *V. parahaemolyticus* isolates obtained in that year. These O3:K6 strains carry the *tdh* gene, but not the *trh* gene, and show a unique DNA fingerprint when examined by an arbitrarily primed-polymerase chain reaction (AP-PCR) technique. Matsumoto et al. (2000) developed a molecular typing method, known as group-specific PCR (GS-PCR) that could detect nucleotide variations within the 1,364-bp *toxRS* region that was unique to the new O3:K6 clone. Using this technique, 119 *V. parahaemolyticus* O3:K6 isolates from Taiwan, Laos, Japan, Thailand, Korea, and the United States obtained during 1997-1999 were GS-PCR positive. Therefore, these strains were characterized as pandemic strains.

Examination of recent clinical strains using this method led to finding other pandemic strains belonging to non-O3:K6 serotypes e.g., O1:K25, O1:KUT, O4:K68 (Bhuiyan, et al. 2002; Chowdhury et al. 2000 Matsumoti et al. 2000). These serotypes might have diverged from the O3:K6 clone.

In Thailand, 86.9% of clinical specimens collected from Hat Yai hospital during September and October 1998 were O3:K6 and GS-PCR positive (Vuddhakul et al. 2000). In addition, 1 out of 114 seafood samples obtained in this area was GS- PCR positive *V. parahaemolyticus*. In the United States, during May–July 1998, 296 cases of *V. parahaemolyticus* infections in Texas and 120 cases of infections from 12 other states were reported (CDC 1998). All available stool samples

yielded *V. parahaemolyticus* serotype O3:K6. Later in 1998, another multistate outbreak of *V. parahaemolyticus* infections occurred; 12 of 23 isolates were serotyped and all of them were O3:K6. Patients were from New York, New Jersey, and Connecticut and infection was due to consumption of raw oysters and clams harvested from Oyster Bay, Long Island, New York (CDC 1999; Daniels et al. 2000). An investigation of food-borne illness in Taiwan, 0.6% of the total *V. parahaemolyticus* isolates in 1995 were serotype K6 but this serotype increased to 50.1% and 83.8% of the total *V. parahaemolyticus* isolates in 1996 and 1997 respectively (Chiou et al. 2000). In Russia, an outbreak of acute enteric disease in Vladivostok in 1997 was reported to be due to *V. parahaemolyticus* serotype O3:K6. In Vietnam, between 1997 and 1999, 523 *V. parahaemolyticus* isolates in Khanh Hoa province were examined. Forty-nine percent of the isolates belonged to the pandemic strains as determined by the GS-PCR assay and the presence of the ORF8, an open reading frame of the f237 phage genome. Eleven O:K serotypes were detected among the pandemic strains (Chowdhury et al. 2004). In Europe, analysis of 13 *V. parahaemolyticus* isolates from clinical samples sent to the National Reference Center, France during 1997-2004 indicated five isolates belonged to the O3:K6 pandemic strains (Quilici et al. 2005). In South America, analysis of clinical isolates of *V. parahaemolyticus* from two outbreaks in Chile from November 1997 to March 1998 and Puerto Montt from January to March 2004 indicated that 19 of 20 isolates from Chile and 23 of 24 from Puerto Montt belonged to the pandemic clonal complex (Gonzalez-Escalona et al. 2005). In the African continent in 2004, 42 isolates of *V. parahaemolyticus* were obtained from patients with diarrhea in Mozambique, 34 isolates (81%) were pandemic strains including O3:K6 and O4:K68 serotypes (Ansaruzzaman et al. 2005).

5. Detection of pandemic *V. parahaemolyticus*

According to outbreak of a new clone of *V. parahaemolyticus* O3:K6 in 1996 in Calcutta and subsequently spread all over the world. Many techniques have been developed to detect this clone including its serovariant. These include AP-PCR technique which generates the unique fingerprinting pattern in comparison with O3:K6 pandemic clone from Calcutta in 1996 (Okuda et al. 1997), GS-PCR which

detects the 7 bases-nucleotide variation within the 1,364 bp *toxRS* region that are unique to the new O3:K6 clone (Matsumoto et al. 2000)**,** ORF8 of bacteriophage detection that detects the gene encoding filamentous phage f237 is unique for the pandemic *V. parahaemolyticus* clone (Nasu et al. 2000), 16 Kb insertion sequence detection which detects VP2905 fragment inserted into 16 kb sequence located in the open reading frame of a histone-like DNA-binding protein, HU-α (Williams et al. 2004; Okura et al. 2005), detection of 23 kb insertion sequence which detect DNA methyltransferase (MTase) gene located in the 22.79 kb pathogenicity island in *V. parahaemolyticus* pandemic strain (Wang et al. 2006).

6. Treatment of *V. parahaemolyticus*

Infection due to *V. parahaemolyticus* usually causes mild symptoms but some patients may develop severe symptoms and need antibiotics such as ceftaxidime and doxycycline or doxycycline in combination with ciprofloxacin or an aminoglycosides (Daniels et al. 2000). Ansaruzzaman et al. (2005) demonstrated that O3:K58, O4:K68, and O4:K13 *V. parahaemolyticus* isolated from Mozambique were susceptible to tetracycline, ampicillin, sulfamethoxazole-trimethoprim, nalidixic acid, furazolidone, erythromycin, and ciprofloxacin, whereas all strains serovar O3:K6 were resistant to ampicillin but susceptible to all other antibiotics.

OBJECTIVES

1. To isolate *E. coli* O157:H7 from Malaysian and Thai beef.
2. To characterize virulence factors of *E. coli* O157:H7 obtained from Malaysian and Thai beef.
3. To investigate relationship among Malaysian and Thai *E. coli* O157:H7 strains using IS-printing and PFGE.
4. To isolate *E. coli* strain that produces high concentration of Shiga toxin and investigate factor that is involved in toxin production.
5. To isolate pandemic strains of *V. parahaemolyticus* from Malaysia to Thai molluscan shellfish.
6. To investigate relationship among Malaysian and Thai *V. parahaemolyticus* pandemic strains using AP-PCR and PFGE.

CHAPTER 2

RESEARCH METHODOLOGY

MATERIALS AND EQUIPMENTS

1. Beef and shellfish samples

Beef and shellfish from Malaysia and Thailand were purchased from local markets in Hat Yai City, Southern Thailand, from March 2008 to Oct 2010. Three kinds of shellfish, namely bloody clam, green mussel and oriental hard clam, were investigated. They were delivered to the laboratory within 1-2 h and processed immediately.

2. Bacterial strains

E. coli O157 strain Thai 12 was obtained from stock culture of Dept. of Microbiology, Fac. of Science, Prince of Songkla University. *E. coli* EDL 933, C0207, 144, E02-15, E07-I8, and E09-A, strains were received from Prof. Mitsuaki Nishibuchi, the Center for Southeast Asian Studies, Kyoto University, Japan. *Salmonella enteritica* serotype Braenderup strain H9812 was provided by Dr. Yoshito Iwade, Mie Prefectural Health and Environmental Research, Mie, Japan.

V. parahaemolyticus used in this study were obtained from stock culture of Dept. of Microbiology, Fac. of Science, Prince of Songkla University and VP6, 48541, VP54, VPS10, VPS27, VPS31, VPS35 were kindly provided by Prof. Mitsuaki Nishibuchi, the Center for Southeast Asian Studies, Kyoto University, Japan.

3. Microbiological media

All microbiological media used were purchased from Difco (U.S.A.); Merck, Germany; Becton Dickinson (U.S.A.) and CHROMagar (Microbiology, Paris, France).

4. Chemicals

All chemicals used in this study are of analytical grade and purchased from Merck, (Germany); Sigma-Aldrich (U.S.A.); Lab-Scan, Analytical Science (POCH. S.A., Poland).

5. PCR reagents and primers

PCR reagents including thermophillic DNA polymerase 10X buffer (Magnesium free buffer containing 100 mM Tris-HCl [pH9.0]), 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate (dNTPs) and *Taq* DNA polymerase in storage buffer A (5 units/µl) were purchased from Promega Corp., Madison, WI, USA. The oligonucleotide primers were synthesized by Operon Technologies (Alameda, U.S.A.) and Invitrogen (U.S.A.).

AP-PCR reagents including dNTPs, 10X *Ex Taq* buffer and *Ex Taq* were purchased from TaKaRa Biochemicals, Tokyo, Japan. Random 10-mers primers were synthesized by Invitrogen (U.S.A.). All primer names and sequences are listed in Table 1

6. Antibiotics

The antibiotics used in this study were purchased from Fluka (Sigma-Aldrich, USA) and Songklanagarind hospital. The antibiotic disks were purchased from Oxoid (Basingstoke, Hampshire, UK).

7. Enzymes

Restriction endonucleases were purchased from TOYOBO, Osaka, Japan. RNase A was purchased from Merck (Germany). Proteinase K was purchased from Sigma (U.S.A.).

8. Other reagents

Reagents	Company
1. Specific anti-O and anti-K antibodies - 9 polyvalent and 65 monovalent K-typing antisera - 11 O-grouping antisera	Denka Seiken, Tokyo, Japan
2. Wizard® plus SV Minipreps DNA purification system	Promega Corporation, Madison, WI, U.S.A.
3. VTEC-RPLA SEIKEN	Denka Seiken, Tokyo, Japan
4.,IS-printing system kit	TOYOBO, Osaka, Japan
5. Immunomagnetic separation kit Dynabeads® anti-*E. coli* O157	Dynal Biotech ASA, Oslo, Norway

9. Equipments and instruments

Instruments	Company
Autoclave	Tomy, Japan
Autopipette	Gilson, France
Contour-clamped homogeneous electric field DRIII Pulsed-field gel electrophoresis machine	Bio-Rad Laboratories, Tokyo, Japan
Electrophoresis apparatus	Bio-Rad Laboratories, U.S.A.
Freezer (4°C, -20°C, -70°C)	Sanyo, Japan
Incubator	Heraeus, Germany
Hot air oven	Venticell
Hot plate and stirrer	Fisher Scientific, U.S.A.
Lamina airflow cabinet, ABS 1200A	ASTEC microflow, UK
Microcentrifuge (Eppendorf 5415 C)	Brinkman Instrument Inc., Germany
4°C Microcentrifuge	Hettich Zentrifugen, Germany
PCR Gene Amp, PCR system 2400	Perkin Elmer, U.S.A.
pH meter	Sartorius, U.S.A.
Power supply, 200/2.0	Bio-Rad Laboratories, U.S.A.
Power supply, PowerPac Basic	Bio-Rad Laboratories, U.S.A.
Balance	Denver Instrument company, U.S.A.
Shaking incubator	Labline Instrument Inc., U.S.A.
UV light transilluminator	San Gabriel Inc., U.S.A.
Waterbath, 1235	Sheldon Manufacturing Inc., U.S.A.
25-cm^2 culture flasks	Iwaki Glass., Tokyo, Japan
Fingerprinting II software	Bio-Rad Laboratories, Tokyo, Japan

10. Miscellaneous

Agarose	Gibco, USA
SeaKem Gold agarose	Lonza, Rockland, ME, U.S.A.
Pulsed-field gel electrophoresis grade agarose	Bio-Rad Laboratories
Nusieve 3:1 agarose	TaKaRa Biochemicals, Tokyo, Japan
1kb DNA ladder	New England Biolabs, Inc., Ipswich, MA
100 bp DNA ladder	New England Biolabs, Inc., Ipswich, MA
Hind III digested of λ DNA	New England Biolabs, Inc., Ipswich, MA

Table 1 Oligonucleotide primer pairs and expected amplification products

Gene	Primer	Amplicon size (bp)	Sequences of primer (5' to 3')	References
stx$_1$	EVT-1	349	CAACACTggATgATCTCAg	Koitabashi
	EVT-2		CCCCCTCAACTgCTAATA	et al. 2006
stx$_2$	EVS-1	404	ATCAgTCgTCACTCACTggT	Koitabashi
	EVS-2		CCAgTTATCTgACATTCTg	et al. 2006
eae	AE19	1,087	CAggTCgTCgTgTCTgCTAAA	Koitabashi
	AE20		TCAgCgTggTTggATCAACCT	et al. 2006
TNP-A	TNPf1	458	CCATgAgCAAATgATgATTg	Koitabashi
	TNPr1		TTTAgTTCTCTTATgCCCAC	et al. 2006
TNP-B	TNPf2	694	CTAAATTCATggAgAgCgTg	Koitabashi
	TNPr2		TTAACgTCAggCACAAAgAg	et al. 2006
TNP-C	TNPf3	268	AACCggAAACgTgTAgAg	Koitabashi
	TNPr2		TTAACgTCAggCACAAAgAg	et al. 2006
TNP-D	TNPf4	549	gAACATATCAAAATCAggC	Koitabashi
	TNPr3		gggAATAggATACCgAAg	et al. 2006
*esc*V	MP-*esc*V-F	535	ggCTCTCTTCTTCTTTATggCTg	Müller et
	MP-*esc*V-R		CCTTTTACAAACTTCATCgCC	al. 2006
*bfp*A	BFP1	326	AATggTgCTTgCgCTTgCTgC	Müller et
	BFP2		gCCgCTTTATCCAACCTggTA	al. 2006
*tox*R	T4	368	gTCTTCTgACgCAATCgTTg	Kim et al.
	T7		ATACgAgTggTTgCTgTgATg	1999
tdh	D3	251	ggTACTAAATggCTgACATC	Tada et al.
	D5		CCACTACCACTCTCATATgC	1992
trh	R2	250	ggCTCAAAATggTTAAgCg	Tada et al.
	R6		CATTTCCgCTCTTCATATgC	1992
16 kb	F2-1-F	385	ggCTgCTATAACATTgAgCAC	Okura et
IS	F2-1-R		gAggACTTgTgAAATCCCATg	al. 2005

Gene	Primer	Amplicon size (bp)	Sequences of primer (5' to 3')	References
23 kb IS	VP0388-F VP0388-R	399	CAAACTTCATTggTTTggTTTT AATATCAATgTgATTgCgACAA	Nishibuchi unpublished data
GS-VP	GS-VP1 GS-VP2	651	TAATGAggTAgAAACA ACgTAACgggCCTACA	Matsumoto et al. 2000
AP-PCR	Primer2	–	gTTCgCTCC	Matsumoto et al. 2000

Table 2 Primers used in IS-printing system

1st set		2nd set	
Primer No.	Size (bp)	Primer No.	Size (bp)
1-01	974	2-01	987
1-02	839	2-02	861
1-03	742	2-03	801
1-04	645	2-04	710
1-05	595	2-05	642
1-06	561	2-06	599
1-07	495	2-07	555
1-08	442	2-08	499
1-09	405	2-09	449
1-10	353	2-10	394
1-11	325	2-11	358
1-12	300	2-12	331
1-13	269	2-13	301
1-14	241	2-14	278
1-15	211	2-15	240
eae	185	2-16	211
1-16	171	*stx*$_2$	181
*hly*A	137	*stx*$_1$	151

METHODS

This study was divided into two parts. The first part was investigation of *E. coli* O157:H7 in beef imported from Malaysia to Thailand and isolation of high concentration of Shiga toxin-producing *E. coli* from Thai beef**.** The second part was determination *V. parahaemolyticus* in Malaysian and Thai shellfish.

