Characterization of Pandemic O1:KUT *Vibrio parahaemolyticus* and Evaluation of Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA) for Clinical Strain Typing

Sutima Preeprem

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Vibrio parahaemolyticus is one of the important causative agents of gastroenteritis, especially O3:K6 serotype which is considered as a pandemic serotype disseminated worldwide. Recently, the outbreaks of *V. parahaemolyticus* infection occurred not only by O3:K6 serotype but also its serovariants, O1:K25 and O1:KUT (untypeable). However, little information of O1:KUT serotype has become available. In order to obtain more knowledge of O1:KUT isolates it is imperative to explore their characteristics, and evaluate the novel method to discriminate the strains. In this study, A total of 46 pandemic *V. parahaemolyticus* isolates (*tdh* gene positive, *trh* gene negative, GS-PCR positive) serotypes O1: KUT (*n*=32), O1:K25 (*n*=8), and O3:K6 (*n*=6) obtained from diarrhea patients in Hat Yai hospital, Songkhla during 2001-2012 were examined for their antimicrobial resistance profiles and virulence characteristics. Resistant towards ampicillin, ciprofloxacin and norfloxacin were found in all (100%), 7 (15%) and 1 (2%) isolates, respectively. Interestingly, more than 50% of the isolates were found to be intermediated susceptibility to ciprofloxacin and/or norfloxacin. The virulence-associated genes encoding type III secretion systems (T3SS1 and T3SS2), and type VI secretion systems (T6SS1 and T6SS2) were widely distributed among the isolates in all serotypes. All isolates were able to produce the similar level of thermostable direct hemolysin (TDH) on the Wagatsuma blood agar. Of 46 isolates, 21 (46%) and 15 (33%) displayed high level of swarming and twitching motilities, respectively. All isolates except one were able to use hemoglobin as the only iron source. More than 80% of the isolates were able to grow in iron-depleted medium under hemoglobin concentration as low as 50 µM. There was no difference in the antimicrobial resistance profiles and virulence characteristics observed among different serotypes.

Due to the limitation of established K antisera, tracking the sources of KUT for epidemiological investigation is not available. Therefore, the effective molecular typing is required in order to discriminate the strains. This study demonstrated that arbitrarily primed PCR (AP2-PCR and AP4-PCR) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) exhibited low resolution for typing among pandemic *V. parahaemolyticus* isolates. The discriminatory power of these methods ranged from 0.79, 0.30, and 0.24 for AP2-PCR, ERIC-PCR, and AP4-PCR, respectively. Pulsed-field gel electrophoresis (PFGE), a gold standard method, showed low ability to discriminate among isolates of the same serotypes. Most of the O1:KUT isolates obtained during 2001-2005 (*n*=12) showed similar PFGE profile. However, A minimum spanning tree (MST) based on PFGE revealed that PFGE profiles of all isolates were associated with their serotype and the period of strain isolation. In order to discriminate among all pandemic isolates, this study develops a multiplex multiple-locus variable-number tandem repeat (MLVA) assay for strain typing. The assay was based on the analysis of 4 variable number of tandem repeat (VNTR) loci. MLVA analysis of *V. parahemolyticus* isolates generated 38 distinct profiles whereas only 16 types were obtained from PFGE. High copy number variations of VNRT alleles in TR1, TR2, TR3, and TR4 loci were ranged from 5–44, 7–24, 5–44, and 7–38, respectively. The Nei's genetic diversity indices (DI) of the 4 VNTR loci ranged from 0.83-0.92. For stability study, some variations in TR2 were found after multiple subcultures and in stress conditions. In this work, MLVA resolved the 12 isolates of O1:KUT obtained in 2001-2005 with identical PFGE patterns into unique profiles. The multiplex MLVA developed in this study has higher discriminatory power (D=0.99) than PFGE (0.89), and is superior to PFGE for distinction pandemic *V. parahaemolyticus* including O1:KUT isolates. In addition, MLVA is more rapid than other typing methods used in this study.

This study highlight the antimicrobial resistant and multiple virulence characteristics among pandemic *V. parahaemolyticus* isolates which will be useful for further epidemiological and clinical investigations of this organism. In addition, the MLVA typing technique developed in this study would significantly contribute to surveillance and outbreak investigation of pandemic *V. parahaemolyticus*.

Keywords: DNA profile, MLVA, O1:KUT, *Vibrio parahaemolyticus*, virulence

ชื่อวิทยานิพนธ์ การตรวจหาลักษณะของ *Vibrio parahaemolyticus* ซีโรไทป์ O1:KUT สายพันธุ์ระบาดและการประเมินประสิทธิภาพของเทคนิค multiple-locus variable-number tandem repeat analysis (MLVA) เพื่อใช้ในการจำแนกสายพันธุ์ของเชื้อที่แยกได้จากผู้ป่วย

ผู้เขียน นางสาวสุธิมา ปรีเปรม

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บทคัดย่อ

Vibrio parahaemolyticus เป็นสาเหตุสำคัญของโรคกระเพาะอาหารและลำไส้อักเสบ โดยเฉพาะอย่างยิ่งเชื้อในซีโรไทป์ O3:K6 ที่เป็นสาเหตุหลักของการระบาดทั่วโลก ปัจจุบันพบว่าสาเหตุของการระบาดเกิดจากซีโรไทป์ O1:K25 และ O1:K untypeable (KUT) ด้วยอย่างไรก็ตามข้อมูลที่เกี่ยวข้องกับ *V. parahaemolyticus* ซีโรไทป์ O1:KUT มีเพียงเล็กน้อย ดังนั้นการศึกษาลักษณะของเชื้อและการประเมินเทคนิคใหม่ในการจำแนกสายพันธุ์ของเชื้อจึงมีความจำเป็น ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อตรวจสอบรูปแบบการดื้อต่อยาปฏิชีวนะและลักษณะที่เกี่ยวกับการก่อโรคของ *V. parahaemolyticus* สายพันธุ์ระบาดจำนวน 46 ไอโซเลท แบ่งเป็น ซีโรไทป์ O1:KUT จำนวน 32 ไอโซเลท O1:K25 จำนวน 8 ไอโซเลท และ O3:K6 จำนวน 6 ไอโซเลท ซึ่งแยกได้จากผู้ป่วยในโรงพยาบาลหาดใหญ่ จังหวัดสงขลา ประเทศไทย ระหว่างปี พ.ศ. 2544-2555 จากการศึกษาพบว่าเชื้อทั้งหมดดื้อต่อยา ampicillin และมีเชื้อจำนวน 7 (ร้อยละ 15) และ 1 (ร้อยละ 2) ไอโซเลท ดื้อต่อยา ciprofloxacin และ norfloxacin ตามลำดับ ที่น่าสนใจ คือ เชื้อมากกว่าร้อยละ 50 มีความไวในระดับปานกลางต่อยา ciprofloxacin และ/หรือ norfloxacin นอกจากนี้ยังพบการกระจายของยีนที่เกี่ยวข้องกับระบบ type III secretion systems (T3 SS1 และ T3 SS2) และระบบ type VI secretion systems (T6SS1 และ T6SS2) ในเชื้อทุกซีโรไทป์ การทดสอบการสร้างสารพิษ thermostable direct hemolysin (TDH) บนอาหารเลี้ยงเชื้อ wagatsuma blood agar พบว่าเชื้อทั้งหมดมีความสามารถในการสร้าง TDH ได้ในระดับเดียวกัน การศึกษาความสามารถในการเคลื่อนที่พบว่าเชื้อจำนวน 21 (ร้อยละ 46) และ 15 (ร้อยละ 33) ไอโซเลท มีความสามารถในการเคลื่อนที่แบบ swarming และ twitching ตามลำดับ ในระดับสูง นอกจากนี้เชื้อจำนวน 45 ไอโซเลท สามารถใช้ฮีโมโกลบินเป็นแหล่งของธาตุเหล็กเพียงอย่างเดียวได้ โดยเชื้อมากกว่าร้อยละ 80 ของทั้งหมดสามารถเจริญในสภาวะที่มีฮีโมโกลบินในระดับความเข้มข้นต่ำเพียง 50 ไมโครโมลาร์ การศึกษานี้ไม่พบความแตกต่างของรูปแบบการดื้อยาปฏิชีวนะ และลักษณะที่เกี่ยวกับการก่อโรคในเชื้อที่มีซีโรไทป์ต่างกัน

ปัจจุบันมีข้อจำกัดของซีรั่มที่ใช้ในการจำแนก K antigen จึงทำให้ไม่สามารถบ่งชี้แหล่งที่มาของเชื้อซีโรไทป์ KUT เพื่อศึกษาทางระบาดวิทยาได้ เทคนิคอณูชีวโมเลกุลจึงมีความจำเป็นในการจำแนกเชื้อสายพันธุ์ดังกล่าว การศึกษานี้แสดงให้เห็นว่า เทคนิค arbitrarily primed PCR (AP2-PCR และ AP4-PCR) และเทคนิค enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) เป็นเทคนิคที่มีความละเอียดต่ำในการจำแนก *V. parahaemolyticus* สายพันธุ์ระบาด โดยค่าประสิทธิภาพในการจำแนกเชื้อของเทคนิค AP2-PCR, ERIC-PCR และ AP4-PCR มีค่าเท่ากับ 0.79, 0.30 และ 0.24 ตามลำดับ การศึกษาเทคนิค pulsed-field gel electrophoresis (PFGE) ซึ่งเป็นเทคนิคมาตรฐานที่ใช้ในการจำแนกสายพันธุ์ของเชื้อ พบว่า PFGE ยังคงมีความสามารถในการจำแนกสายพันธุ์ของเชื้อที่มีซีโรไทป์เดียวกันในระดับต่ำ โดยเชื้อซีโรไทป์ O1:KUT ซึ่งแยกได้ในระหว่าง ปี พ.ศ. 2544-2548 มีรูปแบบ PFGE ที่เหมือนกัน อย่างไรก็ตามแผนภาพ minimum spanning tree (MST) ซึ่งสร้างจากรูปแบบ PFGE บ่งบอกความสัมพันธ์ของซีโรไทป์และช่วงเวลาที่แยกเชื้อได้ ดังนั้นการศึกษานี้จึงได้พัฒนาเทคนิค multiplex multiple-locus variable-number tandem repeat (MLVA) เพื่อใช้ในการจำแนกสายพันธุ์ของเชื้อ โดยตรวจสอบจำนวนซ้ำของลำดับเบสบนโครโมโซมที่มีความผันแปร (variable number of tandem repeat; VNTR) จำนวน 4 ตำแหน่ง จากการใช้เทคนิค MLVA ในการจำแนกสายพันธุ์ของ *V. parahaemolyticus* พบรูปแบบ MLVA ที่แตกต่างกัน 38 รูปแบบ ซึ่งแตกต่างจากเทคนิค PFGE ที่ให้รูปแบบ PFGE ที่แตกต่างกันเพียง 16 รูปแบบเท่านั้น นอกจากนี้ ยังพบว่า จำนวน copy number ของ VNTR ทั้ง 4 ตำแหน่ง มีความหลากหลายสูงโดย TR1, TR2, TR3 และ TR4 มีจำนวน copy number อยู่ระหว่าง 5-44, 7-24, 5-44 และ 7-38 ตามลำดับ ซึ่งค่า Nei's genetic diversity indices (DI) ของ VNTR ทั้ง 4 มีค่าอยู่ระหว่าง 0.83-0.92 การศึกษาความเสถียรพบว่า TR2 มีความผันแปรไปเมื่อผ่านการ subculture และสภาวะเครียด เทคนิค MLVA ที่พัฒนาขึ้นสามารถจำแนกความแตกต่างของเชื้อซีโรไทป์ O1:KUT ทั้ง 12 ไอโซเลทที่มีรูปแบบ PFGE เหมือนกันออกจากกันได้ มีประสิทธิภาพในการจำแนกเชื้อ (D=0.99) สูงกว่าเทคนิค PFGE (0.89) มีความสามารถสูงกว่าเทคนิค PFGE ในการจำแนก *V. parahaemolyticus* ซีโรไทป์ O1:KUT สายพันธุ์ระบาด และมีความรวดเร็วกว่าเทคนิคอื่น ๆ ที่ใช้ในการศึกษาลายพิมพ์ดีเอ็นเอในครั้งนี้

การศึกษานี้แสดงให้เห็นถึงการดื้อต่อยาปฏิชีวนะและลักษณะที่เกี่ยวข้องกับความรุนแรงในการก่อโรคที่หลากหลายของ *V. parahaemolyticus* สายพันธุ์ระบาด โดยเฉพาะเชื้อซีโรไทป์ O1:KUT ซึ่งมีประโยชน์ต่อการศึกษาทางระบาดวิทยาของเชื้อในอนาคต อีกทั้งเทคนิค multiplex MLVA ที่พัฒนาขึ้นยังสามารถนำมาใช้เฝ้าระวังและตรวจสอบการระบาดของเชื้อได้ต่อไป

คำสำคัญ: ความสามารถในการก่อโรค, ซีโรไทป์ O1:KUT, เทคนิค MLVA, รูปแบบดีเอ็นเอ, *Vibrio parahaemolyticus*

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LIST OF ABBREVIATIONS AND SYMBOLS

A	=	Adenine
AMP	=	Ampicillin
AP-PCR	=	Arbitrarily primed PCR
bp	=	Base pair
C	=	Cytocine
CHL	=	Chloramphenicol
CIP	=	Ciprofloxacin
CO	=	Cotrimoxazole
D	=	Discriminatory power
DI	=	Nei's genetic diversity indices
dNTPs	=	Deoxynucleotide triphosphates
ERIC-PCR	=	Enterobacterial repetitive intergenic consensus PCR
G	=	Guanine
GS-PCR	=	Group-specific PCR
kb	=	Kilobase
KP	=	Kanagawa phenomenon
KUT	=	K untypeable
LB	=	Luria Bertani
min	=	Minute
MLVA	=	Multiple-locus variable-number tandem repeat analysis
mm	=	Millimeter
mM	=	Millimolar
NaCl	=	Sodium chloride
Na_2HPO_4	=	Sodium hydrogen phosphate

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

NOR	=	Norfloxacin
OD	=	Optical density
PCR	=	Polymerase chain reaction
PFGE	=	Pulsed-field gel electrophoresis
s	=	Second
t	=	Time-consuming
T	=	Typeability
T	=	Thymine
T3SSs	=	Type III secretion systems
T6SSs	=	Type VI secretion systems
TCBS	=	thiosulfate citrate bile salts sucrose agar
TDH	=	Thermostable direct hemolysin
TET	=	Tetracycline
TRH	=	TDH-related hemolysin
TSA	=	Tryptic soy agar
TSB	=	Tryptic soy broth
VNTR	=	Variable-numbers of tandem repeat
°C	=	Degree Celsius
µM	=	Micromolar
µl	=	Microliter
%	=	Percentage

CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

Vibrio parahaemolyticus is one of the major causes of gastroenteritis in humans. Cases are often associated with eating raw or undercooked shellfish or even cooked foods that have been cross-contaminated with raw shellfish (Yeung and Boor, 2004). Recently, the number of *V. parahaemolyticus* infections in Thailand has been gradually increasing (AESR, 2015). *V. parahaemolyticus* O3:K6 serotype that was positive for group-specific PCR (GS-PCR) targeted to *toxRS* regions and thermostable direct hemolysin (*tdh*) gene but not the *tdh*-related hemolysin (*trh*) gene was shown to be responsible for pandemic outbreaks of diarrhea in various parts of the world including Asia, America, Africa and Europe (Okuda *et al.*, 1997, Daniels *et al.*, 2000, Martinez-Urtaza *et al.*, 2004, Ansaruzzaman *et al.*, 2005, AESR, 2015). *V. parahaemolyticus* serotypes O4:K68, O1:K25, and O1:K untypeable (KUT) were reported as the pandemic serotypes which originated form O3:K6 clone by alteration of their O and K antigens (Chowdhury *et al.*, 2000, Matsumoto *et al.*, 2000, Bhuiyan *et al.*, 2002). In Thailand, these serotypes have been reported to continually detect among patient since 2000 (Wootipoom *et al.*, 2007, Thongjun *et al.*, 2013). Due to the variation of O1:KUT pandemic isolates, further characterization are needed in order to obtain more epidemiological knowledge among these isolates.

Pathogenicity of *V. parahaemolyticus* depends on multiple factors. Thermostable direct hemolysin (TDH), type III secretion systems (T3SSs), and type VI secretion systems (T6SSs) are recognized as virulence-associated factors among clinical isolates of *V. parahaemolyticus* (Ceccarelli *et al.*, 2013, Zhang and Orth, 2013). TDH is responsible for cytoxicity and hemolytic activity (Vuddhakul, 2008, Wang *et al.*, 2015). T3SSs are responsible for host cell cytotoxicity and enterotoxicity (Calder *et al.*, 2014). T6SSs play an important role in environment fitness (Salomon *et al.*, 2013, Wang *et al.*, 2013b) and necessary for the adhesion to host cells (Wang *et al.*, 2015). Besides those factors, *V. parahaemolyticus* exhibit different types of bacterial surface motility, swarming and twitching which contribute bacteria to

survival in the environment and enhance colonization (Fraser and Hughes, 1999). Iron acquisition was reported to correlate with the ability of infection among *V. parahaemolyticus* isolates (León-Sicairos *et al.*, 2015). Under iron-limited condition, *V. parahaemolyticus* produces the siderophore vibrioferrin to chelate iron of host cell.