Part I

1.1 Investigation of *E. coli* O157:H7 in beef imported from Malaysia to Thailand

1.1.1 Bacterial isolation (Koitabashi et al. 2008)

Malaysian beef (imported) and Thai beef (domestic) were purchased from a fresh market in Hat Yai city, southern Thailand. Samples were collected between March and September 2008. Fifty grams of beef were homogenized in tryptic soy broth (TSB). The liquid portion after homogenized was supplemented with novobiocin (20 µg/ ml) and incubated at 37ºC for 6 h as first enrichment. One milliliter from the first enrichment was inoculated into 10 ml of TSB and incubated at 42ºC for 2 h as a second enrichment (Koitabashi et al., 2008). Immunomagnetic separation for isolation of *E. coli* O157 was performed by mixing 1 ml of the second enrichment culture and 20 µl of immunomagnetic beads (IMBs). The mixture was mixed by inversion for 30 min. The IMBs were harvested using magnetic concentrator, Dynal (Dynal Biotech ASA, Oslo, Norway). Subsequently, IMBs were washed with phosphate buffer saline (PBS), pH 7.4 for 3 times. After washing, 50 µl of PBS were added to the beads. Ten-fold dilution of the beads was performed and 100 µl of 10^{-1} dilution were spread on CHROMagar® O157 plates (CHROMagar Microbiology, Paris, France). Plates were incubated at 37ºC. Three to five mauve colonies from each plate were selected and determined for specific characteristics of *E. coli* O157 (Fig. 8).

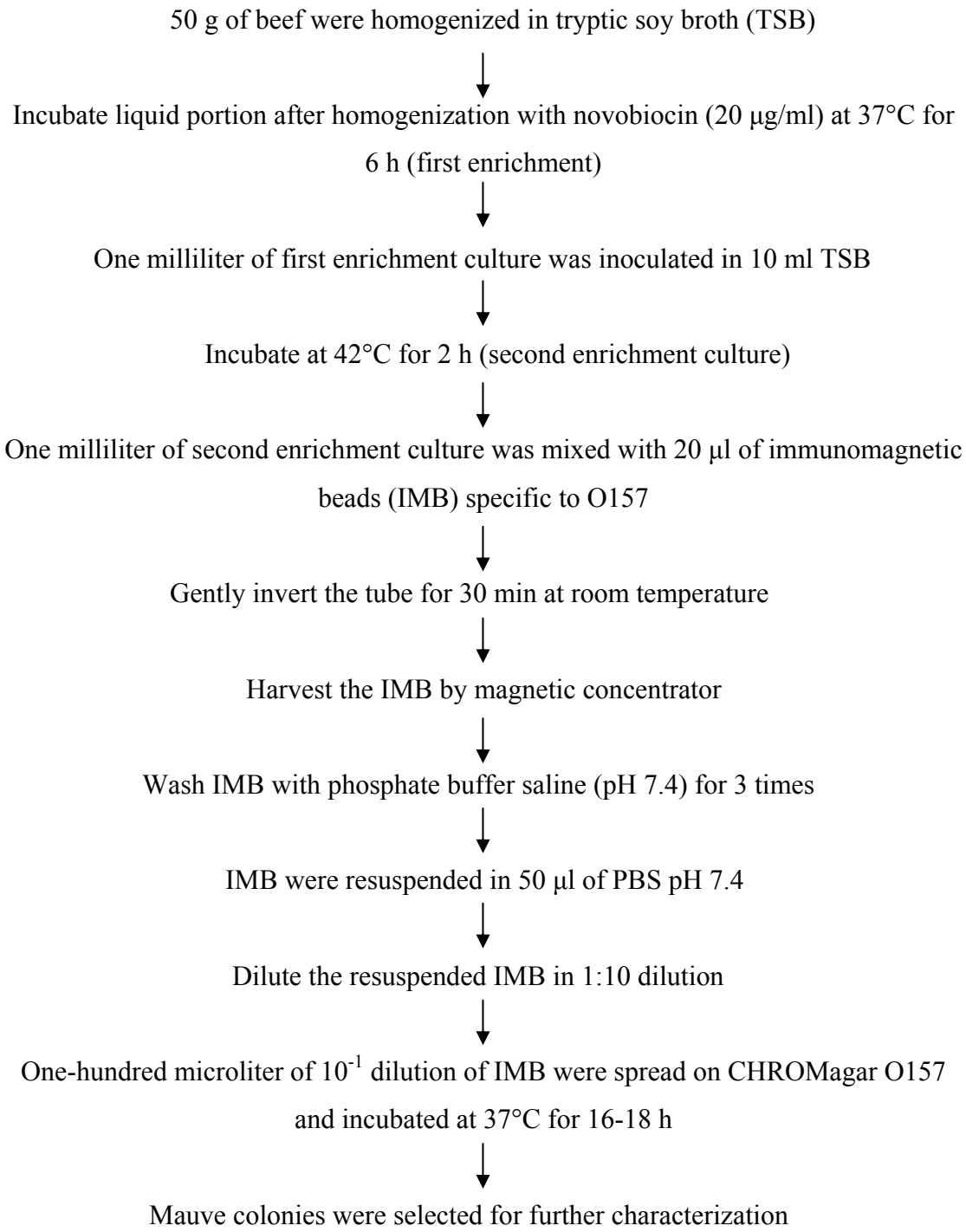

Figure 8 Protocol for isolation of *E. coli* O157 from beef sample

1.1.2 Virulence genes determination

1.1.2.1 Preparation of DNA

The test isolate was grown in LB broth with shaking (160 rpm) at 37°C overnight. One milliliter of the broth culture was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as DNA template for PCR amplification

1.1.2.2 Shiga toxin (*stx*) gene and *eae* gene detection

stx and *ea*e genes were examined by PCR method using primers EVT-1 and EVT-2 for stx_1, EVS1 and EVS-2 for stx_2, AE19 and AE20 for *eae* gene. Briefly, the reaction was performed in 25 µl reaction mixture containing 1X Go*Taq* reaction buffer, 0.4 µM of each primer, 0.1 mM of dNTPs, 0.5 unit of Go*Taq* DNA polymerase, and 1.0 µl of solution containing DNA. The PCR condition for stx_1 and *eae* gene is as followed: the reaction was pre-heated at 95ºC for 3 min. Subsequently, the 35 cycles of PCR were carried on, which was composed of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min. The final extension was performed at 72ºC for 7 min. For stx_2 gene detection, the annealing temperature was changed to be 50ºC. The amplicon sizes (349 bp for stx_1 and 404 bp for stx_2 and 1,087 bp for *eae* gene) were detected using 1.0 % agarose gel electrophoresis.

1.1.2.3 Locus of enterocyte effacement pathogenicity island detection (Müller et al. 2006)

Locus of enterocyte effacement (LEE) pathogenicity island was detected by PCR target to *esc*V gene. Primer pair MP-*esc*V-F and MP-*esc*V-R was used for amplification. Briefly, the reaction was performed in 25 µl reaction mixture containing 1X Go*Taq* reaction buffer, 0.4 µM of each primer, 0.16 mM of dNTPs, 0.5 units of Go*Taq* DNA polymerase, and 1.0 µl of DNA. The PCR condition was pre-

heated at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 45ºC for 30 sec, extension at 72ºC for 1 min. The final extension was performed at 72ºC for 7 min.

1.1.2.4 Investigation of 60-MDa plasmid (Radu et al. 1998)

Investigation of 60-MDa plasmid was performed using plasmid extraction kit, Wizard® plus SV Minipreps DNA purification system (Promega Corporation, Madison, USA). Briefly, five milliliters of overnight culture were centrifuged for 5 min at 10,000 x g and the supernatant was discarded. Two hundred and fifty microliters of cell resuspension solution [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A] was added and mixed with 250 µl of cell lysis solution [0.2 M NaOH, 1% SDS]. The tube was incubated at room temperature until the cell suspension became clear. Ten microliters of alkaline protease were added into the solution and the tube was further incubated at room temperature for 5 min. Then, 350 µl of Neutralization solution [4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, final pH ~ 4.2] was immediately added and tube was mixed invertly. The mixture was centrifuged at 14,000 x g for 10 min at room temperature. The lysate was applied to the spin column. After centrifugation at 14,000 x g for 1 min the flow-through liquid was discarded. The column was washed twice with column wash solution [162.8 mM potassium acetate, 22.6 mM Tris-HCl (pH 7.5), 0.109 mM EDTA (pH 8.0)] and nuclease-free water was added to the column, after final spining, the eluted plasmid DNA was obtained and was determined by eletrophoresis in 0.8% agarose gel.

1.1.2.5 Determination of bundle forming pilli (Müller et al. 2006)

Typical EPEC possess bundle forming pilli which was encoded by *bfp*A gene. Investigation of this gene was performed by PCR technique using primers BFP1 and BFP2 (Table 1). Briefly, the reaction was performed in 25 µl reaction mixture containing 1X Go*Taq* reaction buffer, 0.8 µM of each primer, 0.2 mM of dNTPs, 0.5 units of Go*Taq* DNA polymerase, and 1.0 µl of DNA. The PCR condition

was pre-heated at 94ºC for 1 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min. The final extension was performed at 72ºC for 7 min.

1.1.3 Investigation of toxin production

1.1.3.1 Determination of Stx2 production by Toxin-non-producing PCR (TNP-PCR) (Koitabashi et al. 2006)

Four separated PCR reactions designated as TNP-A, TNP-B, TNP-C and TNP-D targeted to the *Q* gene and its surrounding region of EHEC were performed using primer pairs TNPf1 and TNPr1, TNPf2 and TNPr2, TNPf3 and TNPr2 and TNPf4 and TNPr3 respectively. The reaction was carried out in a total of 20 µl. The final concentration of each component is as followed; 4 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1X *Taq* DNA polymerase buffer, and 0.5 unit of *Taq* DNA polymerase. Two microliters of DNA (extracted by boiling as described above) were used as a template for PCR. The PCR condition for TNP-PCR was pre-heated at 96ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min. The final extension was performed at 72ºC for 7 min. Amplicon sizes of 458 (TNP-A), 694 (TNP-B), 268 (TNP-C) and 549 (TNP-D) were detected by electrophoresis in 1.5 % agarose gel. *E. coli* O157:H7 strain Thai-12 was used as a positive control.

1.1.3.2 Determination of Stx production by reverse passive latex agglutination (RPLA) assay (Koitabashi et al. 2006)

Quantitative analysis for Stx2 was evaluated by RPLA assay using Verotox-F Seiken commercial kit (Denka Seiken Co., Ltd) according to manufacturer's specification. In brief, the test strains were cultured in 5 ml of Casamino acid-Yeast extract (CA-YE) medium (composed of 2% Casamino Acids, 0.6% yeast extract, 0.25% NaCl, 0.87% K_2HPO_4 , 0.005% $MgSO_4$, 0.0005% $MnSO_4$, 0.0005% $FeCl_3$, pH8.5) at 37ºC for 20 h. The culture was pelleted at 15,000 x g for 5

min and the supernatant was used directly for Stx2 detection. The agglutination test was carried out in a 96-well V-bottom microtiter plate. Twenty five microliters of diluent (containing 0.5% w/v bovine serum albumin and 0.1% w/v sodium azide as a preservative) were added into the wells. The supernatant was diluted by serial two-fold dilution and 25 µl of VT2 sensitized latex was subsequently added into all wells. This experiment was done in parallel with Stx1 or Stx2 positive control toxin. For negative control, no toxins were added. The plate was kept at room temperature for 20 h before reading. The reciprocal of the highest dilution of the test sample, giving positive reaction was defined as RPLA titer. The test was examined in duplicate.

1.1.4 Serotyping

In order to determine somatic O-antigen (O157), bacteria was inoculated on LB agar plates and incubated at 37ºC for 16-18 h. Single colony (~2 mm diameter) was inoculated into tryptic soy broth (TSA) and incubated at 37ºC for 5 h without shaking. The cells were collected by centrifugation and then re-suspended in 0.85% normal saline solution (NSS) and was heated at 121ºC for 15 min by autoclaving. The heat-killed cell suspension was employed for agglutination test by reacting with antiserum specific to O157 antigen (Denka Seiken, Tokyo, Japan).

To examine H-antigen, motility of O157 strains were enhanced by passing the strains three to four times through heart infusion-based semisolid medium (Difco). Subsequently, the test strains were grown in TSB as described above. The cells were fixed by adding formalin at a final concentration of 1% (Radu et al. 1998). Then, the cell suspension was prepared as described above and agglutination was performed using a set of antisera for H-serotyping.

1.1.5 Antibiotic susceptibility analysis

Antibiotic susceptibility of the test strains was performed using disk diffusion method. Antibiotic-loaded paper disks were dispensed on Mueller-Hinton agar plates inoculated with a bacterial lawn. After incubation at 37ºC for 14 to 18 h, the diameter of the inhibition zone was recorded and interpreted according to the

reference provided by the manufacturer. Twelve antibiotic disks were used, cephalothin (30 μg), ceftriazone (30 μg), ampicillin (10 μg), trimethoprim/sulfamethoxazole (1.25 μg), norfloxacin (10 μg), colistin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), cefipime (30 μg) gentamicin (10 μg) and imipenam (10 μg). *E. coli* ATCC 25922 was used as a standard strain.

1.1.6 Molecular typing

1.1.6.1 DNA fingerprinting by IS-printing analysis (Ooka et al. 2009b)

DNA preparation was performed using alkaline-boiling method. Briefly, a single colony of the test strains on LB agar was picked up and inoculated into the tube. Then, 0.2 M NaOH and 0.1 % SDS were added, the tube was boiled for 10 min and immediately cooled down on ice. The tube was centrifuged at 15,000 x g for 5 min and supernatant was collected and used as DNA template for IS-printing analysis. The primers used in IS-printing system were shown in Table 3.

1.1.6.2 DNA fingerprinting by Pulsed-field gel electrophoresis (PFGE) (Hunter et al. 2005)

The test strain was grown in 5 ml of LB broth and incubated at 37°C for overnight without shaking. Five hundred microliters of the cultures were taken to a new 1.5 ml tube and centrifuged at 10,000 x g for 5 min. The cell pellet was gently resuspended in 150 μl of suspension buffer [100 mM Tris, 100 mM EDTA. pH 8.0] and was subjected to heat at 50°C and mixed with warm 1% SeaKem Gold agarose in TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0]. This mixture was carfully loaded into plug mold to avoid bubbles formation. After the gel was set , the plug was removed and put into 2 ml tube containing 500 μl of (20 mg/ml) proteinase K-containing lysis buffer [50 mM Tris, 50 mM EDTA, pH 8.0 supplemented by 1% Sarcosyl]. Subsequently, the tube was placed in 50°C water bath for overnight to lyze bacterial

cells. After that the plug was transferred to a new 2 ml tubes and washed 3 times with TE buffer. The plug was placed on a steriled petri-dish and cut with sharp spatula or coverslip to obtain a piece of 5x3 mm. This plug slice was incubated with 500 µl 1X Medium (M) buffer (TOYOBO, Osaka, Japan) for 1 h at room temperature. The M-buffer was discarded and the new 300 µl of M-buffer containing 50 units of *Xba*I restriction enzyme (TOYOBO, Osaka, Japan) was added. The tube was incubated at 37°C for 3 h and was fixed with comb teeth by adding one to two drops of 1% PFGE-grade agarose. The comb was then set in a tray and was loaded with 1% Pulse-Field Certified agarose (Bio-Rad Laboratories). After the comb was removed, this gel was transferred to PFGE chamber and run in 0.5X TBE buffer on a CHEF-DRIII system (Bio-Rad). Electrophoresis was performed at 6 V/cm with a field angle of 120º at 14ºC. The pulse times were 2.2 to 54.2 s for 19 h. After electrophoresis the gel was stained with ethidium bromide and DNA was visualized with a UV transilluminator. *Salmonella enterica* serotype Braenderup H9812 was digested by *Xba* I and used as a standard size marker (Hunter et al. 2005). The diagram illustrated PFGE protocol was shown in Appendix, part IV.

1.2 Isolation of high concentration of Shiga toxin-producing *E. coli* from Thai beef

1.2.1 Isolation and characterization of bacteria

Local Thai beef samples were purchased from various fresh markets in Hat Yai city, Thailand during May 2010-October 2010. Isolation of bacteria, virulence genes examination, serotyping and drug susceptibitity were performed using the same technique as described in sections 1.1.1, 1.1.2, 1.1.3, 1.1.3.1, 1.1.4, and 1.1.5.

1.2.2 Q_{933}/Q_{21} examination (Koitabashi et al. 2006)

To investigate correlation between Stx production and type of *Q* gene, PCR amplification of *Q* gene was performed using two primer pairs, qEf-1 and qEr-2, and qDf-1 and qDr-2, which are specific to *Q* gene of EDL933 and Thai-12 *E. coli*

strains, respectively (Koitabashi et al. 2006). PCR was performed in 20 µl volume consisting of 20 ng of chromosomal DNA, 2.5 mM $MgCl_2$, 0.1 µM each primer pair, 0.4 mM each dNTPs, 0.5 U of *Taq* DNA polymerase (Promega, U.S.A.), and 1X *Taq* DNA polymerase buffer (Promega, U.S.A.). Amplification was performed with a single cycle at 94^{o}C for 2 min, followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec, and extension at 72^{o}C for 2 min, and a final extension at 72^{o}C for 7 min.

1.2.3 Arbitrarily Primed PCR (AP-PCR) analysis (Okuda et al. 1997)

To evaluate DNA profiles of the test strains, AP-PCR was developed. DNA was extracted by Puregene DNA extraction kit (Gentra system, USA). In brief, 0.5 ml of overnight culture was harvested by centrifugation at 9,000 x g for 1 min. Cell pellet was re-suspended in 300 µl lysis buffer and incubated at 80ºC for 5 min. RNase A was added and the solution was further incubated at 37ºC for 15-60 min. After that 100 µl of protein precipitation solution was added and the tube was mixed vigorously. Then the tube was centrifuged at 20,000 x g for 10 min and the supernatant containing DNA was collected. Genomic DNA was precipitated by adding isopropanol to the supernatant at the ratio of 1:1. The tube was centrifuged at 15,000 x g for 10 min and DNA was washed with 70% ethanol. Rehydration of genomic DNA pellet was performed by adding 30 µl of rehydration buffer and incubated at 65ºC for 1 h.

AP-PCR was performed using primer 2 (5'-GTTTCGCTCC-3') as described previously (Vuddhakul et al. 2000). Briefly, amplification was performed in a 30-µl mixture composed of 0.33 mM dNTPs (TaKaRa Biochemicals, Tokyo), 25 ng DNA template, 2.5 U Ex *Taq* (TaKaRa), 0.83 pmol primer, and 1X Ex *Taq* buffer (TaKaRa). The PCR was performed in a thermal cycler (Program Temp Control System PC-808, Astec Co., Japan). The thermocycle was started with a cycle at 95^{o}C for 4 min, followed by 45 cycles of denaturation at 95^{o}C for 1 min, annealing at 36^{o}C for 1 min, and extension at 72^{o}C for 2 min, in which a transition time of 5 min was set between the denaturation and annealing, annealing and extension, and extension and

denaturation steps. The thermocycle finished with one cycle at 72°C for 7 min. The amplification products were analyzed by electrophoresis in a 1.5% agarose gel. *E. coli* O157 EDL933 and Thai-12 were used as control strains.

Part II Determination *V. parahaemolyticus* in Malaysian and Thai shellfish

2.1. Bacterial isolation

Both Thai and Malaysian shellfish samples including bloody clam, green mussle, and oriental hard clam, were purchased from Klong-rean market in Hat Yai city in 2008. Twenty five grams of sample were homogenized in alkaline peptone water (pH 8.6) and incubated for 6 h. One milliliter of supernatant was subjected to react with antiserum against K6 or K25 antigen of *V. parahaemolyticus*. The tubes was gently inverted every 5 min for 20 min before adding anti-immunoglobulin coated immunomagnetic beads (Dynal Biotech ASA, Oslo, Norway). The tube was gently inverted every 5 min for 30 min and immunomagnetic beads were recovered by a magnetic concentrator. The beads were washed twice with sodium phosphate buffer (pH 7.4) and resuspended in 100 µl of the same buffer. The suspension was spread on CHROMagar Vibrio and incubated at 37ºC for 16-18 h. Mauve colonies were examined for the presence of *V. parahaemolyticus* by re-agglutination with K6 or K25 antiserum.

2.2 Confirmation and virulence gene determination

2.2.1 DNA preparation

The test isolate was grown in LB broth containing 1% NaCl with shaking (160 rpm) at 37°C overnight. One milliliter of the broth culture was centrifuged, and the bacterial cells were washed with sterile saline (0.85% NaCl) and then suspended in it. The cell suspension was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as the template for PCR amplification.

2.2.2 *toxR* investigation

V. parahaemolyticus was confirmed by PCR targeted to the *toxR* gene, using primers T4 and T7 (Kim et al. 1999). Briefly, in 20 µl reaction mixture consisted of 1.5 µl DNA, 2.0 µl of 10X buffer, 1.6 µl of 25 mM $MgCl_2$, 1.6 µl of 2.5 mM dNTPs, 2.0 µl of each primer (2 µM), 0.1 µl of *Taq* DNA polymerase (5.0 U/ µl), and 3.2 µl of deionized water. PCR was performed in GeneAmp thermocycle using the conditions as followed: 5 min of hot start at 96ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 63ºC for 1.5 min, and extension at 72ºC for 1.5 min. The final extension was 72ºC for 7 min. PCR product was electrophoresed in 1.5% agarose gel to detect 368 bp amplicon.

2.2.3 *tdh* and *trh* genes detection

PCR was carried out using primers D3 and D5 for the *tdh* gene and primers R2 and R6 for *trh* gene amplification (Tada et al. 1992). Briefly, in 20 µl reaction mixture consisted of 1.5 µl DNA template, 1X buffer, 2mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 0.5 U of *Taq* DNA polymerase. Amplification was performed with a pre-heat at 96ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 7 min. PCR product was subjected to electrophoresis to detect 251 bp and 250 bp amplicons of *tdh* and *trh* respectively.

2.3 Investigation of pandemic markers

To investigate whether *V. parahaemolyticus* isolated from shellfish belonged to pandemic strain, three pandemic markers were determined using PCR technique.

2.3.1 Group Specific PCR (GS-PCR)

GS-PCR was carried out using primers GS-VP1 and GS-VP2 (Matsumoto et al. 2000). In brief, PCR mixture consisted of 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 1X reaction buffer, 0.2 µM of each primer, 0.5 U of *Taq* DNA polymerase, and 2.5 µl of DNA template in a total volume of 20 µl. Amplification was performed with a single cycle at 96ºC for 5 min, followed by 25 cycles of denaturation at 96ºC for 1 min, annealing at 45ºC for 2 min, and extension at 72ºC for 3 min. The final extension was applied at 72ºC for 7 min. Electrophoresis was performed to detect 651 bp amplicon.

2.3.2 23 kb insertion sequence

The primer pair VP0388-F and VP0388-R was used for detection of 23 kb insertion sequence. PCR mixture was composed of 0.2 mM dNTPs, 0.5 µM of each primer. 0.5 unit of Go*Taq* DNA polymerase, 1X Go*Taq* reaction buffer, and 2.0 µl of DNA template in a total volume of 20 µl. Amplification was performed with a single cycle at 95ºC for 3 min, followed by 30 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. The final extension was applied at 72ºC for 10 min. The amplicon size of 399 bp was separated by electrophoresis using 1.5% agarose gel.

2.3.3 16 kb insertion sequence

To detect gene located in 16 kb insertion sequence, PCR was carried out using F2-1F and F2-1R primers. The reaction mixture composed of 0.2 mM dNTPs, 0.5 µM of each primer. 0.5 unit of Go*Taq* DNA polymerase, 1X Go*Taq* reaction buffer, and 2.0 µl of DNA template in a total volume of 20 µl. Amplification was performed with a single cycle at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 30 sec. The final extension was applied at 72ºC for 7 min. The amplicon size 385 bp was separated by electrophoresis using 1.5% agarose gel.

2.4 Serotyping

The O:K serotype of the test isolate was determined by the slide-agglutination test using anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan). Briefly, the test isolate was grown in tryptic soy broth containing 3% NaCl at 37 ^{o}C for 18 h, and the bacterial cells were suspended in saline (3% NaCl). The bacterial cell suspension was subjected to agglutination with specific anti-K antibodies for the K serotype determination. For the O serotype determination, the bacterial cell suspension was autoclaved at 121^{o}C for 30 min. Autoclaved bacterial cells were subjected to agglutination with specific anti-O antibodies.

2.5 Molecular typing

2.5.1 DNA fingerprinting by AP-PCR

DNA was extracted by Puregene DNA extraction kit (Gentra system, USA) as described in section 1.2.3. AP-PCR was performed using primer 2 (5'-GTTTCGCTCC-3') as described previously in section 1.2.3.

2.5.2 DNA fingerprinting by PFGE

PFGE was performed in the same manner as described previously in section 1.1.6.2 except DNA of *V. parahaemolyticus* was digested with *Not* I and *Sfi* I restriction enzymes (TOYOBO Co. Ltd., Osaka, Japan) instead of *Xba*I and pulse times were 10 to 35 s for 18 h.

CHAPTER 3

RESULTS

Part I

1.1 Investigation of *E. coli* O157:H7 in beef imported from Malaysia to Thailand

1.1.1. Characteristics of *E. coli* isolates from beef

In this study, 31 beef samples from Malaysian exported to Thailand and 36 beef samples from Thai (domestic) were examined. Immunomagnatic technique was performed to effectively isolate *E. coli* O157 strains. After the initial screening, 15 strains were isolated from eight Malaysian samples, and six strains were isolated from four Thai beef samples. However, the results of subsequent repeated agglutination tests indicated that one of the Malaysian isolate belonged to serotype O116:H31 and the remaining isolates belonged to O157:H7 (Table 3). Therefore, a total of 20 strains of the O157:H7 serotype from 12 beef samples were obtained. Sample no. 13 yielded three O157:H7 strains and one O116:H31 strain (Table 4). All O157:H7 strains but one were stx_1^- stx_2^+ eae^+ virulence gene type; the exceptional strain exhibited stx_1^- stx_2^- eae^+ genotype (Table 5, strain M2). Accordingly, 13 Malaysian and 6 Thai strains were *E. coli* O157:H7 possessing the stx_2 and *eae* genes. The O116:H31 strain, M7, was stx_1^+ stx_2^+ eae^- genotype (Table 5). All O157 strains possessed the *escV* gene, suggesting that they harbored the LEE pathogenicity island. Plasmid analysis revealed that all O157:H7 strains contained a 60-MDa plasmid unique to EHEC (Fig. 9). The 19 strains of stx_2^+ *E. coli* O157 including two strains of stx_1^- stx_2^- (M2) and stx_1^+ stx_2^+ (M7) were examined for the inability to produce Stx2 by the TNP-PCR method. All isolates were positive in all four PCR tests (TNP-A, TNP-B, TNP-C and TNP-D) except the M2 and M7 strains (Table 4).This indicated their inability to produce Stx2 due to possessing the *Q* gene and surrounding nucleotide sequences unique to the Stx2-non-producing strains. Confirmation of Stx2

production from these strains using RPLA method revealed that most of the strains did not produce Stx2 or produced in an undetectable level. Strain M10 exhibited an RPLA titer of only 1:4 and the titer of the other 18 O157 strains were below the detection limit (< 2) whereas the titer of a positive control, strain EDL933, was > 1:128 (Table 4).

Enterohemolysin gene was detected in all *E. coli* isolates. The possession of enterohemolysin gene was correlated with the presence of 60-MDa plasmid (Table 5).

Table 3 Enterohemorrhagic *Escherichia coli* O157 isolated from Malaysian and Thai beef using immunomagnetic separation technique

Source of sample	No. of positive sample/ no. of test sample (percentage)	No. of total isolate	O157:H7/ Non-O157	No. of positive isolate		
				Virulence genes		
				stx_1	stx_2	*eae*
Malaysia	8/31 (22.6%)	15	14/1*	1	14	14
Thailand	4/36 (11.1%)	6	6/0	0	6	6

* Serotype O116:H31

Resistance of the 19 strains of stx_2^+ eae^+ *E. coli* O157:H7 to 12 commonly used antibiotics was examined. There were similar antibiotic resistance patterns between the Malaysian and Thai strains. Two of six (33.3%) Thai strains were resistant to one or two antibiotics and five of thirteen (38.5%) Malaysian strains were resistant to one to three antibiotics (Table 6). One Malaysian (M14) and one Thai (T3) strain shared the same pattern of antibiotic resistance. Strain M7 which is *E. coli* O116:H31 showed the resistance to Ceftriazone (CRO), Ampiciliin (AMP), and Cephalothin (KF) (Appendix, part V).

Table 4 Stx2 production detected by TNP-PCR and RPLA assay

Source (Strain name)	Sample no. (no. of isolates)	TNP-PCR				Stx2 detection (Titer)
		TNP-A	TNP-B	TNP-C	TNP-D	
Malaysia (M1)	9 (1)	+	+	+	+	<2
(M2)	10 (1)	-	-	-	-	<2
(M3)	11 (1)	+	+	+	+	<2
(M4-6)	13 (3)	+	+	+	+	<2
(M7)	13 (1*)	-	-	-	-	<2
(M8-9)	17 (2)	+	+	+	+	<2
(M10)	29 (1)	+	+	+	+	4
(M11-13)	30 (3)	+	+	+	+	<2
(M14-15)	31 (2)	+	+	+	+	<2
Thailand (T1)	5 (1)	+	+	+	+	<2
(T2)	6 (1)	+	+	+	+	<2
(T3)	24 (1)	+	+	+	+	<2
(T4-6)	29 (3)	+	+	+	+	<2
USA (EDL933)		-	-	-	-	>128

* One strain obtained from sample 13 (designated as M7) was serotype O116:H31

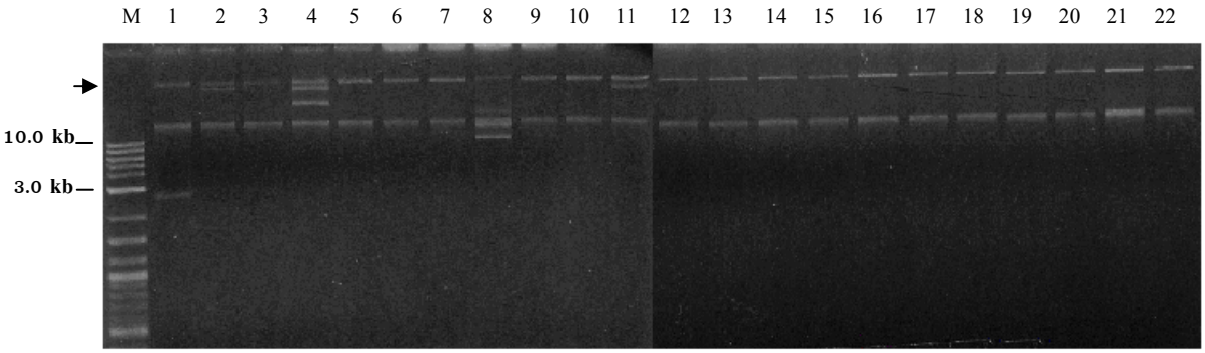

Figure 9 Illustration of 60-MDa plasmid obtained from Malaysian and Thai O157:H7 and O116:H31 strains. Lane M, 2 log DNA ladder; Lane1, EDL933 (control strain); Lane 2-16 are M1-M15, respectively. Lane 17-22 are T1-T6, respectively. The 60-MDa plasmids are indicated by an arrow. The DNA bands at lower position (above 10 kb marker) are contaminated chromosomal DNA.