Many molecular typing techniques have been developed to gain a better understanding of genetic relatedness among strains of *V. parahaemolyticus*. Several PCR-based typing methods, such as arbitrarily primed PCR (AP-PCR) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) have been demonstrated for *V. parahaemolyticus* strain typing. Although these techniques are affordable, rapid, and easy-to-perform, they are occasionally associated with low discriminatory power and low reproducibility (Wong and Lin, 2001, Sabat *et al.*, 2013, Oyarzabal and Kathariou, 2014). A standard method for epidemiological investigation of this pathogen is pulsed-field gel electrophoresis (PFGE). However, this technique requires expensive specialized equipment, time-consuming, and PFGE banding patterns are sometimes difficult to interpret. Multiple-locus variable-number tandem repeat analysis (MLVA) has been developed for typing of bacterial pathogens base on the detection of variable number of tandem repeats (VNTR) at multiple VNTR loci (Lindstedt, 2005). MLVA results can be easily interpreted allowing interlaboratory comparisons. The five previously reported MLVA typing protocols differ in the number of VNTR loci that were used, including 7 (Ansede-bermejo *et al.*, 2010), 8 (Kimura *et al.*, 2008, Jiang *et al.*, 2016), 10 (Harth-Chu *et al.*, 2009), and even 12 (Lüdeke *et al.*, 2015). So far, 19 VNTRs has been used for typing of *V. parahaemolyticus* (Kimura *et al.*, 2008, Harth-Chu *et al.*, 2009, Ansede-bermejo *et al.*, 2010, Lüdeke *et al.*, 2015, Jiang *et al.*, 2016). MLVA typing of O1:KUT isolates has been rarely reported, and the stability of VNTR markers has not been evaluated.

To improve our understanding about O1:KUT isolates, this study aimed to determine the various virulence-associated characteristics of *V. parahaemolyticus* isolates from clinical samples. Additional studies were aimed to identify novel potential VNTR loci and develop a multiplex MLVA typing scheme using fewer VNTR loci than previously protocols.

LITERATURE REVIEWS

Vibrio parahaemolyticus

General characteristics

Vibrio parahaemolyticus belongs to the family Vibrionaceae. It is a Gram negative, curve-rod shaped, halophilic bacterium, and motile by a single polar flagella (Su and Liu, 2007). Like other members of the genus *Vibrio*, this species is oxidase positive and facultative aerobic bacterium. It requires sodium chloride (NaCl) for growth with the optimal concentration around 2%–4%. The optimal growth temperature is 30–35°C, but can survive at 4°C for 3 weeks. The pH range for growth is 4.8–11 with the optimum of pH 7.8–8.6. The % mol G + C of DNA is 46 –47 (T_m) (Farmer and Janda, 1984, Vuddhakul, 2008).

Antigenic structure and biological classification

V. parahaemolyticus strains are differentiated serologically based on the major classes of bacteria antigen, including somatic (O) and capsular (K) which are associated with the bacterial lipopolysaccharide and extracellular layer or capsule, respectively (Farmer and Janda, 1984). Currently, *V. parahaemolyticus* can be classified into more than 80 serotypes which compose of 12 O antigens and more than 60 K antigens **(Table 1.1)** (Farmer and Janda, 1984, Kaysner and Angelo DePaola, 2004). The pandemic strain of *V. parahaemolyticus* serotype O3:K6 was first reported in Calcutta, India. Then, this strain was detected in many part of the world (Yeung and Boor, 2004). In addition, other pandemic serotypes O1:K25 and O1:KUT have been reported to be originated from O3:K6 serotype (Yeung and Boor, 2004).

Table 1.1 Antigenic scheme of *V. parahaemolyticus*[a] (Kaysner and Angelo DePaola, 2004).

O group	**K type**
1	1, 25, 26, 32, 38, 41,56, 58, 64, 69
2	3, 28
3	4,5,6,7,27,30,31,33,37,43,45,48,54,57,58,59,65
4	4,8,9,10,11,12,13,34,42,49,53,55,63,67
5	5,15,17,30,47,60,61,68
6	6,18,46
7	7,19
8	8,20,21,22,39,70
9	9,23,44
10	l9,24,52,66,71
11	36,40,50,51,61
12	52

[a] The antigenic scheme was first established by Sakazaki *et al*. (Sakazaki *et al.*, 1963) and later extended by the Commission of the Serotyping of *V. parahaemolyticus* (Japan); K antigens, 2,14,16, 29,35, and 62 were excluded by the Commission (Hugh and Feeley, 1972). K types 4, 5, 6, 7, 8, 9, and 19 occur with more than one O group.

Epidemiology

V. parahaemolyticus was first isolated in 1950 from a major food poisoning outbreak in Japan (272 people were infected, and 20 died*)* which caused by the consumption of "Shirasu" (partially boiled juvenile sardines) (Kaneko and Colwell, 1973). Since then, *V. parahaemolyticus* has been recognized as a common cause of food poisoning outbreaks in Japan, and throughout Asia (Daniels *et al.*, 2000). In 1997, a large outbreak of *V. parahaemolyticus* food poisoning was related to raw oyster consumption, and was occurred along the Pacific coast (McCarter, 1999, Daniels *et al.*, 2000). This bacterium has been the most common *Vibrio* species isolated from outbreaks and sporadic causes of gastroenteritis throughout the world (Daniels *et al.*, 2000). The serotype O3:K6, the major pandemic strain, was reported in Calcutta India in 1996, and subsequently isolated in many part of the world (Farmer and Janda, 1984, Kaysner and Angelo DePaola, 2004, Yeung and Boor, 2004). In 1998, The United States reported the outbreaks of O3:K6 strain through the consumption of raw oyster (Su and Liu, 2007). However, most of the outbreaks in Europe are associated with shrimp consumption (Su and Liu, 2007). The O1:K25 and O1:KUT strains isolated from 1997 onwards originate from the same clone as the O3:K6 (Laohaprertthisan *et al.*, 2003, Nair *et al.*, 2007). Recently, an outbreak of *V. parahaemolyticus* involves the new O1:KUT.

Pathogenesis & pathology

V. parahaemolyticus is an important cause of gastroenteritis (Nair *et al.*, 2007). Gastroenteritis is associated with the consumption of raw seafood, especially raw oyster. The infection dose of *V. parahaemolyticus* is around 10^5-10^7 organisms and incubation time of infection is about 15 hours (rang from 4 - 96 hours) (Nair *et al.*, 2007, Vuddhakul, 2008). The common symptoms of gastroenteritis include diarrhea, abdominal pain, nausea, vomiting, headache, and low-grade fever. Sometimes the diarrhea is bloody and is described as "meat washed" because the stool is reddish watery (Nair *et al.*, 2007). Moreover, *V. parahaemolyticus* causes wound infections and septicemia. The wound infections can occur when the open wound exposes to seawater (Daniels *et al.*, 2000).

Virulence factors

Various virulence factors possessed by *V. parahaemolyticus* were identified, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), type III secretion systems (T3SSs), and type VI secretion systems (T6SSs). Other characteristics such as swarming motility, twitching, and iron acquisition were also reported to be related to the virulence of this bacterium **(Fig. 1.1)**.

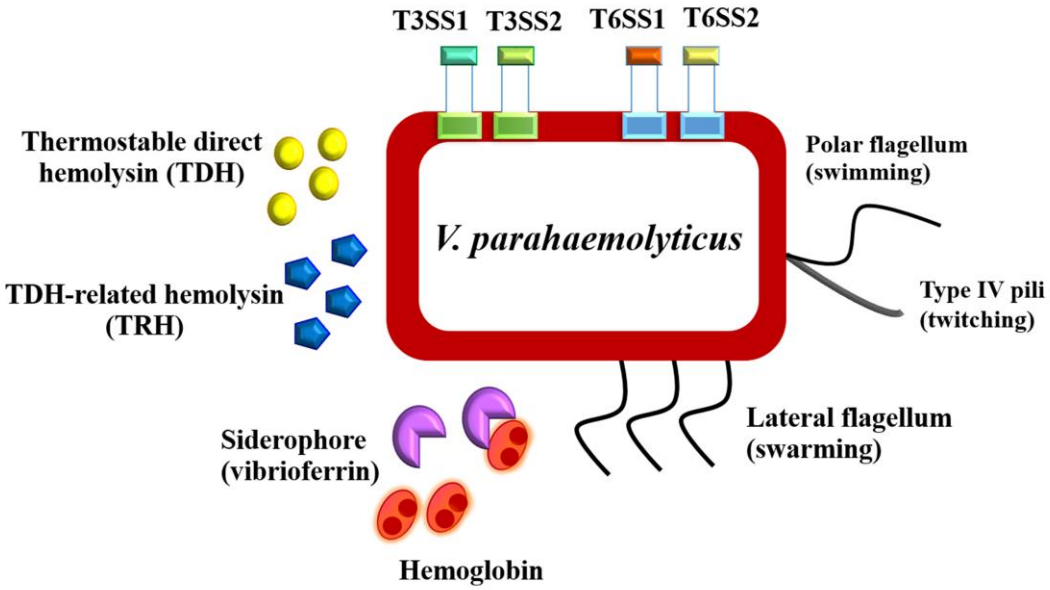

Fig. 1.1 Virulence factors of *V. parahaemolyticus.*

Thermostable direct hemolysin (TDH)