Table 5 Characteristics of *E. coli* isolated from Malaysian and Thai beef

Strains [a]	*stx*$_1$	*stx*$_2$	*eae*	*escV*	enterohemolysin[b]
M1	-	+	+	+	+
M2	-	-	+	+	+
M3	-	+	+	+	+
M4	-	+	+	+	+
M5	-	+	+	+	+
M6	-	+	+	+	+
M7	+	+	-	-	+
M8	-	+	+	+	+
M9	-	+	+	+	+
M10	-	+	+	+	+
M11	-	+	+	+	+
M12	-	+	+	+	+
M13	-	+	+	+	+
M14	-	+	+	+	+
M15	-	+	+	+	+
T1	-	+	+	+	+
T2	-	+	+	+	+
T3	-	+	+	+	+
T4	-	+	+	+	+
T5	-	+	+	+	+
T6	-	+	+	+	+

[a]M, Malaysian ; T, Thai

[b]enterohemolysin was detected by IS-printing technique

Table 6 Antibiogram pattern of stx_2^+ O157 isolates from Malaysian and Thai beef

Antibiogram pattern*	Malaysian (13)	Thai (6)
KF	1 (M8)	0
CR	0	1 (T4)
CR, KF	1 (M14)	1 (T3)
CR, AMP	1 (M15)	0
CR, AMP, KF	1 (M12)	0
KF, AMP, AK	1 (M9)	0
Total	5 (38.5%)	2 (33.3%)

*The antibiotics examined were cephalothin (KF), ceftriazone (CR), ampicillin (AMP), trimethoprim/sulfamethoxazole, chloramphenicol, tetracycline, norfloxacin, colistin, amikacin (AK), ciprofloxacin, ceftacidime, cefipime, gentamicin, and imipenam. Both resistance and intermediate reaction are judged as resistance in this study, and only these reactions are listed in these antibiograms.

1.1.2 DNA fingerprinting by IS-printing

The DNA fingerprints of the 19 strains of stx_2^+ eae^+ *E. coli* O157:H7 isolated in this study were examined. stx_2^- eae^- *E. coli* O157:H7 (M2), stx_2^+ eae^- *E. coli* O116: H31 (M7) isolated in this study; and stx_2^+ eae^+ TNP-PCR-positive strains of *E. coli* O157 previously isolated from beef, bovine feces, and humans in Thailand, Japan, and China ; and a standard *E. coli* O157 strain, EDL933 were included for comparison (Table 7). Two sets of primers were used and both of them provided similar results (Fig. 10 & 11). The DNA profiles revealed 19 distinct patterns that could be classified into seven groups, I to VII, with the O116: H31 strain (M7) being located in the most distantly related group VII (Fig. 12). All the current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 (T1-T6) were in group III and showed 95 - 100% similarity, whereas high degrees of heterogeneity (25 - 100% similarity) were observed among the current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7 (M1-M15) (Fig. 12).However, most of the current Malaysian and Thai strains were also found in group III (Fig. 12). This demonstrated the close relationship among Thai and Malaysian *E. coli* O157:H7.

In this experiment, *E. coli* O157:H7 strain E07-I8 from China and strain Thai-12 identified as Stx2-non-producing strain, were also classified into the same group as the most Thai and Malaysian *E. coli* O157:H7 strains (group III, Fig. 12). This indicated relationship among Stx2-non-producing strains.

1.1.3 DNA fingerprinting by pulsed-field gel electrophoresis (PFGE)

The DNA fingerprints of the same set of the test strains were investigated using PFGE. Chromosomal DNA was digested with *Xba* I to generate DNA fragments range between ca. 600 kb and 30 kb (Fig. 13). This technique generated 25 distinct DNA patterns that were clustered into 11 groups designated as group I to XI with the O116: H31 strain (M7) located in the most distantly related group XI (Fig. 14). The tendency of the distribution of the test strains was similar to that of the O157 IS-fingerprinting result. All current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 (T1 - T6) were in group IX (82 - 100 % similarity), and the DNA profiles of two of the six strains (T4 and T5) were identical. The current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7 showed more heterogeneity (52 - 100 % similarity), and 15 of the 19 strains were classified into seven groups (groups I, II, III, V, IX, X and XI). Thus, only two, M14 and M15, of the current 19 Malaysian strains were in the same group (group IX) as the current Thai strains. The results indicated that all current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 were related to some of the current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7.

PFGE showed higher resolution in strain classification over IS-printing system. Malaysian strains M11, M12, and M13 and Thai strains Thai-1 and Thai-13 and strain T2, T3, and T6 showed identical fingerprint pattern by IS-printing system but these strains can be differentiated by PFGE (Fig. 12 and Fig. 14). The close relationship among Thai and Malaysian *E. coli* O157:H7 strain examined by IS-printing and PFGE analysis indicated the possibility of *E. coli* O157:H7 might be transferred from Malaysia to Thailand through beef trade. Moreover, these two methods also demonstrated relationship of Stx2-non-producing *E. coli* O157:H7 isolates in Asia.

Table 7 Standard strains of *E. coli* O157:H7 used for DNA fingerprinting analysis

Strains	Origin	Date of isolation	*stx*$_1$	*stx*$_2$	*eae*	TNP-PCR	References
EDL933	Human, USA	1982	+	+	+	-	Riley et al. 1982
Thai-1	Beef, Thailand	May-Oct 1988	+	+	+	+	Vuddhakul et al. 2000
Thai-12	Beef, Thailand	May-Oct 1988	-	+	+	+	Vuddhakul et al. 2000
Thai-13	Bovine fece, Thailand	May-Oct 1988	+	+	+	+	Vuddhakul et al. 2000
144	Human, Japan	2003	-	+	+	+	Koitabashi et al. 2006
C0207	Human, Japan	2003	-	+	+	+	Koitabashi et al. 2006
E02-15	Beef, China	Aug 2004-Jul 2005	-	+	+	+	Koitabashi et al. 2008
E07-I8	Beef, China	Aug 2004-Jul 2005	-	+	+	+	Koitabashi et al. 2008
E09-A	Beef, China	Aug 2004-Jul 2005	-	+	+	+	Koitabashi et al. 2008

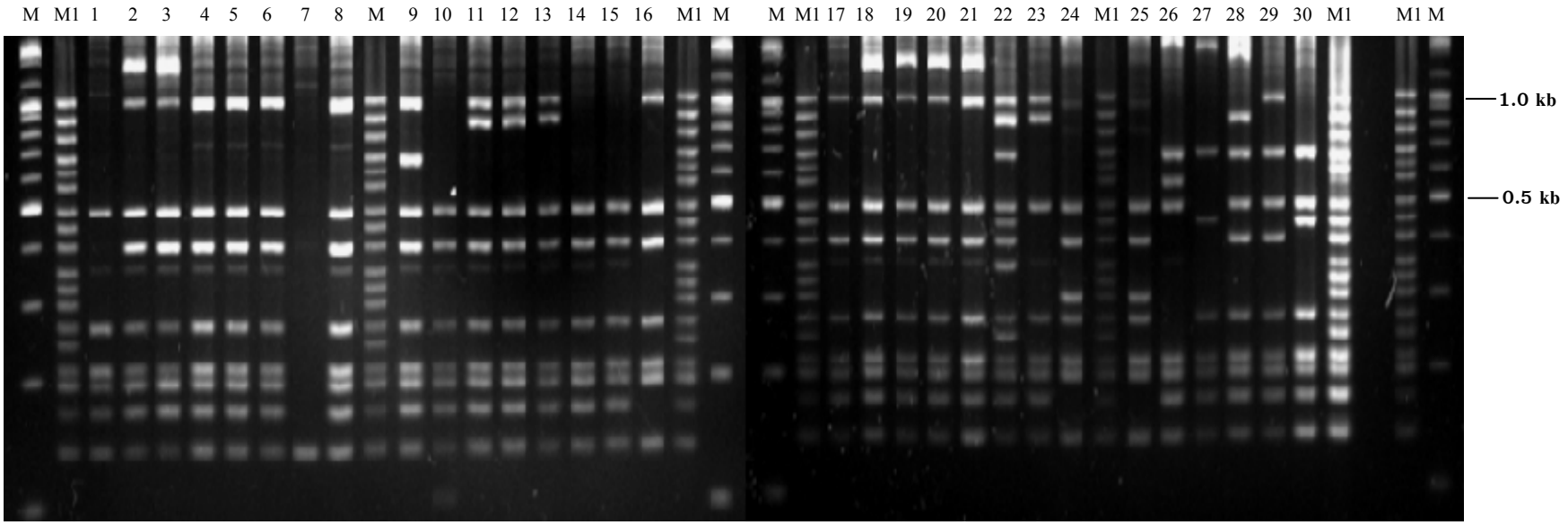

Figure 10 IS-printing analysis (first set primer) of *E. coli* O157:H7 and O116:H31. Lane: M, 2 log DNA ladder; lane M1, first set standard; lane 1-15, M1-M15 ; lane 16-21, T1-T6; lane 22, EDL933; lane 23, Thai-12; lane 24, Thai-1; lane 25, Thai-13; lane 26, 144; lane 27, C0207; lane 28, E02-15; lane29, E07-I8; lane30, E09-A.

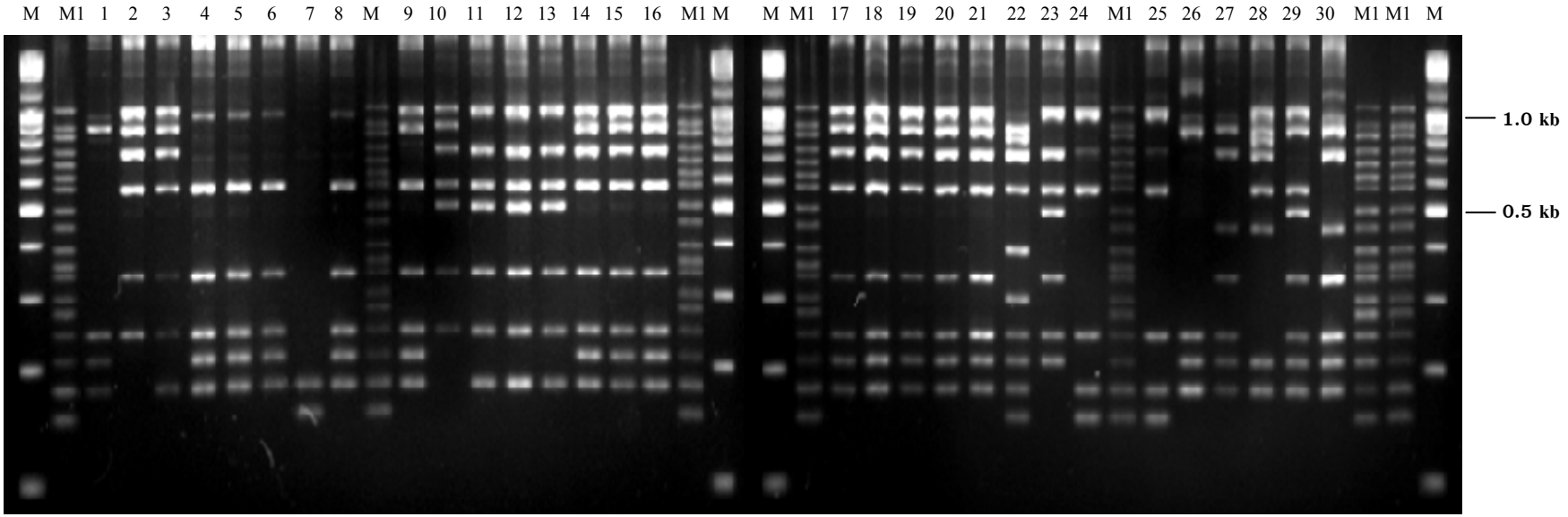

Figure 11 IS-printing analysis (second set primer) of *E. coli* O157:H7 and O116:H31. Lane: M, 2 log DNA ladder ; lane M1, second set standard; lane 1-15, M1-M15 ; lane 16-21, T1-T6; lane 22, EDL933; lane 23, Thai-12; lane 24, Thai-1; lane 25, Thai-13; lane 26, 144; lane 27, C0207; lane 28, E02-15; lane 29, E07-I8; lane 30, E09-A.

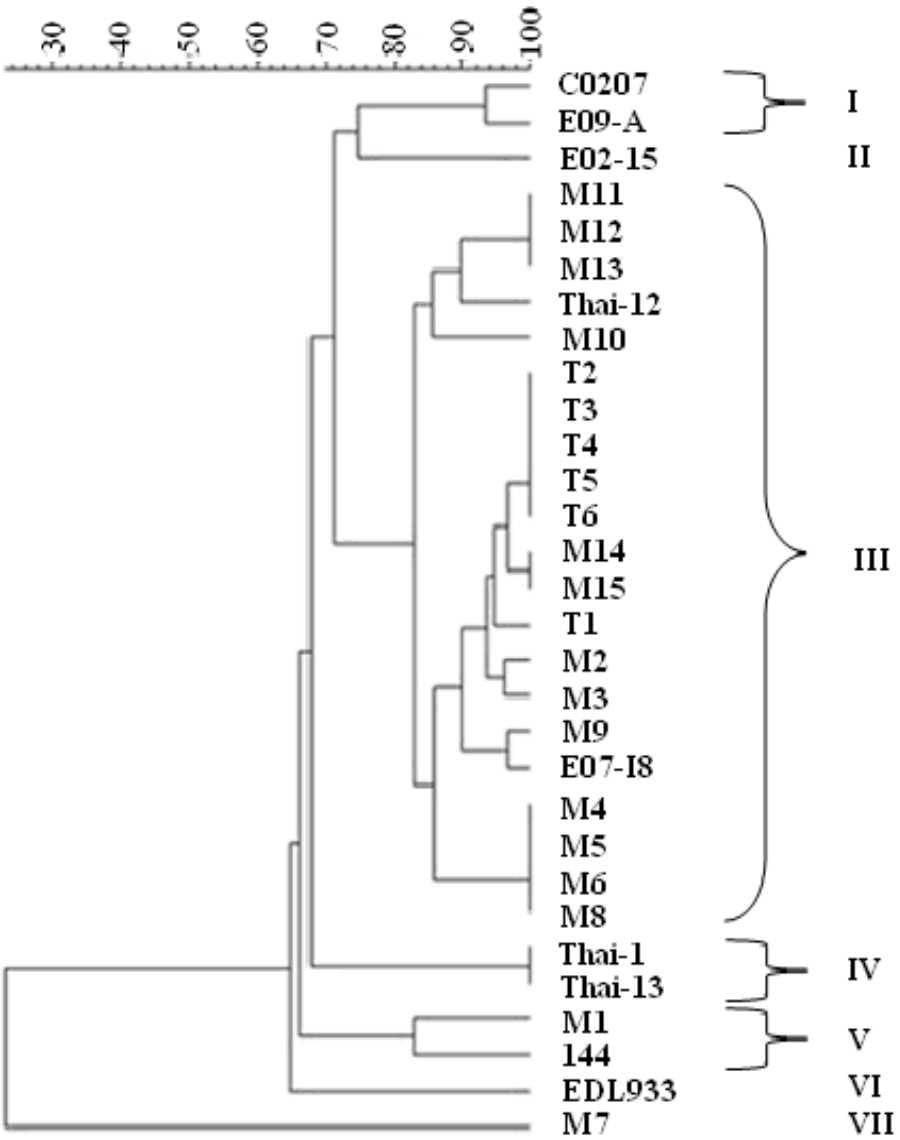

Figure 12 IS-printing-based dendrogram profile of 20 strains of *E. coli* O157:H7 and one O116:H31. This dendrogram was analyzed in parallel with 9 standard strains of *E. coli* O157:H7 isolated from the past. All 30 strains could be classified to be 7 subgroups (subgroup I to VII) at 80% similarity. Most of current Thai and Malaysian strains including 2 strains of Stx2-negative strains (Thai-12 and E07-I8), were classified into subgroup III. The rest of Malaysian strains, M1 and M7, were classified into subgroups V and subgroup VII, respectively.