TDH is a major virulence factor of *V. parahaemolyticus* that causes cytoxicity and hemolytic activity (Vuddhakul, 2008, Wang *et al.*, 2015). This toxin enters to host membrane by forming a tetrameric pore complex in the host membrane **(Fig. 1.2)**. These pores allow ions to flow across the membrane (Broberg *et al.*, 2011). TDH is encoded by *tdh* gene and co-regulated by T3SS2 genes. The *tdh* gene is identified into five subgroups (*tdh*1-5). A subgroup *tdh*2 is responsible for the extracellular TDH production and hemolytic activity (Vuddhakul, 2008). In 1980, TDH was firstly demonstrated to cause a hemolytic reaction on Wagatsuma's agar (Kanagawa phenomenon; KP). The KP-positive *V. parahaemolyticus* isolates have been predicted as pandemic strains (Broberg *et al.*, 2011, Zhang and Orth, 2013).

TDH-related hemolysin (TRH)

TRH is responsible for cytoxicity and enterotoxicity which is similar to TDH (Vuddhakul, 2008, Broberg *et al.*, 2011, Zhang and Orth, 2013) **(Fig. 1.2)**. TRH is encoded by *trh* gene which shares 68 % sequence homology to *tdh* gene. *trh* gene is divided into two subgroups, including *trh*1 and *trh*2 genes. The *trh*1 gene can cause hemolytic in human, rabbit, sheep, and calf erythrocytes, while the *trh*2 gene is able to cause weakly hemolytic in human and rabbit erythrocytes. TRH can be produced by KP-negative isolates. However, some KP-negative isolates containing both TDH and TRH, produce TDH less than that of KP-positive strain (Vuddhakul, 2008).

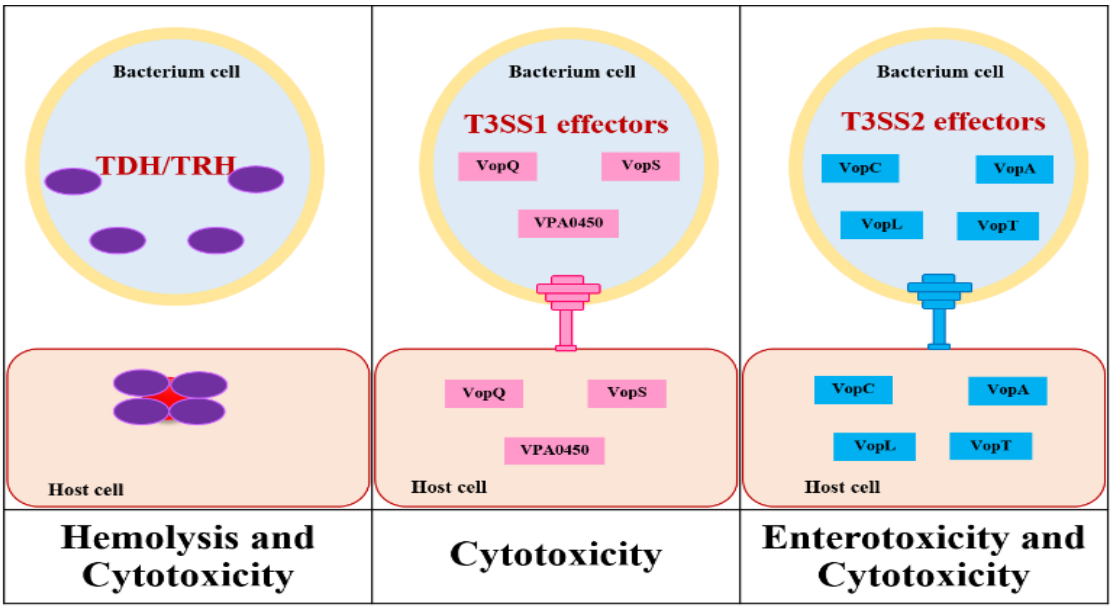

Fig. 1.2 Toxins and T3SS secreted effectors of *V. parahaemolyticus* (modified from Broberg *et al.*, 2011).

Type III secretion systems (T3SSs)

T3SSs, including T3SS1 and T3SS2, are important virulence factors of *V. parahaemolyticus*. These systems are needle-like bacterial structure use for translocate effector proteins directly into host cell (Salomon *et al.*, 2013). The effectors proteins enter to host cell by needle structure. VcrD1 (*vcrD1*) and VcrD2 (*vcrD2*) are the basal structure of T3SS1 and T3SS2, respectively (Calder *et al.*, 2014) **(Fig. 1.3)**. T3SS1-effectors are responsible for cytotoxicity, whereas

T3SS2-effectors are responsible for cytotoxicity and enterotoxicity (Calder *et al.*, 2014, Okada *et al.*, 2014).

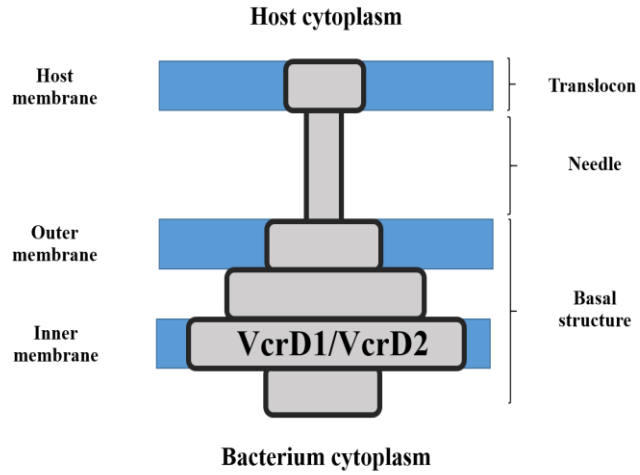

Fig. 1.3 Needle-like apparatus of T3SS (modified from Calder *et al*., 2014).

T3SS1 is detected in both clinical and environmental isolates (Vuddhakul, 2008). Currently, three effector proteins are characterized, including VopQ, VopS and VPA0450 **(Fig. 1.2)**. VopQ or Vibrio outer protein Q has been reported to cause host cell digestion and prevent bacterial cell from phagocytosis. VopQ is a 53 kDa protein with no known conserved structural domain. This protein protects *V. parahaemolyticus* cell from phagocytosis and accelerates host cell death by disrupting membrane and vacuolar trafficking which led to host cell cytotoxicity (Sreelatha *et al.*, 2013). VopS causes cell-rounding by inhibition of host cell structure assembly. VP0450 induce membrane blubbing by disruption of actin-binding protein of the host cell membrane (Zhang and Orth, 2013) **(Fig. 1.4).**

T3SS2 is detected in *tdh*$^{+}$ *V. parahaemolyticus* (KP-positive) (Vuddhakul, 2008). It can be used to indicate the pathogenic strain and is responsible for enterotoxicity (Vuddhakul, 2008, Broberg *et al.*, 2011). T3SS2-effectors include VopC, VopA, VopL, and VopT **(Fig. 1.2)**. VopC is homologous to cytotoxic necrotizing factor 1 (CNF1) which is an exotoxin of pathogenic strain of *E. coli.* VopA is associated with the prevention of cytokines from host. VopT is homologous to the ADP-ribosyltransferase domain of the *Pseudomonas aeruginosa* and VopT is responsible for cytotoxicity. VopL is homologous to VopF in *V. cholerae* which induce the formation of actin stress fibers (Broberg *et al.*, 2011) **(Fig. 1.5)**.

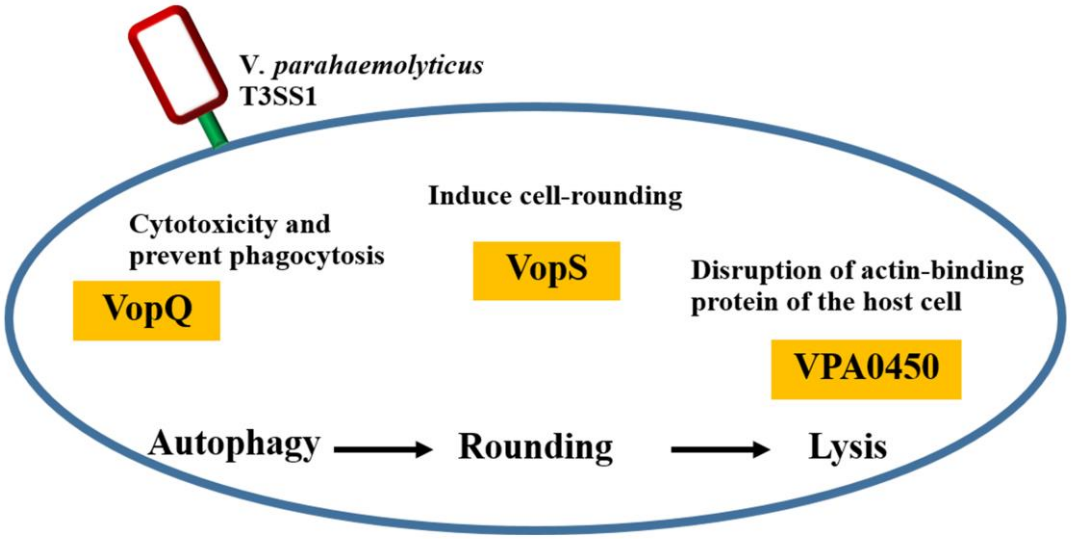

Fig. 1.4 Roles of T3SS1 secreted effectors (modified from Zhang and Orth, 2013).

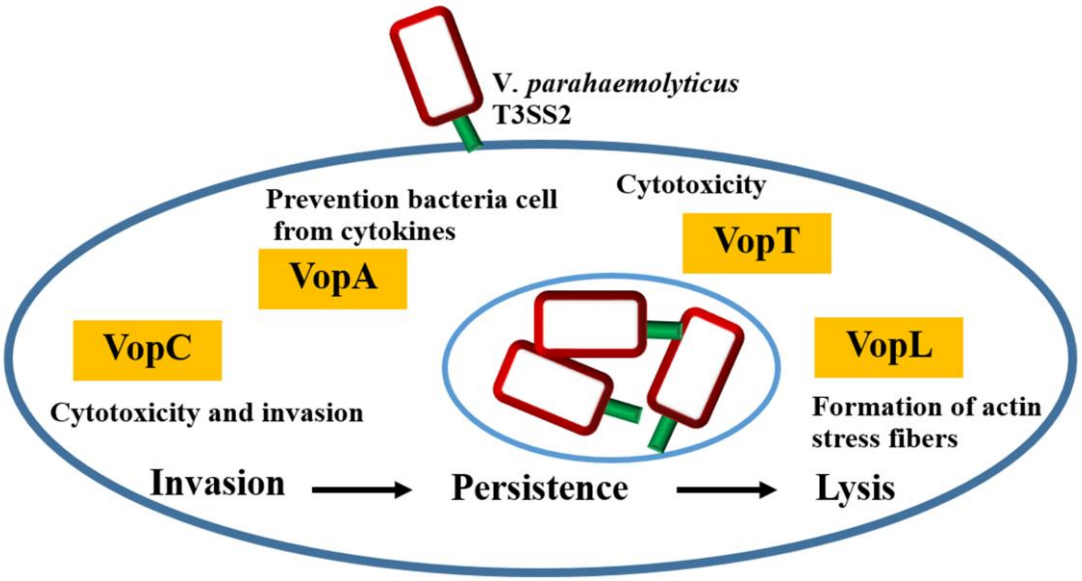

Fig. 1.5 Roles of T3SS2 secreted effectors (modified from Zhang and Orth, 2013).

Type VI secretion systems (T6SSs)

T6SSs are responsible for the translocation of the effector proteins into the membrane and cytoplasm of eukaryotic cells, similar to T3SSs (Salomon *et al.*, 2013). These systems include T6SS1 and T6SS2. Those are found on chromosome 1 (VP1386–VP1414) and chromosome 2 (VPA1025–VPA1046), respectively. T6SS1 has been reported to play a role against other bacteria whereas T6SS2 is responsible for adhesion to host cell (Ceccarelli *et al.*, 2013). The VipA1 (*vipA1*) and VipA2 (*vipA2*) structures are the essential structure of T6SS1 and T6SS2 (Salomon *et al.*, 2013). T6SS1 is found in clinical isolates, while T6SS2 is found in all *V. parahaemolyticus* strains. The expression of these genes is regulated by quorum sensing (Salomon *et al.*, 2013, Wang *et al.*, 2013b). Moreover, they depend on salt condition. T6SS1 is highly expressed under high-salt conditions, whereas T6SS2 is active under low salt conditions (Wang *et al.*, 2013b). Both T6SSs are associated with the adhesion to host cell. Notably, T6SS1 contributes to survival in marine environment when compete with other bacteria (Salomon *et al.*, 2013).

Swarming motility

V. parahaemolyticus is able to swim by single flagellum with speed up to 60 μm/s (Broberg *et al.*, 2011). Rotation of flagellum depends on the energy from the sodium motive force, that associated with salt condition (Broberg *et al.*, 2011). A switch of swimmer to swarmer cell occurs under stress condition such as an increase in viscosity of the growth environment, and growth under iron-limiting conditions. A swarming motility is associated to the lateral flagella and it contributes the bacteria to survival in the environment and enhances colonization (Fraser and Hughes, 1999).

Twitching motility

Twitching motility is a movement of bacteria on surface including agar gels, epithelial cells, plastics, glass, and metals (Mattick, 2002). It occurs by the extension and retraction of type IV pili (Mattick, 2002). Twitching motility play a role in an attachment, biofilm formation, and infection which are important to pathogenesis of bacteria such as *P. aeruginosa*, *E. coli*, *V. chlorae* as well as *V. parahaemolyticus* (Watnick and Kolter, 1999, Mattick, 2002).

Iron acquisition

Iron is essential for bacteria as it is an important co-factor in several metabolic reactions. Pathogenic bacteria use siderophore to chelate the iron from host. Under iron-limited condition, *V. parahaemolyticus* produces the siderophore vibrioferrin (León-Sicairos *et al.*, 2015) which is regulated by *fur* gene (Vuddhakul, 2008, Zhang and Orth, 2013). Vibrioferrin is associated with iron transportation. It can chelate free ferric ion or iron that bound to tranferrin and lactoferrin from host cell.

Laboratory diagnosis

V. parahaemolyticus can be isolated from cultures of stool, wound, and blood. For isolation from stool, the selective medium, thiosulfate citrate bile salts sucrose (TCBS) agar, is widely use. *V. parahaemolyticus* produces green colony on TCBS agar. Other biochemical tests are used to identify this organism include oxidase, arginine dihydrolase, gelatinase, citrate, and Voges-Prokauer test. It cannot grow under 0% NaCl but can grow on 7% NaCl. Furthermore, it is sensitive to the vibriostatic agent O/129 (Hara-Kudo *et al.*, 2003, Cabanillas-Beltrán *et al.*, 2006).

Treatment

Gastroenteritis caused by *V. parahaemolyticus* infection can be treated with oral rehydration solution (ORS). The antibiotic is rarely necessary, since the symptom of gastroenteritis is usually mild and self-limited (Daniels *et al.*, 2000, Nair *et al.*, 2007). However, in case of severe diarrhea, wound infections, or septicemia, antibiotics such as tetracycline, fluoroquinolones, and aminoglycosides could be taken to against bacteria (Daniels *et al.*, 2000).

Prevention and control

V. parahaemolyticus infection can be prevented by cooking seafood promptly and thoroughly. While, wound infections can be prevented by avoiding the exposure of the open wound to seawater (Daniels *et al.*, 2000).

DNA fingerprinting analysis

Arbitrarily primed PCR (AP-PCR)

AP-PCR or randomly amplified polymorphic DNA analysis (RAPD) is one of the DNA fingerprint methods based on PCR. This approach employs arbitrarily primer which is a single, short, and random oligonucleotide primer to amplify discrete fragments of genomic DNA (Penner *et al.*, 1993, Kumar and Gurusubramanian, 2011). The discrete fragments generate by PCR at low annealing temperature. The length of the amplified fragments depends on the homology of template DNA and primer **(Fig. 1.6)** (Kumar and Gurusubramanian, 2011). Recently, AP-PCR is applied in gene mapping, molecular evolutionary genetics, and molecular genotyping. This method is simple and rapid which amplifies the DNA fragments by PCR and separate by gel electrophoresis. However, it has poor reproducibility and low discriminatory power (Penner *et al.*, 1993, Kumar and Gurusubramanian, 2011).