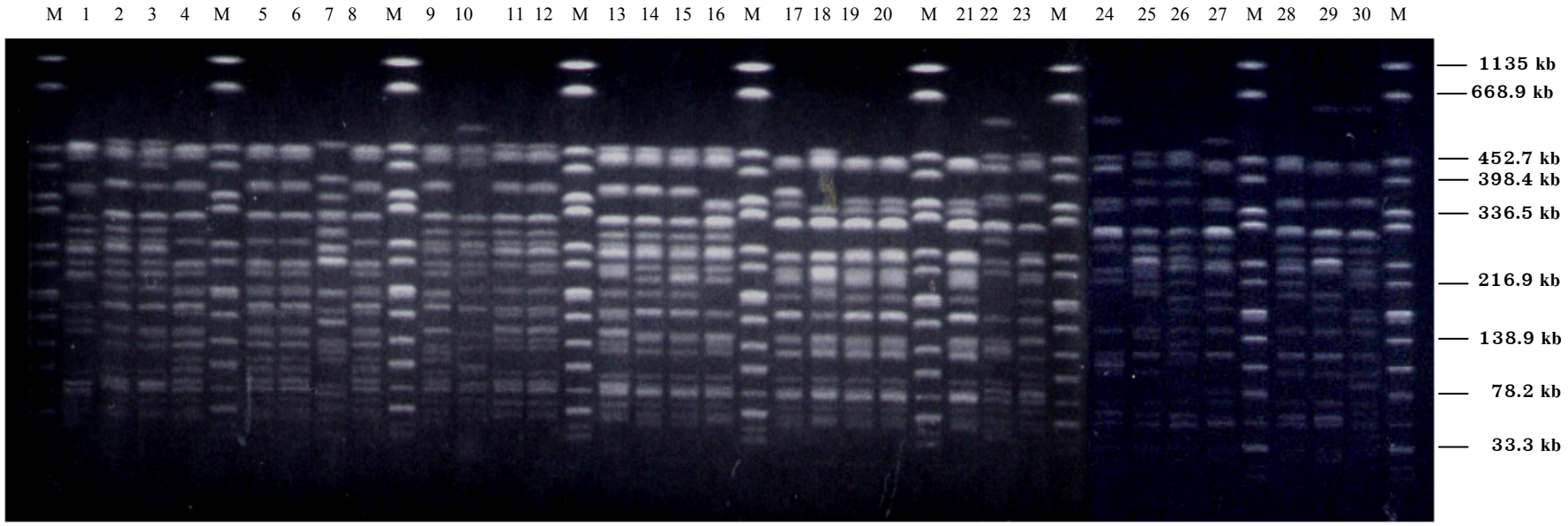

Figure 13 Pulsed-field gel electrophoresis of *E. coli* O157:H7 and O111:H31**.** Lane: M, *Salmonella enterica* serovar Braenderup standard size marker for PFGE; lane 1-15, M1-M15 ; lane 16-21, T1-T6; lane 22, Thai-1; lane 23, Thai-12; lane 24, Thai-13; lane 25, C0207; lane 26, 144; lane 27, E02-15; lane 28, E07-I8; lane 29, E09-A; lane 30, EDL933.

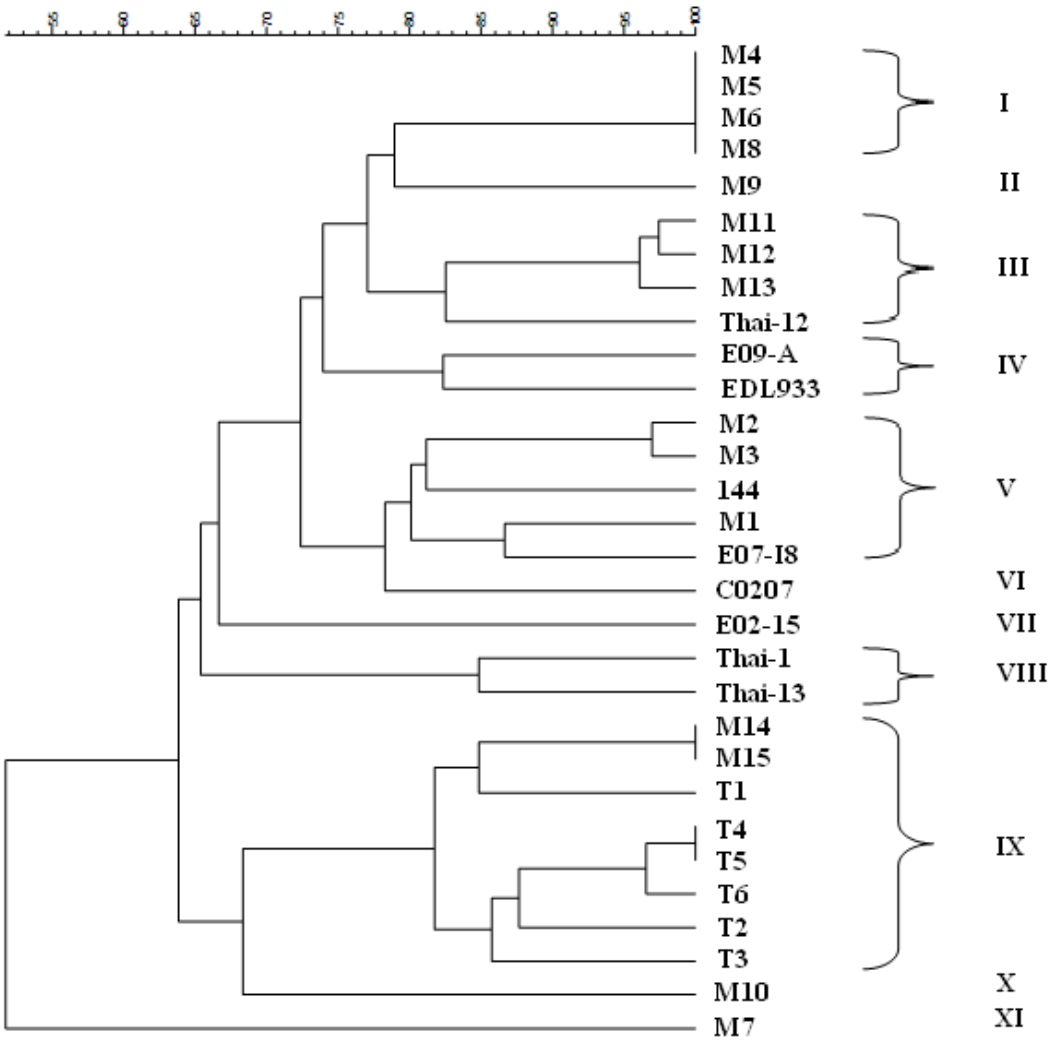

Figure 14 A dendrogram generated from pulsed-field gel electrophoresis profile of 20 strains of *E. coli* O157:H7 and one O116:H31 strain. This dendrogram was analyzed in parallel with 9 standard strains of *E. coli* O157:H7 isolated from the past. All 30 strains could be classified to be 11 subgroups (subgroup I to XI) at 80% similarity. Thai and Malaysian *E. coli* O157:H7 showed the relatedness at subgroup IX, suggesting the close relationship between Thai and Malaysian *E. coli* O157:H7 strains.

1.2 Isolation of high concentration of Shiga toxin-producing *E. coli* from Thai beef

In Thailand, infection due to Shiga toxin-producing *E. coli* STEC is very rare (Bettelheim et al., 1990; Kalnauwakul et al., 2007). Therefore, to confirm that most of STEC isolates in Thailand are Shiga toxin non-producing *E. coli*. An extra 10 Thai beef samples were collected from various markets in Hat Yai city. The samples were processed using the same methods as described in section 1.1.1, Chapter Research Methodology. In all 10 samples, 163 mauve colonies were selected and characterized.

A total of 6 stx^+ *E. coli* were detected in 4 out of 10 beef samples (40%) (Table 8). One isolate (0.6%) possessed stx_1 alone (designated as PSU 5023). Five isolates (3.1%) (designated as PSU 5026-5030) harbored only stx_2 gene. The *eae* gene was demonstrated in 4 out of 6 strains (66.7%) (Table 8). Serotyping using O157 antiserum indicated that 4 out of 6 strains were O157 and the rest of 2 isolates were non-O157 (PSU 5023 and PSU 5030). TNP-PCR was used to assess the ability of the test strains in producing Stx. It was shown that 4 stx_2^+ isolates (PSU 5026-5029) were positive, indicating that they were unable to produce toxin. However, PSU 5023 and PSU 5030 showed TNP-PCR negative (Table 8). The direct detection of Stx production was performed by RPLA assay. The results showed that PSU 5023 could produce a high concentration of Stx1 (titer = 1: 2,048), while PSU 5026-5030 could not or produced in an undetectable level (titer < 1: 2).

Antibiogram pattern using 8 antibiotic disks [Ceftrizone (30 μg), trimethoprim/sulfamethoxazole (1.25 μg), norfloxacin (10 μg), amikacin (30 μg), gentamicin (10 μg), Ciprofloxacin (5 μg), Ceftazidime (30 μg), imipenam (10 μg)] illustrated that all strains were susceptible to all antibiotics tested (Table 8).

Since PSU 5023 produced Stx1 in high amount, characterization of antiterminator Q protein of PSU 5023 was performed by PCR using primer pairs specific to *Q* gene of bacteriophage 933W (Q_{933}) and bacteriophage Thai-12 (Q_{21}). A 567 bp of Q_{933} gene amplicon was detected in PSU 5023 strain, suggesting that PSU 5023 might have *Q* gene similar to phage 933W (Fig. 15).

To determine whether the stx_1^+ PSU 5023 strain originated from a neighboring country, the DNA profile of this strain was compared to $stx_1^+stx_2^+$ *E. coli* derived from Malaysia (M7) using the AP-PCR technique. However the DNA profile of PSU 5023 was different from the DNA profile of the Malaysian strain and both of them were different from the DNA profiles of *E. coli* EDL933 and Thai-12 strains (Fig. 16). This indicated that their origins were different.

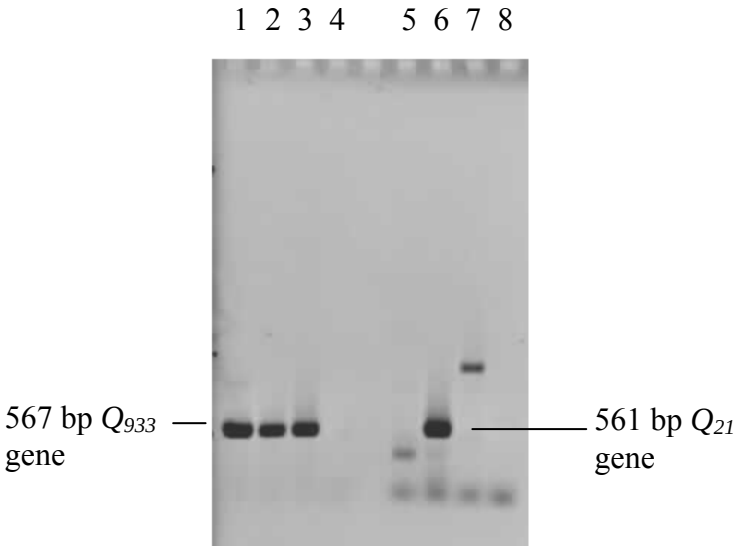

Figure 15 PCR amplification of the *Q* gene region using the EDL933 *Q* gene, lane 1-4 and the Thai-12 (Q_{21}) gene primer pair, lanes 5-8. Lane 1, 5: *E. coli* O157:H7 EDL933; lane 2, 6: *E. coli* O157:H7 Thai-12; lane 3, 7: Stx1-producing *E. coli* PSU 5023; lane 4, 8: stx_2^+ *E. coli* PSU 5030. The bands which are lower and upper the *Q* gene are non-specific amplification (lane 5 and 7).

Table 8 Characterization of Shiga toxin-producing *E. coli* isolates from Thai beef samples

Sample no.	No. of positive isolates (sample strain)	O157/ non-O157	Virulence genes			Antibiogram pattern	TNP-PCR	RPLA (titer)
			stx$_1$	*stx*$_2$	*eae*			
5	1 (PSU 5023)	0/1	+	-	-	[a]S	-	2,048
6	1 (PSU 5026)	1/0	-	+	+	S	+	< 2
7	3 (PSU 5027-5029)	3/0	-	+	+	S	+	< 2
9	1 (PSU 5030)	0/1	-	+	-	S	-	< 2

[a]S, Susceptible to all antibiotics tested

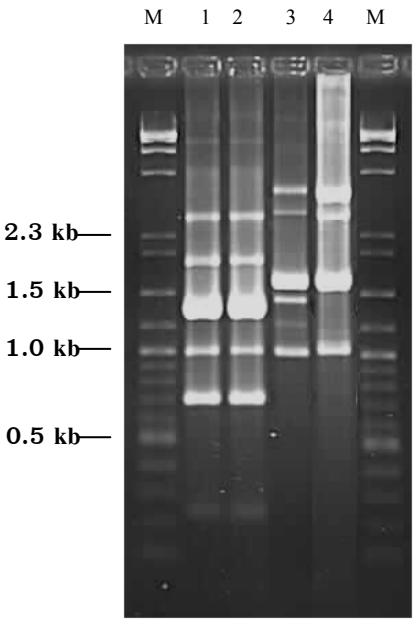

Figure 16 AP-PCR analysis of Thai stx_1^+ *E. coli* PSU 5023 and Malaysian, stx_1^+ stx_2^+ *E. coli* Lane M, mixture of λ/ Hind III and 100 bp ladders; lane 1: *E. coli* O157:H7 EDL933; lane 2: *E. coli* O157:H7 Thai-12; Lane 3: PSU 5023; lane 4: Malaysian strain

Part II Determination of *V. parahaemolyticus* in Malaysian and Thai molluscan shellfish

2.1. Characteristics of *V. parahaemolyticus* isolated from molluscan shellfish

A total of 24 molluscan shellfish samples including bloody clam (Thai and Malaysian bloody clam), green mussel, and oriental hard clam, were investigated for *V. parahaemolyticus*. Green mussel, and oriental hard clam were harvested in Thailand and were included in this study for comparison of *V. parahaemolyticus* pandemic strains. Using immunomagnetic separation technique with K6 and K25 antisera against capsular antigen of pandemic strains, a total of 802 suspected colonies on CHROMagar Vibrio were obtained and were re-investigated by agglutination with K6 and K25 antisera (Table 9). Forty two colonies were agglutinated with either anti-

K6 or anti-K25 antisera and all of them were *V. parahaemolyticus* after confirmation by PCR targeted to the *toxR* gene. To investigate whether they were pandemic strains, virulence genes (*tdh* and *trh*), and pandemic markers (GS-PCR, 23 kb insertion sequence and 16 kb insertion sequence) were determined by PCR. It was found that *V. parahaemolyticus* pandemic strains were detected in 6 out of 24 samples (25.0%) because only 15 isolates were positive for *tdh*, GS-PCR and 16 kb inserted sequence (Table 9 & 10).