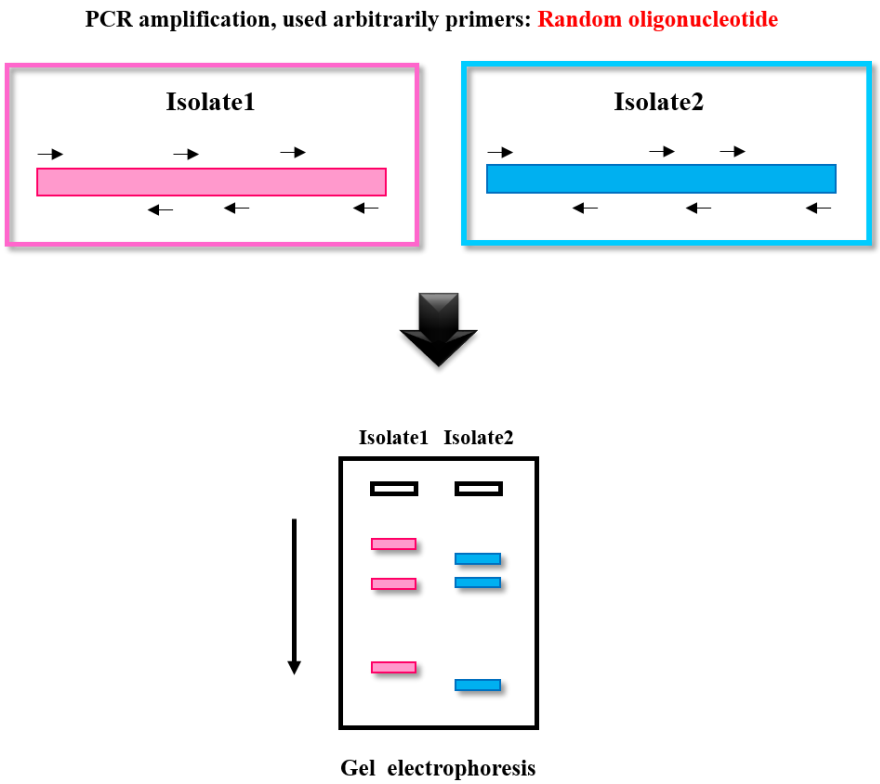

Fig. 1.6 Arbitrarily primed-PCR (AP-PCR).

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

ERIC-PCR is the molecular typing technique which detect enterobacterial repetitive intergenic consensus sequence; (ERICs): 5'GTGAATCCCCAGGAGCTTACATAAGTAAGTGACTGGGGTGAGC G-3' (De Bruijn, 1992, Shuan Ju Teh *et al.*, 2011). ERICs is the repetitive unit of non-coding, intergenics sequence of enteric bacteria. Moreover, ERICs can be found in many Gram-negative and Gram-positive bacteria such as *V. cholerae*, *V. parahaemolyticus*, and *Staphylococcus aureus* (Khan *et al.*, 2002, Vuddhakul, 2008, Shuan Ju Teh *et al.*, 2011, Ye *et al.*, 2012). The primers are composed of ERIC 1R primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 primer (5'-AAGTAAGTGA CTGGGGTGAGCG-3') **(Fig. 1.7)** (De Bruijn, 1992). This technique is a simple and high reproducibility method.

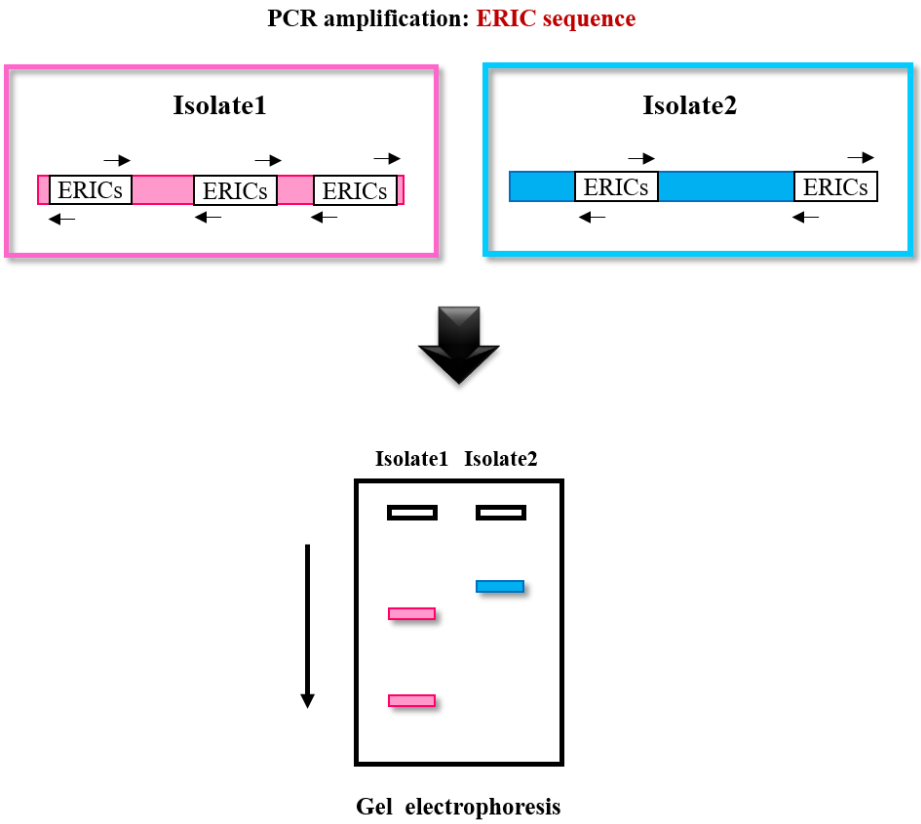

Fig. 1.7 Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR).

Pulsed-field gel electrophoresis (PFGE)

PFGE is a powerful technique used to differentiate both Gram-negative and Gram-positive pathogenic bacteria. It utilizes the restriction enzyme to digest the total genomic DNA and separates DNA by gel electrophoresis which periodically changes direction **(Fig. 1.8)** (Tenover *et al.*, 1997). This method exhibited high discriminatory power and high reproducibility. Nevertheless, PFGE requires special equipment, high cost, and time consuming (Shuan Ju Teh *et al.*, 2011).

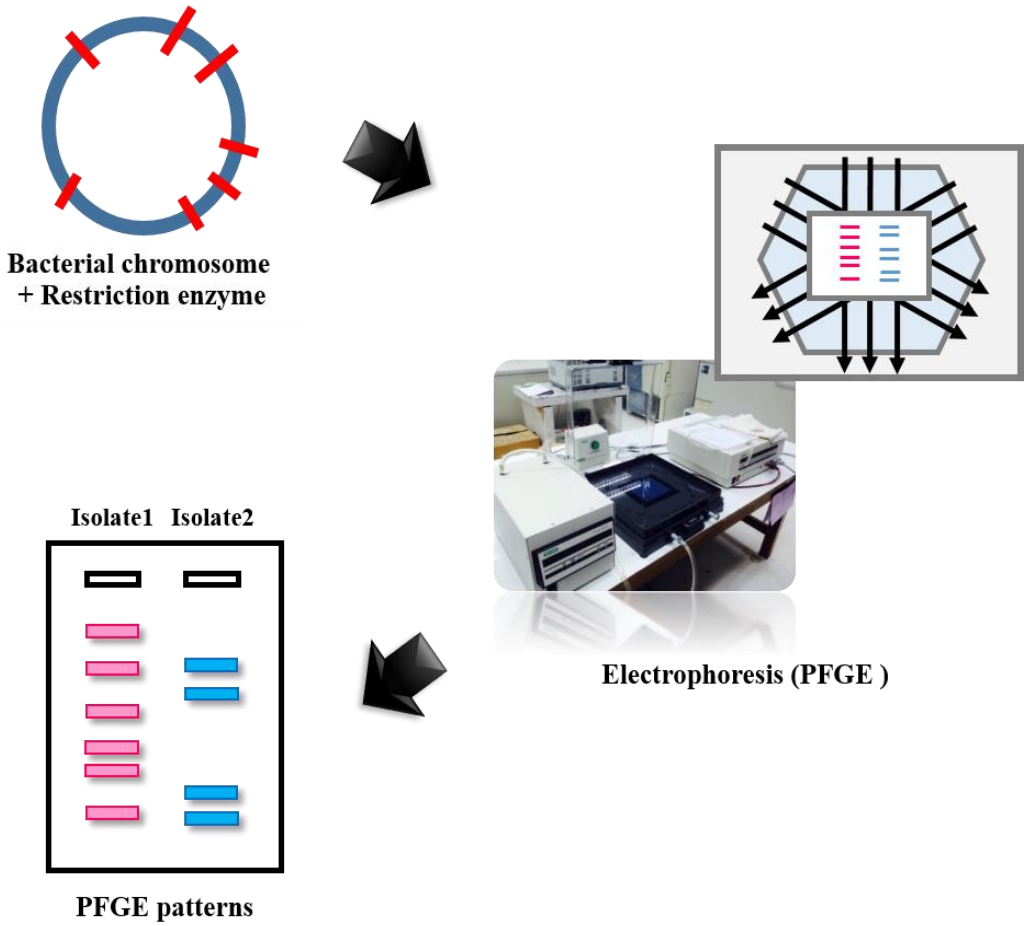

Fig. 1.8 Pulsed-field gel electrophoresis (PFGE).