All of them were serotype O3:K6 in which 3, 1, 8 and 3 isolates were obtained from Thai bloody clam, Malaysian bloody clam, green mussel and oriental hard clam respectively (Table 9). Although 9 isolates were positive for GS-PCR and serotype O3:K6 (Table 10), they were not classified as pandemic strain because *tdh* virulence gene was not observed in these isolates.

Table 9 Molluscan shellfish samples which pandemic strains were isolated

Molluscan shellfish sample sets			No. of colonies		
Species	No. of examined/ no. of positive samples	Antiserum used in IMS	No. of examined	No. of agglutinated[a]	No. of pandemic strains
Bloody clam (Thai)	7/3	Anti-K6	172	7	3
		Anti-K25	129	4	0
Bloody clam (Malaysian)	7/1	Anti-K6	167	10	1
		Anti-K25	195	4	0
Green mussel	5/1	Anti-K6	35	8	8
		Anti-K25	73	2	0
Hard clam	5/1	Anti-K6	21	5	3
		Anti-K25	10	2	0
Total	24/6		802	42	15

[a]colony re-agglutination with anti-K6 and anti-K25 antibodies

Table 10 Characteristics of K6 and K25 strains isolated in this study

Serotype	Results of PCR					Molluscan Bivalve sample[a]	No. of isolated strains
	GS-PCR	*tdh*	*trh*	23kb insertion sequence	16kb insertion sequence		
O3:K6	+	+	-	+	+	GM	8
	+	+	-	+	+	HC	3
	+	+	-	+	+	BC(T)	2
	+	+	-	+	+	BC(M)	1
	+	+	-	-	+	BC(T)	1
	+	-	-	-	-	BC(T)	1
	+	-	-	-	-	BC(M)	6
	+	-	-	-	-	HC	2
	-	-	-	Not detected	Not detected	BC(T)	3
	-	-	-	Not detected	Not detected	BC(M)	3
O1:K25	-	-	-	Not detected	Not detected	BC(T)	4
	-	-	-	Not detected	Not detected	BC(M)	2
	-	-	-	Not detected	Not detected	GM	2
	-	-	-	Not detected	Not detected	HC	2
OR:K25	-	-	-	Not detected	Not detected	GM	2
							Total 42

[a]GM,Green mussel; HC, Hard clam; BC, Bloody clam; T, Thai; M, Malaysian

2.2 DNA fingerprinting by AP-PCR

To investigate correlation of *V. parahaemolyticus* pandemic strains obtained from Thai and Malaysian molluscan shellfish, AP-PCR technique was performed. A total of 15 pandemic strains were determined (Table 11). DNA profiles of all *V. parahaemolyticus* isolates (PSU 4056/1-8) from a green mussel were identical indicated that these pandemic strains were originated from the same clone

(Fig. 17). Two identical DNA profiles were demonstrated in 3 isolates (PSU 4070/1-3) of *V. parahaemolyticus* from an oriental hard clam, suggesting that this shellfish sample harbored 2 different clones of pandemic strains. In addition, 2 different clones of pandemic strains were also detected in isolates from a Thai bloody clam (PSU4063/1-2) because non-identical DNA profiles were detected. DNA profiles of the rest pandemic strains (one from Malaysian isolates [PSU 4067] and another from Thai isolate [PSU 4041]) were different (Fig. 17). Interestingly, DNA profile of PSU 4063/2 obtained from Thai bloody clam was identical to DNA profile of PSU 4067 obtained from Malaysian bloody clam.

Table 11 The relationship of pandemic *V. parahaemolyticus* strains analyzed by AP-PCR

Strain (PSU)	Serotype	Source
4056/1	O3:K6	Green mussel
4056/2	O3:K6	Green mussel
4056/3	O3:K6	Green mussel
4056/4	O3:K6	Green mussel
4056/5	O3:K6	Green mussel
4056/6	O3:K6	Green mussel
4056/7	O3:K6	Green mussel
4056/8	O3:K6	Green mussel
4070/1	O3:K6	Hard clam
4070/2	O3:K6	Hard clam
4070/3	O3:K6	Hard clam
4063/1	O3:K6	Bloody clam (Thai)
4063/2	O3:K6	Bloody clam (Thai)
4067	O3:K6	Bloody clam (Malaysian)
4041	O3:K6	Bloody clam (Thai)

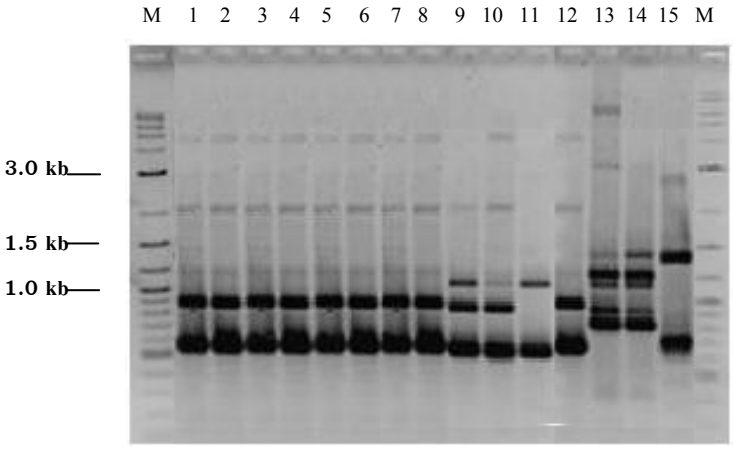

Figure 17 AP-PCR analysis of pandemic strains of *V. parahaemolyticus* from Thai and Malaysian shellfish samples. The strains were tested by primer 2. Lane: M, 2 log DNA ladder; lane 1, PSU 4056/1; lane 2. PSU 4056/2; lane 3, PSU 4056/3; lane 4, PSU 4056/4; lane 5, PSU 4056/5; lane 6, PSU 4056/6; lane 7, PSU 4056/7; lane 8, PSU 4056/8; lane 9 ; PSU 4070/1; lane 10, PSU 4070/2; lane 11, PSU 4070/3 ; lane 12, PSU 4063/1; lane 13, PSU 4063/2; lane 14, PSU 4067 ; lane 15, PSU 4041.

2.3 DNA fingerprinting by PFGE

PFGE was performed to confirm correlation between some pandemic strains of *V. parahaemolyticus* from Thai and Malaysian clinical and environmental samples (isolates from 1998-2008). Some non-pandemic strains of *V. parahaemolyticus* from Hong Kong (2005), Thailand (2005), and U.S.A. (1997 and 2003) were included for comparison (Table 12 and Fig. 18).

A total of 23 *V. parahaemolyticus* strains were analyzed by PFGE using *Not* I and *Sfi* I restriction endonucleases. They were 17 pandemic strains (tdh^+ and GS-PCR positive), and 6 non-pandemic strains (tdh^- and GS-PCR negative, tdh^+ and GS-PCR negative and tdh^- and GS-PCR positive) (Table 12). Twenty distinguishable DNA profiles were demonstrated and they were classified into 6 groups based on 80% similarity of DNA profile (group I to VI) (Fig. 18). All Thai and Malaysian *V. parahaemolyticus* pandemic strains isolated from clinical and

environmental samples between 1998 and 2008 were in group IV. Interestingly, within this group DNA profiles of four isolates obtained in 2001 and 2008, one Thai clinical isolates (PSU 2050) and two Thai environmental isolates (PSU 4063/2 and PSU 474) and one Malaysian isolate (PSU 4067), were identical. This indicated that they were derived from the same origin, although two Thai isolates were from bloody clam and green mussel, respectively, and one Malaysian isolate was from bloody clam. DNA profiles of the rest pandemic strains (13 isolates) were 83-98% similarity.

DNA profile of one environmental non-pandemic isolate obtained in 2008 from Thailand (*tdh*$^-$ and GS-PCR positive, O3:K6-PSU4059) was 81% similar to environmental non-pandemic isolate obtained in the same year from Malaysia (*tdh*$^-$ and GS-PCR positive, O3:K6-PSU4049). In addition, each DNA profile of isolate obtained in 2005 from Thailand (*tdh*$^-$ and GS-PCR negative, O2:K28-VPS27) and Hong Kong (*tdh*$^+$ and GS-PCR negative, O4:K8- VPS10) was 69% similarity. Although DNA profiles of *V. parahaemolyticus* isolates from U.S.A. were closely related, they were different from all Asian both pandemic and non-pandemic strains.

Table 12 *V. parahaemolyticus* strains used to campare with the current strains

Strains	Sources	Country	Year of isolation	Serotype	Virulence gene		GS-PCR
					tdh	*trh*	
PSU 4063/2	*E (BC)	Thailand	2008	O3:K6	+	-	+
PSU 4059	E (HC)	Thailand	2008	O3:K6	-	-	+
PSU 4049	E (BC)	Malaysia	2008	O3:K6	-	-	+
PSU 4067	E (BC)	Malaysia	2008	O3:K6	+	-	+
VPS27	-	Thailand	2005	O2:K28	-	-	-
PSU 474	E (GM)	Thailand	2001	O3:K6	+	-	+
PSU 435	E (GM)	Thailand	2001	O3:K6	+	-	+
PSU 358	E (HC)	Thailand	2001	O3:K6	+	-	+
PSU 228	E (GM)	Thailand	2000	O3:K6	+	-	+
PSU 46	E (BC)	Thailand	1998	O3:K6	+	-	+
PSU 2050	*C	Thailand	2008	O3:K6	+	-	+
PSU 3864	C	Thailand	2008	O3:K6	+	-	+
2643	C	Thailand	2006	O3:K6	+	-	+
26	C	Thailand	2005	O3:K6	+	-	+
197	C	Thailand	2001	O3:K6	+	-	+
TCVP70	C	Thailand	1999	O3:K6	+	-	+
VP47	C	Thailand	1998	O3:K6	+	-	+
VP54	C	Malaysia	2005	O3:K6	+	-	+
48541	C	Malaysia	2001	O3:K6	+	-	+
VP6	C	Malaysia	1999	O3:K6	+	-	+
VPS10	-	Hong Kong	2005	O4:K8	+	-	-
VPS35	-	U.S.A.	2003	O6:K18	-	-	-
VPS31	-	U.S.A.	1997	O4:K12	+	+	-

* C, Clinical; E, Environmental

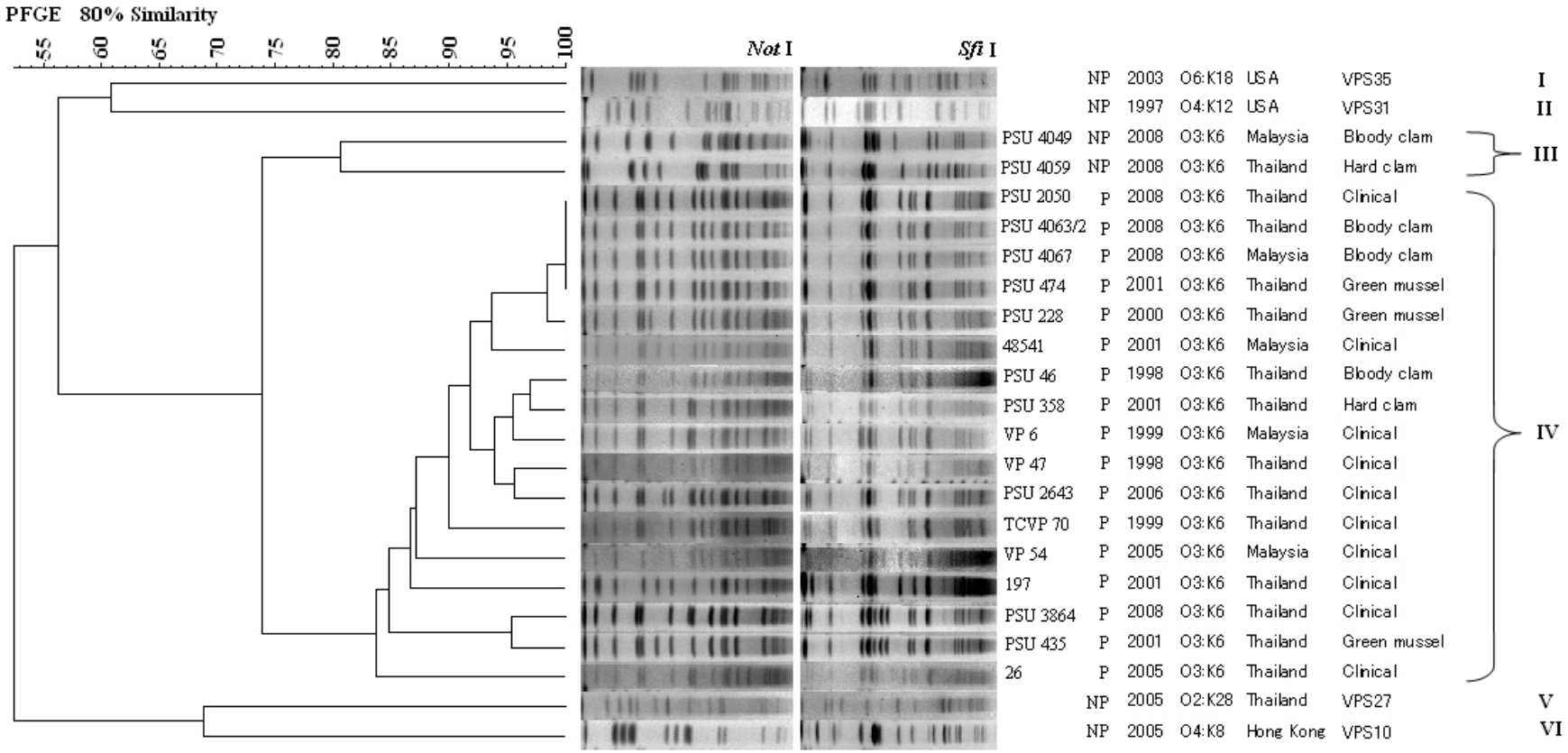

Figure 18 A dendrogram generated from PFGE profile of 23 strains of *V. parahaemolyticus*. P, Pandenic strain; NP, Non-pandemic strain

CHAPTER 4

DISCUSSION

Part I Transfer of *Escherichia coli* O157:H7 from Thai-Malaysian border through beef trade

E. coli O157:H7 was isolated from eight (25.8%) of 31 samples of Malaysian beef exported to Thailand and four (11.1%) of 36 samples of Thai beef. The isolated *E. coli* O157:H7 strains were 20 in total and 19 harbored the *stx*$_2$ and *eae* genes (Table 3). The successful isolation of these O157 strains from food can be attributable, in part, to the isolation procedure including an immunomagnetic separation technique followed by selection using the CHROMagar O157 medium. These results suggested that the sanitary conditions in slaughter houses in Malaysia, and Thailand were not good and resulted in contamination of beef with *E. coli* O157:H7. Thus, traded beef can be a vehicle for the international transmission of *E. coli* O157:H7.