Multiple-locus variable-number tandem repeat analysis (MLVA)

MLVA is a DNA fingerprint method based on PCR technique using multiple sets of primers (Lindstedt *et al.*, 2012) **(Fig. 1.9)**. This method is important for investigation of the outbreaks of pathogenic bacteria (Lindstedt *et al.*, 2012, Okada *et al.*, 2012). It utilizes the primers that are specific to various variable-number tandem repeat (VNTR) of bacteria (Okuda *et al.*, 1997, van Belkum *et al.*, 1998, Kjeldsen *et al.*, 2014). VNTR is a tandem repeat of short nucleotide sequence which is high variable in the genomes of bacterial pathogens (Okada *et al.*, 2012). Some VNTR is related to the gene function of bacteria such as lipopolysaccharide biosynthesis (*Haemophilus influenzae)*, clumping factor and fibrinogen receptor (*S. aureus*), cell division protein (*V. parahaemolyticus*) and other genes **(Table 1.2)** (van Belkum *et al.*, 1998, Kimura *et al.*, 2008, Okada *et al.*, 2012). The difference of size and copy number of repeat at each loci indicate the variation among bacteria (Oyarzabal and Kathariou, 2014). The variability of VNTR copy number involves in the slipped-strand mispairing of DNA repair systems which is introduced or deleted individual repeat units **(Fig. 1.10)** (van Belkum *et al.*, 1998). Moreover, DNA recombination of multiple loci including of homologous of repeat motif and the occurrence of mutation are the causes of variation of VNTR (van Belkum *et al.*, 1998).

MLVA method was first applied to study the human mapping, then it has been developed for genotyping other organisms. The result of MLVA is easier to compare than gel pattern obtained by other genotyping methods; therefore, this technique is simple and rapid (Okada *et al.*, 2012, Oyarzabal and Kathariou, 2014). In addition, it has high discriminatory power which is able to differentiate among strains of the same species (Okada *et al.*, 2012). Recently, MLVA has been successfully applied to study the variation of pathogenic bacteria such as *V. cholerae* O1, *V. parahaemolyticus* O3:K6, *E. coli*, and *S. enterica* (van Belkum *et al.*, 1998, Lindstedt *et al.*, 2004, Kimura *et al.*, 2008, Okada *et al.*, 2012). This method provides insights more than PFGE technique. Hence, it is useful for tracing the emergence and studying the in-depth epidemiology of pathogenic bacteria (Okada *et al.*, 2012).

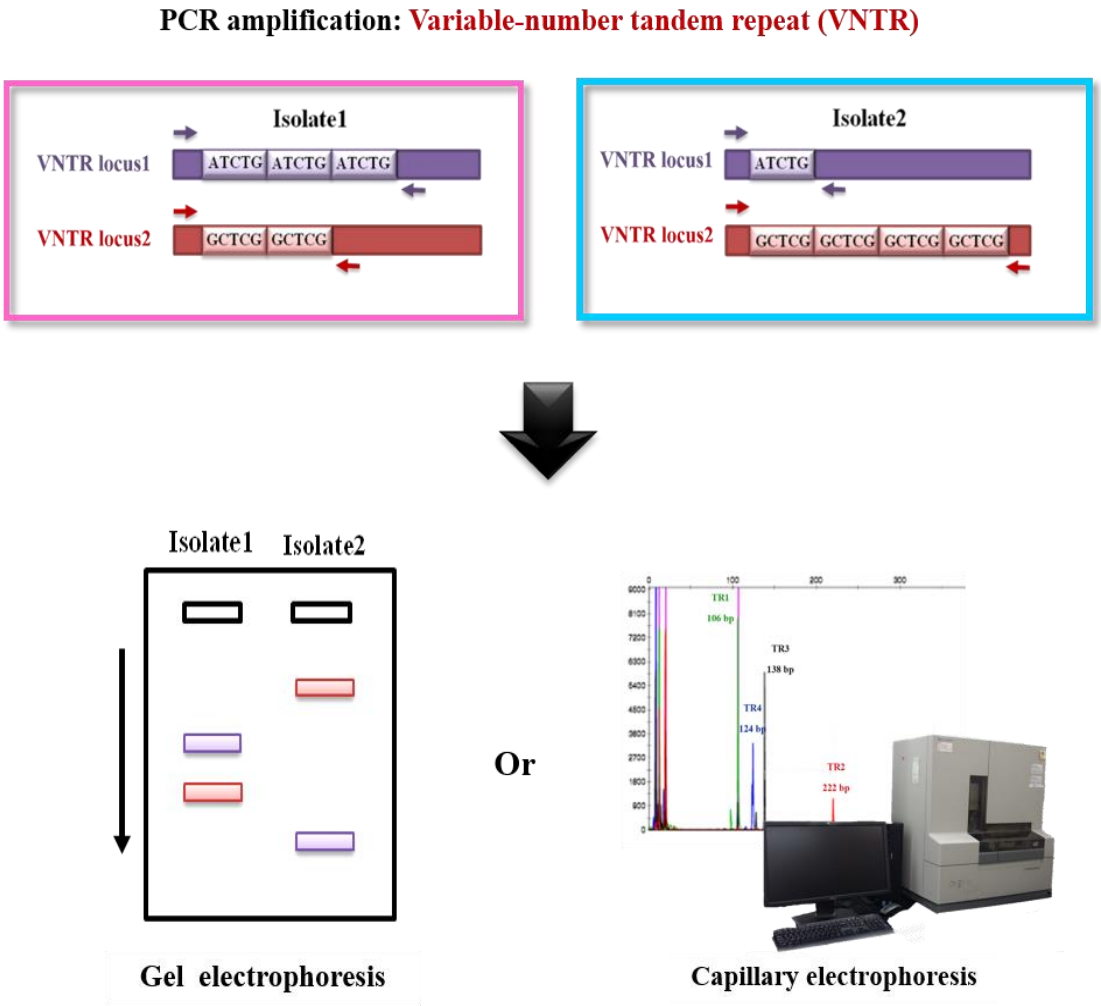

Fig. 1.9 Multiple-locus variable-number tandem repeat analysis (MLVA).

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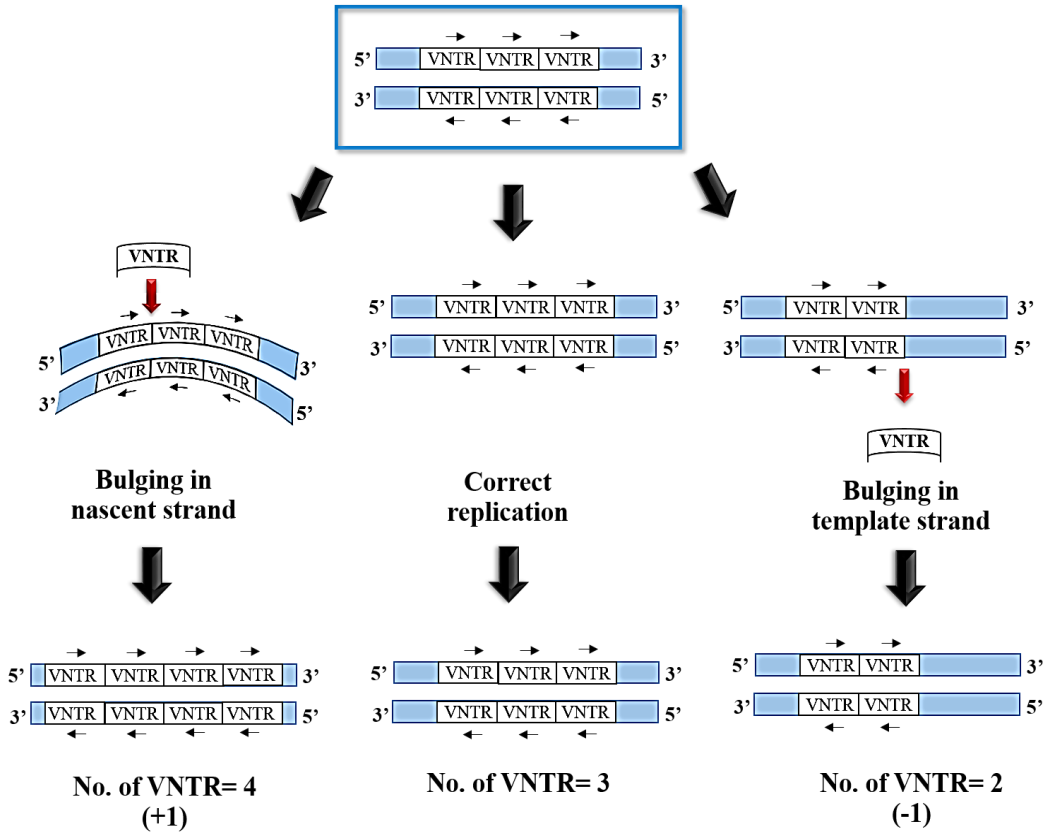

Fig. 1.10 The mechanism of slipped-strand mispairing during replication process, which results in shortening or lengthening of VNTR (modified from van Belkum *et al.*, 1998).