IS*629* is most frequently detected in *E. coli* O157 Sakai strain (Ooka et al. 2009a). O157 IS-printing is a simple and rapid technique for the detection and differentiation of *E. coli* O157:H7 (Ooka et al. 2009b). The technique is based on the variability in the genomic location of IS*629* among the *E. coli* O157:H7 strains. Two sets of primers were designed, and each set generates polymorphisms at 16 loci of IS*629* in the chromosome and plasmid of *E. coli* O157:H7. This technique is useful because primers for specific detection of the *eae* and *hlyA* genes can also be included in the first set of primers, and primers for detection of the *stx*$_1$ and *stx*$_2$ genes can be added to the second set of primers (Table 2). Thus, they are used to confirm genotypes of *E. coli* O157:H7 strains. In this study, the DNA profiles of all 20 strains of *E. coli* O157:H7 isolates including *E. coli* serotype O116:H31 from Thai and Malaysian beef and reference strains were determined by O157 IS-printing (Table 5 & 7). The O157 IS-printing generated seven DNA profile groups. The DNA profiles of all *E. coli* O157:H7 isolates including the reference strains were closely related (65 -

70% similarity) and were classified into groups I – VI, whereas the DNA profile of *E. coli* O116:H31 (strain M7) was classified into group VII that showed only about 25% similarity to the O157 strain (Fig. 12). This suggested that O157 IS-printing specifically differentiated *E. coli* O157 from other *E. coli* strains. All DNA profiles of the TNP-PCR-positive O157 isolates from Asia (groups I - V) were similar and they were distinguishable from a TNP-PCR-negative strain isolated in the United States (EDL933), indicating that these *E. coli* O157 strains may be prevalent and transferred among Asian countries (Fig. 12).

In this study, DNA fingerprints of the test strains were also analyzed using the PFGE method. The result was consistent with that of O157 IS-printing. However, the PFGE method exhibited higher discriminatory power than O157 IS-printing. The *E. coli* O157:H7 isolates from Malaysian beef were classified into six groups by the PFGE method, whereas they were separated into only two groups by O157 IS-printing (Figs. 12 & 14). In addition, PFGE could classify eight test strains into three clusters: three Malaysian isolates (M11, M12 and M13) in group III; three Thai isolates (T2, T3 and T6), in group IX; and two Thai control strains (Thai 1 and Thai 13) in group VIII (Fig. 14), whereas IS-printing failed to differentiate these eight strains (Fig. 12). The PFGE method had a higher resolution than O157 IS-printing because DNA profiles were generated from the entire genome of the tested organisms, but DNA profiles from O157 IS-printing were created from only IS*629* which clustered in the 1.4 Mb specific sequences of Sakai O157 strain (Ooka et al. 2009a).

As described above, DNA profiles of the Malaysian strains were diversified. This may be because beef was imported from different sources in Malaysia to maintain a constant supply to a large Muslim population in southern Thailand. The PFGE analysis demonstrated a wide range of similarity (65 -85%) among the Malaysian and Thai isolates (Fig. 14). Of these strains M11, M12 and M13, isolated from Malaysian beef, and Thai 12, isolated previously from bovine feces in Thailand (a reference strain), were classified into group III. Two Malaysian strains, M14 and M15, and all Thai strains (T1 - T6) isolated in the present study were classified into group IX. The close relationships between particular Malaysian and Thai strains suggested a possible transfer of *E. coli* O157:H7 from Malaysia to Thailand.

The antibiograms of two strains of *stx*$_2^+$ *eae*$^+$ *E. coli* O157:H7, M14 from Malaysia and T3 from Thailand, were identical (Table 6). This phenotypic observation supported the result of the DNA fingerprinting analysis, suggesting that these two strains were closely related because they were classified in the same group by O157 IS-printing (Fig. 12, group III) and PFGE (Fig. 14, group IX). Koitabashi et al. (2006) showed that most of *E. coli* O157:H7 isolated from Asia produced no or low levels of Stx2 because they had a unique *Q* gene which produced a weak antitermination Q protein. In addition, they also had a defect of their *stx*$_2$ promoter causing little or no production of Stx2. Those strains were named as Stx2-negative strains. The results of TNP-PCR and RPLA indicated that all of the *E. coli* O157:H7 strains isolated from Thai and Malaysian beef in the present study were assigned to the Stx2-negative group (Table 4). However, the production of Stx2 *in vivo* is needed to confirm that these strains were not harmful to humans.

One strain of *E. coli* serotype O116:H31 obtained from Malaysian beef (M7) harbored *stx*$_1$ and *stx*$_2$ genes but lacked of *eae*. Thus, this strain was classified as STEC and was found to be the most distantly related to another *E. coli* strains in this study either by IS-printing system or by PFGE. This may suggest the relationship among *E. coli* O157:H7 strains.

In conclusion, the data obtained in this study suggested that there has been a beef trade-mediated transfer of *stx*$_2^+$ *eae*$^+$ *E. coli* O157:H7 from Malaysia to Thailand. Although the *E. coli* O157:H7 strains isolated in this study were of the Stx2-negative type, some of the Stx2-negative strains were reported to produce low levels of Stx2 in vitro (Koitabashi et al. 2006). Trans-border transfer of food contaminated by pathogens between Malaysian and Thai is a public health concern.

Although Stx2-negative strains are preference in Asia (Koitabashi et al. 2006) including the *E. coli* O157:H7 strains obtained in this study, Shiga toxin-producing *E. coli* is believed to be existed and contaminated in beef. In order to demonstrate STEC that has an ability to produce a high level of toxin from domestic beef in southern Thailand, ten beef samples obtained from various markets in Hat Yai were investigated. Six strains of *stx*$^+$ *E. coli* were isolated from these domestic beef. Five strains produced no Stx or produced in an undetectable level. However, one strain of non-O157 *E. coli* (designated as PSU 5023) possessed *stx*$_1$ gene and was

capable of producing high amount of Stx1 (titer = 1: 2,048). This strain was TNP-PCR negative which indicated that its *Q* gene was different from other Stx2-negative strains. Therefore, an investigation of the *Q* gene of PSU 5023 was performed by PCR. This strain was positive for Q_{933} and exhibited a 567 bp amplicon size but was negative for Q_{21} (Fig. 15).

In *E. coli* O157:H7, stx_2 was located downstream of the *Q* gene of 933W phage (Plunkett et al., 1999). Koitabashi and colleagues (2006) demonstrated that *Q* gene of Thai O157:H7 strains were homologous to *Q* gene of Φ21 but not to the 933W phage and this caused most of Thai O157 strains unable to produce Stx2. Katsushi and colleagues (2000) also demonstrated that in Sakai strain stx_1 gene was located downstream of *Q* gene in the prophage genome, suggesting that production of Stx1 was regulated by *Q* gene. Thus, the presence of Q_{933} in PSU 5023 might affect stx_1 gene and caused high production of Stx1. Further study by deletion of Q_{933} and determination of Stx1 production may clarify this.

Koitabashi and colleagues (2006) showed that *E. coli* O157:H7 strain 98-16 isolated from patient in Japan produced Stx1 in titer of 32 and *E. coli* O157:H^- strains Thai-1 and Thai-13, which carried a combination of stx_1 and stx_2, produced Stx1 in titer of 128 and 64, respectively. The level of Stx1 toxin produced by strain PSU 5023 in this study is 16 to 64-fold higher than those strains obtained from Japan and Thailand which indicates that PSU 5023 may be extremely hazardous to human.

Most of the non-O157 strains are often overlooked in clinical microbiology laboratories because most laboratories use sorbitol MacConkey agar and serotyping to identify *E. coli* O157:H7. In this study, we employed an immunomagnetic separation technique using anti-O157 antiserum to separate EHEC O157 and accidentally obtained this Stx1-producing *E. coli* strain from beef because the colony of this non-O157 on CHROMagar was not different from other EHEC O157 strains. Thus, non-O157 STEC pathogenic strains may be present in the Thai environment but as they produce only a mild infection (Hashimoto et al., 1999), they are overlooked by microbiologists, and are therefore, not recognized.

In this study, all *E. coli* isolates showed susceptibility to all antibiotics employed, so it was unlikely that they had a human origin.

Part II Transfer of *V. parahaemolyticus* from Malaysia to Thailand through imported seafood

Since the emergence of pandemic strain of *V. parahaemolyticus* in Calcutta, India in 1996, several infections due to pandemic *V. parahaemolyticus* have been reported worldwide (Daniels et al. 1998; Chowdhury et al. 2000; Vuddhakul et al. 2000; Wootipoom et al. 2007; Bhoopong et al. 2007; Ansaruzzaman et al. 2008). *V. parahaemolyticus* is an important food-borne pathogen causing gastroenteritis in southern Thailand (Vuddhakul et al. 2000; Wootipoom et al. 2007). However, information of predominant of these bacteria in this area has not been clearly understood. It was found that some molluscan shellfish harboured pathogenic *V. parahaemolyticus* strains. Since bloody clam, a kind of mollusk, has been imported from Malaysia to southern Thailand. In this study, domestic and imported bloody clam from Malaysia were investigated for *V. parahaemolyticus* using immunomagnetic separation specific to K6 and K25. Green mussel and oriental hard clam were included for comparison. In addition, they were sold nearby bloody clam and cross-contamination might occur. The percentage of pandemic *V. parahaemolyticus* isolated from shellfish in this study was 1.9% (15 out of 802 isolates) (Table 9 & 10)

In this study, although 8 isolates of *V. parahaemolyticus* obtained from green mussel, AP-PCR indicated that they were homogenous or identical strains (Fig. 17; lane 1 to 8). However, the pandemic strains (1-3 isolates) obtained from hard clam, Thai and Malaysian bloody clam showed distinct DNA fingerprintings (Fig. 17). This suggested that different strains of pandemic *V. parahaemolyticus* exist in Thai environment.

DNA fingerprinting generated by PFGE clearly elucidated correlation of *V. parahaemolyticus* isolates from Malaysia and Thailand. Pandemic *V. parahaemolyticus* PSU 2050 isolated from a clinical sample of Thailand, PSU 474 isolated from Thai green mussel and PSU 4063/2 isolated from Thai bloody clam were identical to *V. parahaemolyticus* PSU 4067 isolated from Malaysian bloody clam. Non-pandemic *V. parahaemolyticus*, PSU 4049 isolated from Malaysian bloody

clam was 81% similarity to *V. parahaemolyticus* PSU 4059 isolated from Thai hard clam.

AP-PCR is a useful method in comparison of the relationship among *V. parahaemolyticus* pandemic strains (Okuda et al. 1997). Ansaruzzaman and colleagues (2008) compared pandemic strains of *V. parahaemolyticus* serotype O3:K6 and serotype O4:K68 isolated in Mozambique between 2004 and 2005 using AP-PCR, PFGE, and Multi locus sequence typing (MLST) analysis. It was found that all O3:K6 and O4:K68 pandemic strains provided identical AP-PCR pattern. In contrast, PFGE was capable to differentiate those strains. This suggested the high resolution power of PFGE. PFGE is the most reliable tool for analysis diversity of bacterial strains (Kam et al. 2008). In this study, PFGE analysis showed high relatedness (>80% similarity) in O3:K6 pandemic group isolated from Thailand and Malaysia even they were isolated at different time. However, DNA profiles of pandemic strains were different from DNA profiles of non-pandemic O3:K6 strains, PSU 4049 and PSU 4059 (GS-PCR positive, *tdh* negative, *trh* negative), including non-pandemic of other serotype strains (O2:K28 from Thailand, O4:K8 from Hong Kong, O4:K12 and O6:K18 from U.S.A.). This indicated the unique clone of pandemic strains and confirmed that pandemic strains of *V. parahaemolyticus* have been derived from the same origin.

Pandemic *V. parahaemolyticus* was first demonstrated in patients in southern Thailand in 1998 (Vuddhakul et al. 2000). Later on, Wootipoom and colleagues (2007), reported infections by pandemic *V. parahaemolyticus,* in Hat Yai hospital during 2000 to 2003 were between 64.1 and 69.7%. In this study, *V. parahaemolyticus* PSU 474 and PSU 4063/2 which were isolated from Thai shellfish, 2001 and 2008, and PSU 4067 which was isolated from shellfish imported from Malaysia in 2008 showed indistinguishable PFGE fingerprinting pattern with PSU 2050 which was the clinical isolates in the same year. These indicated the possibility of pandemic strain of *V. parahaemolyticus* transferred from Malaysia to southern Thailand through imported bloody clam.

From this study, it is concluded that an STEC isolate can produce toxin and it is not serotype O157, thus prevalence of STEC other than O157 should be investigated to clarify the incidence of pathogenic STEC in this area and other parts of Thailand. In addition, the role of Q_{933} gene in Stx1 production should be confirmed by

deletion of this gene and investigation of Stx1 production in mutant strain. Furthermore, pathogenicity of this strain in animal should be demonstrated to confirm its virulence in human.

In this study, it was also clearly that pathogenic *V. parahaemolyticus* were transferred from Malaysia to Thailand through seafood. Therefore, the seafood should be strictly and frequently examined and the public health warning should be issued for prevention and control of this organism that would endanger Thai population.

CHAPTER 5

CONCLUSIONS

E. coli O157:H7 and *V. parahaemolyticus* are important enteric bacteria that usually infect people through the consumption of beef and seafood, respectively. *E. coli* O157:H7 causes hemorrhagic colitis and hemolytic uremic syndrome and the most important virulence factor is Shiga toxin (Stx) whereas *V. parahaemolyticus* causes gastroenteritis and its virulence factors are thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH). Southern part of Thailand shares the border with Malaysia, and many kinds of foods which may be pathogens vehicle have been transported through this border. In this study, we demonstrated that both *E. coli* O157 and pandemic strains *V. parahaemolyticus* were contaminated in imported beef and bloody clam from Malaysia because we could isolate 13 strains of *E. coli* O157 harbor stx_2 gene from Malaysian beef. Using IS-printing and PFGE analysis, most Malaysian and Thai *E. coli* O157:H7 strains were more than 80% similarity. Thus, it is possible that these *E. coli* strains may have been transferred and existed in this area for a period of time.

In addition, we were able to isolate one STEC that produced high amount of Stx1 (titer = 1: 2,046) from Thai beef. Moreover, we showed that this high production of Stx1 may be due to the presence of Q_{933} in this bacterial strain. This information has never been reported in Thailand and it is important for public health warning.

Finally, we could also isolate pandemic strains of *V. parahaemolyticus* from bloody clam imported from Malaysia and found that DNA profile of one Malaysian isolate (PSU 4067) obtained in 2008 was identical to DNA profiles of one Thai clinical isolates (PSU 2050). This indicates that people in this area are likely to be infected with pandemic *V. parahaemolyticus* by consuming Malaysian bloody clam.

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APPENDIX

Part I Culture media

1.1 Alkaline peptone water (APW)

Peptone (Difco)	10	g
NaCl	10	g
dH_2O	900	ml

Adjust pH to 8.6 and bring the volume to 1,000 ml. Sterilize by autoclaving at 121ºC, 15 ppi for 15 min.

1.2 Tryptic soy broth

Dissolve 30 g of Tryptic soy broth powder in 1000 ml of distilled water. Boil the mixture 1-2 min before autoclaving at 121ºC, 15 ppi for 15 min.

1.3 Chromagar Vibrio

Dissolve 74.7 g of CHROMagar Vibrio powder in 1,000 ml of distilled water. Boil the mixture 1-2 min before plating in steriled petri-dish. The agar plates are kept in dark place.

1.4 Chromagar O157

Dissolve 29.2 g of CHROMagar O157 powder in 1,000 ml of distilled water. Boil the mixture 1-2 min before plating in steriled petri-dish. The agar plates are kept in the dark place.

1.5 CA-YE medium (1L)

Casamino acids	20	g
Yeast extract	6	g
NaCl	2.5	g
K_2HPO_4	8.71	g
Trace salt mixture	1	ml

Trace salt mixture

5% $MgSO_4$

0.5% $MnCl_2$

0.5% $FeCl_3$

Dissolve the salts above in 0.001 N H_2SO_4 (0.0005 M)

1.6 Luria-Bertani (LB)

Tryptone	10	g
Yeast-extract	5	g
NaCl	5	g

Bring the volume to 1,000 ml by distilled water. Sterilize by autoclaving at 121ºC, 15 ppi for 15 min.

1.7 One percent PFGE-grade agarose

-Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 or 15 wells)

-Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (≥ 15 wells)

Part II Buffer

2.1 One molar Sodium phosphate buffer at 25ºC

pH	Volume of 1 M Na_2HPO_4 (ml)	Volume of 1 M NaH_2PO_4(ml)
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
8.0	93.2	6.8

Sterilized by autoclaving at 121ºC, 15 ppi for 15 min.

2.2 1M EDTA, pH8.0

Dissolve 186.12 g of Na_2EDTA in 800-900 ml of distilled water. Adjust pH to 8.0 with 10N NaOH. Add distilled water to make 1 liter. Sterilize by autoclaving at 121ºC, 15 ppi for 15 min.

2.3 Cell suspension buffer for PFGE (100mM Tris; 100mM EDTA, pH8.0)

- 10ml of 1 M Tris, pH8.0
- 20ml of 0.5 M EDTA, pH8.0

Dilute to 100 ml with sterile ultrapure water (milliQ)

2.4 Cell lysis buffer for PFGE (50mM Tris; 50mM EDTA, pH8.0 + 1% Sarcosyl)

- 25ml of 1 M Tris, pH8.0
- 50ml of 0.5 M EDTA, pH8.0
- 50ml of 10% Sarcosyl (N-Lauroylsarcosine, Sodium salt)

Dilute to 500 ml with sterile ultrapure water (milliQ)

2.5 Washing buffer for PFGE (TE buffer; 10mM Tris; 1mM EDTA, pH8.0)

- 10ml of 1 M Tris, pH8.0
- 2ml of 0.5 M EDTA, pH8.0

Dilute to 1000 ml with sterile ultrapure water (milliQ)

2.6 6X DNA loading buffer (0.25% bromophenol blue and 4.0% (w/v) sucrose)

Weigh 25 mg bromophenol blue and 4 g of glucose. Bring the volume to 10 ml with distilled water. Stored at 4ºC.

2.7 Tris-borate-EDTA (TBE), 10X

This buffer is composed by;

Tris base	108	g
Boric acid	55	g
Sterile distilled water	800	ml
0.5 M EDTA, pH 8.0	40	ml
Sterile distilled water to	1,000	ml

Part III Antibiotics and enzyme preparation

3.1 Novobiocin

Novobiocin powder was dissolved in 1 ml of sterile distilled water at a concentration of 100 mg/ml. The solution was divided into small aliquots and kept at -20°C until used.

3.2 Proteinase K (20mg/ml)

Purchase as a lyophilized powder and dissolved at a concentration of 20 mg/ml in sterile 50 mM Tris (pH8.0), 1.5 mM calcium acetate. Divide the stock

solution into small aliquots and store at -20ºC. Each aliquot can be thawed and refrozen several times but should then be discarded. Unlike much cruder preparation of protease (e.g., pronase), protenase K need not be self-digested before use.

3.3 RNase A (100 mg/ml)

Mix 1 g of RNase A with 10 ml of 10 mM Tris pH 7.5, 15 mM NaCl. Heat to 100ºC for 15 min. Allow the solution to be cooled down to room temperature (overnight). Store at -20ºC.

Part IV Protocol for Pulsed-field gel electrophoresis (Fine description)

PFGE protocol for *E. coli* O157:H7 and *V. parahaemolyticus*

<u>Day 1 Preparation and lysis of the plug</u>

Scrape bacteria from -80ºC stock and inoculated on L-agar and TCBS agar for *E. coli* O157:H7 and *V. parahaemolyticus*, respectively.

↓

Inoculated single colony into 5 ml L-broth or 10 ml TSB+2% NaCl for *E. coli* O157:H7 and *V. parahaemolyticus*, respectively.

↓

Incubate bacteria at 37ºC overnight without shaking

↓

Take 500 µl of overnight culture to 1.5 ml tube and centrifuge at 11,000 x g for 5 min to harvest cells

↓

Discard supernatant and then add 150 µl cell suspension buffer

↓

Resuspend bacterial cells by gently pipetting up and down

↓

Incubate at 50 ºC* so that it can be mixed with 150 µl of 1% Seakem Gold agarose (embedding agarose) prepared by TE buffer

↓

Bacterial cells-agarose mixture is rapidly loaded into plug mold (Bubbles are not allowed to be formed)

↓

Waiting for agarose solidification for 15 min

↓

In the meantime, Proteinase K solution will be carried out by mixing 500 µl cell lysis buffer and 20 µl of Proteinase K (20 mg/ml). If 20 samples will be carried out, so multiply the volumn of either cell lysis buffer or Proteinase K by 21.

↓

Take the plug from plug mold carefully by thin-wide tip spatula and completely sink the plug in Proteinase K solution in 2 ml tube.

↓

Incubate the tube at 50 ºC water bath overnight

*Do not incubate bacterial cell at 50 ºC over 15 min, preventing cell lysis.

Day 2 Washing the plug

After lysis by Proteinase K, bring the plug into new 2 ml tube containing 1 ml TE buffer for washing step

↓

Wash with TE buffer for 3 times, 30 min in each time without shaking

↓

After 3 times washing, add TE buffer for the forth time and can be kept at 4ºC for up to 6 months or until used

Day 3 Restriction digestion of the plug slice

Take the plug from 4 ºC and place the plug on sterile petri-dish

↓

Use clean-sharp spatula or cover slip to slice the plug to be 5x3 mm (LxW) (the long size of the plug slice should be equal to the long side of the comb)

↓

Put the plug slice into 2 ml tube containing 500 µl of 1X *Xba* I buffer of *Not* I buffer for *E. coli* and *V. parahaemolyticus*, respectively(M buffer for *E. co*li and H buffer for *V. parahaemolyticus* , TOYOBO)

↓

Incubate for 60 min at room temperature

↓

Discard 1X *Xba* I buffer by pipetting (do not damage the plug slice) and then add new 300 µl 1X *Xba* I buffer containing *Xba* I restriction enzyme (Master mix of 1X *Xba* I

buffer and 50 units of *Xba* I restriction enzyme, in this case, 6 µl of 8 unit/µl *Xba* I from TOYOBO company were used.)

↓

Incubate the tube at 37ºC water bath for 4 h.

↓

Pour all contents including plug slice into sterile petri dish and then use spatula to bring the plug slice to the comb teeth.

↓

Use tissue paper to adsorb the excess liquid on the comb teeth, then drop 1 drop of 1% PFGE-grade agarose to fix the plug slice with the comb teeth. Then wait for 5 min

↓

Pour 1% PFGE-grade agarose into the tray and put the comb in the tray. Let the agarose gel to be solidified at room temperature for 15 min and then place the tray at 4ºC for 10 min.

↓

Place the agarose gel in PFGE machine (the details of the program are mentioned below). Let the gel be cooled down to 14ºC for 10 min before start the machine.

Electrophoresis condition for *E. coli* O157:H7 recommended by PulseNet

-Running time 19 h
-Voltage (V/cm) 6.0
-Angle 120º
-Initial switch time 2.2 S
-Final switch time 54.2 S

Electrophoresis condition for *V. parahaemolyticus* recommended by PulseNet

-Running time 18 h

- Voltage (V/cm) 6.0

-Angle 120 º

-Initial switch time 10 S

-Final switch time 35 S

Part V Bacterial strains used in this study

Escherichia coli

Strian	PSU no.	Virulence genes			Serotype	Antibiogram patterns[1]	Source
		stx$_1$	*stx*$_2$	*eae*	O :H		
M1	4153	-	+	+	157 :7	-	Malaysia
M2	4154	-	-	+	157 :7	-	Malaysia
M3	4155	-	+	+	157 :7	-	Malaysia
M4	4156	-	+	+	157 :7	-	Malaysia
M5	4157	-	+	+	157 :7	-	Malaysia
M6	4158	-	+	+	157 :7	-	Malaysia
M7	4159	+	+	+	116 :31	CRO, AMP, KF	Malaysia
M8	4160	-	+	+	157 :7	KF	Malaysia
M9	4161	-	+	+	157 :7	KF, AMP, AK	Malaysia
M10	4162	-	+	+	157 :7	-	Malaysia
M11	4163	-	+	+	157 :7	-	Malaysia
M12	4164	-	+	+	157 :7	CRO, AMP, KF	Malaysia
M13	4165	-	+	+	157 :7	-	Malaysia
M14	4166	-	+	+	157 :7	CRO, KF	Malaysia
M15	4167	-	+	+	157 :7	CRO, AMP	Malaysia
T1	4168	-	+	+	157 :7	-	Thailand
T2	4169	-	+	+	157 :7	-	Thailand
T3	4170	-	+	+	157 :7	CRO, KF	Thailand
T4	4171	-	+	+	157 :7	CRO	Thailand
T5	4172	-	+	+	157 :7	-	Thailand
T6	4173	-	+	+	157 :7	-	Thailand
EDL933	4189	+	+	+	157 :7	ND[2]	U.S.A.
Thai-1	4195	+	+	+	157 :7	ND	Thailand
Thai-12	4197	-	+	+	157 :7	ND	Thailand
Thai-13	4198	+	+	+	157 :7	ND	Thailand
144	4194	-	+	+	157 :7	ND	Japan
C0207	4191	-	+	+	157 :7	ND	Japan
E02-15	-	-	+	+	157 :7	ND	China
E07-I8	-	-	+	+	157 :7	ND	China
E09-A	-	-	+	+	157 :7	ND	China
-	5023	+	-	-	ND	-	Thailand

[1]CR, ceftriazone; AMP, Ampicillin; KF, cephalothin ; AK, Amikacin

[2]ND, No data

V. parahaemolyticus

PSU no.	Virulence genes		GS-PCR	16 kb	23 kb	Serotype	Source
	tdh	*trh*				O:K	
4041	+	-	+	+	-	3:6	Bloody clam, Thailand
4045	-	-	-	-	-	3:6	Bloody clam, Thailand
4049	-	-	+	-	-	3:6	Bloody clam, Malaysia
4050	-	-	+	-	-	3:6	Bloody clam, Malaysia
4051	-	-	+	-	-	3:6	Bloody clam, Malaysia
4052	-	-	+	-	-	3:6	Bloody clam, Malaysia
4053	-	-	+	-	-	3:6	Bloody clam, Malaysia
4056/1	+	-	+	+	+	3:6	Green mussel, Thailand
4056/2	+	-	+	+	+	3:6	Green mussel, Thailand
4056/3	+	-	+	+	+	3:6	Green mussel, Thailand
4056/4	+	-	+	+	+	3:6	Green mussel, Thailand
4056/5	+	-	+	+	+	3:6	Green mussel, Thailand
4056/6	+	-	+	+	+	3:6	Green mussel, Thailand
4056/7	+	-	+	+	+	3:6	Green mussel, Thailand
4056/8	+	-	+	+	+	3:6	Green mussel, Thailand
4059	-	-	+	-	-	3:6	Hard clam, Thailand
4060	-	-	+	-	-	3:6	Hard clam, Thailand
4061	-	-	+	-	-	3:6	Bloody clam, Thailand
4062	-	-	+	-	-	3:6	Bloody clam, Thailand
4063/1	+	-	+	+	+	3:6	Bloody clam, Thailand
4063/2	+	-	+	+	+	3:6	Bloody clam, Thailand
4064	-	-	-	-	-	3:6	Bloody clam, Malaysia
4067	+	-	+	+	+	3:6	Bloody clam, Malaysia
4068	-	-	+	-	-	3:6	Bloody clam, Thailand
4069	-	-	+	-	-	3:6	Bloody clam, Malaysia
4070/1	+	-	+	+	+	3:6	Hard clam, Thailand
4070/2	+	-	+	+	+	3:6	Hard clam, Thailand
4070/3	+	-	+	+	+	3:6	Hard clam, Thailand
4080	-	-	-	-	-	3:6	Bloody clam, Malaysia
4081	-	-	-	-	-	3:6	Bloody clam, Malaysia
4042	-	-	-	NT[1]	NT	1:25	Bloody clam, Thailand
4043	-	-	-	NT	NT	1:25	Bloody clam, Thailand
4044	-	-	-	NT	NT	1:25	Bloody clam, Thailand
4046	-	-	-	NT	NT	1:25	Bloody clam, Thailand
4047	-	-	-	NT	NT	1:25	Bloody clam, Malaysia
4048	-	-	-	NT	NT	1:25	Bloody clam, Malaysia
4054	-	-	-	NT	NT	1:25	Green mussel, Thailand
4055	-	-	-	NT	NT	1:25	Green mussel, Thailand

PSU no.	Virulence genes		GS-PCR	16 kb	23 kb	Serotype O:K	Source
	tdh	*trh*					
4058	-	-	-	NT	NT	1:25	Hard clam, Thailand
4065	-	-	-	NT	NT	1:25	Bloody clam, Malaysia
4075	-	-	-	NT	NT	1:25	Hard clam, Thailand
4082	-	-	-	NT	NT	1:25	Bloody clam, Malaysia

[1]NT, Not tested

VITAE

Name Mr. Pharanai Sukhumungoon

Student ID. 5010230039

Education Attainment

Degree	Name of institution	Year of graduation
B. Sc. (Microbiology)	Prince of Songkla University	2004
M. Sc. (Molecular Genetics and Genetic Engineering)	Mahidol University	2007

Scholarships Awards during Enrollment

Prince of Songkla University Graduate study scholarship 2007-2010

Grant-in-aid from Japan Society for the Promotion of Science, KAKENHI 191010

List of publications and proceedings

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