Table 1.2 The VNTR sequence of pathogenic bacteria.

Pathogenic bacteria	VNTR sequence	Function	Reference
Haemophilus influenzae	CAAT	Lipopolysaccharide biosynthesis	(van Belkum *et al.*, 1998)
Staphylococcus aureus	GAXTCXGAXTCXGAXAGX	Clumping factor and fibrinogen receptor	(van Belkum *et al.*, 1998)
Neisseria meningitidis	CTCTT	Opacity surface proteins	(van Belkum *et al.*, 1998)
V. parahaemolyticus O3:K6	CTTCTG	Cell division protein	(Kimura *et al.*, 2008)
V. parahaemolyticus	TGTGTC	Putative hemolysin	(Harth-Chu *et al.*, 2009)
V. cholerae	AACAGA	Cell division protein	(Olsen *et al.*, 2009)
Listeria monocytogenes	TATTTTTATTTAAAAATG	Argininosuccinate synthase	(Murphy *et al.*, 2007)
Salmonella Typhimurium	TGCGATGTC	Putative mannitol dehydrogenase enzyme	(Lindstedt *et al.*, 2004)

OBJECTIVES

1. To determine the antimicrobial resistant profiles and various virulence-associated characteristics of *V. parahaemolyticus* O1:KUT isolates from clinical samples.
2. To identify novel potential VNTR loci and develop a multiplex MLVA typing scheme for *V. parahaemolyticus* O1:KUT typing.

CHAPTHER 2

RESEARCH METHODOLOGY

MATERIALS AND EQUIPMENTS

1. Bacterial strains

Forty-six isolates of pandemic *V. parahaemolyticus* including O1:KUT (*n*=32), O1:K25(*n*=8), and O3:K6 (*n*=6) were kindly provided by Prof. Dr. Varaporn Vuddhakul, Department of Microbiology, Faculty of Science, and Prince of Songkla University. All isolates were obtained from diarrhea patients in Hat Yai hospital, Songkhla between 2001-2012. In addition, non-pandemic *V. parahaemolyticus* isolates (*n*=20) were included only in MLVA experiment.

2. Microbiological media

Microbiological media used in this study were purchased from Difco (USA), Merck (Germany), and HiMedia (India).

3. Chemicals

Chemicals used in this study were purchased from Merck (Germany), Bio-Rad Laboratories (USA), and Bio Basic (Canada).

4. Antimicrobial susceptibility disks

Antimicrobial susceptibility disks used in this study were purchased from Oxoid (United Kingdom).

5. PCR reagents and primers

PCR reagents included 10X buffer, 2.5 mM dNTPs, and *Taq* polymerase were purchased from New England Biolabs (USA). Oligonucleotides primers were synthesized by Eurofins Genomics (Germany).

Molecular typing (AP-PCR and ERIC-PCR) were performed with 10X *Ex Taq* buffer, 2.5 mM dNTPs, and *TaKaRa ExTaq*^{TM} polymerase (TaKaRa, Tokyo, Japan). Oligonucleotides primers were synthesized by Eurofins Genomics, Germany.

Reagents for MLVA study included 10X buffer, 2.5 mM dNTPs, Go *Taq* polymerase were purchased from Promega, Madison (USA). The 5'fluorescent oligonucleotides primers were synthesized and purified by HPLC (Bio Basic, Canada)

6. Bacterial typing antisera

Anti-O and anti-K antibodies were obtained from Denka Seiken, Tokyo (Japan)**.**

7. Miscellaneous

Reagent	Company
DNA extraction kit	Geneaid, Taiwan
High melting agarose	Bio-Rad Laboratories, USA
Lamda ladder PFGE marker	New England Biolabs, USA
NotI restriction enzyme	ThermoFisher scientific, USA
1 kb DNA Ladder	New England Biolabs, USA
100 bp DNA Ladder	GenDireX, Taiwan

8. Equipments and instruments

Equipments / instruments	Company
Applied Biosystems 3100 genetic analyzer	Applied Biosystems, USA
Autoclave	Tomy, Japan
Autopipette	ThermoFisher scientific, Finland
Bioprofile image analysis system	Vilber Lourmat, France
Bionumeric software	Applied Maths, Belgium
Centrifuge	Eppendoff, Germany
Freezer (-20°C and -70°C)	Sanyo, Japan
Gel Documentation	Syngene, USA
Gel electrophoresis apparatus	Cleaver Scientific, United Kingdom
Hat air oven	BINDER, Germany
Incubator	Sanyo, Japan
Laminar airflow cabinet	Astec microflow, United Kingdom
McFarland Densitometer	BioSan, Latvia
Mini-centrifuge	Cubeé Gene Reach, Japan
Mini dry block heater	Major Science, Taiwan
NanoDrop	Maestrogen, Taiwan
PCR Thermocycle	Bio-Rad Laboratories, USA
PFGE electrophoresis apparatus	Bio-Rad Laboratories, USA
pH meter	Sartorius, Germany
Power supply	Bio-Rad Laboratories, USA
Refrigerator	Sanden intercool, Thailand
Refrigerated Microcentrifuge	Hettich, Germany
Shaker incubator	New brunswick scientific, USA
Water bath	Julabo, Germany

METHODS

This study was divided into three parts:

Part I: Characterization of pandemic *V. parahaemolyticus* **(Fig. 2.1)**

Part II: Molecular typing of pandemic *V. parahaemolyticus* **(Fig. 2.2)**

Part III: Development of MLVA for strain typing **(Fig. 2.3)**

Part I: Characterization of pandemic *V. parahaemolyticus*

In order to characterize pandemic *V. parahaemolyticus*, antimicrobial susceptibility test, detection of secretion genes (T3SS1, T3SS2, T6SS1, T6SS2), detection of thermostable direct hemolysin production, swarming motility, twitching motility, iron acquisition were performed as shown in **Fig. 2.1**.

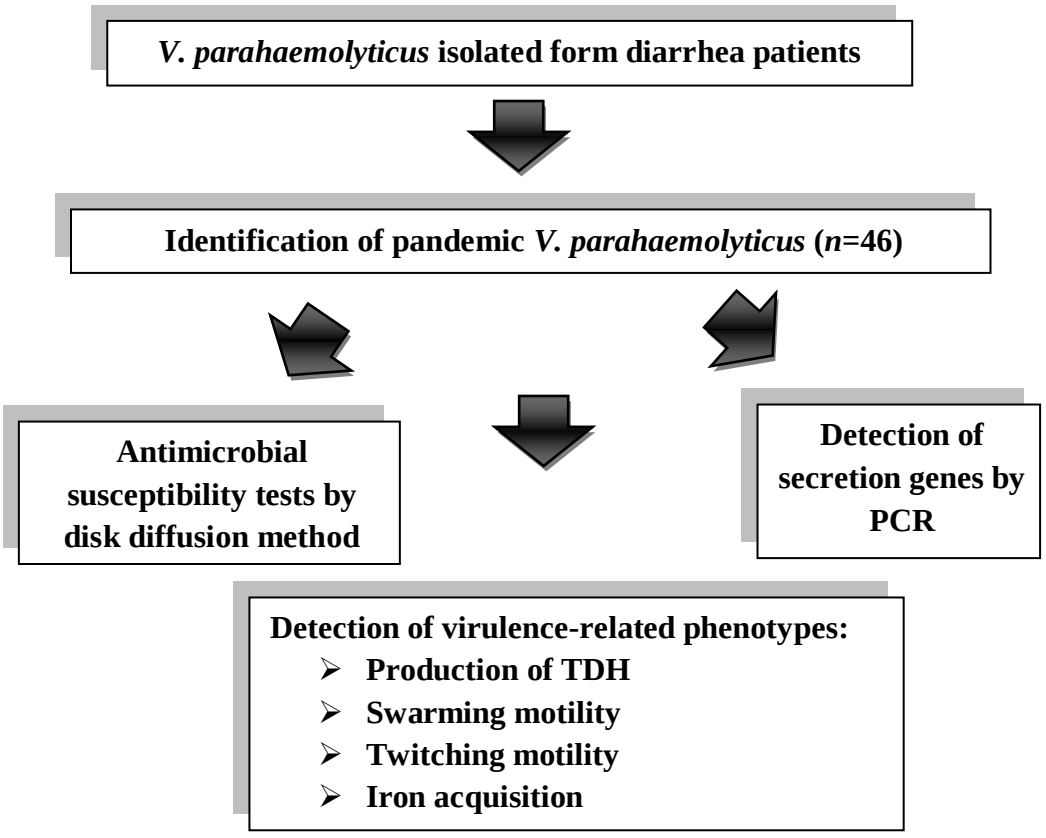

Fig. 2.1 Diagram represents the method of Part I: Characterization of pandemic *V. parahaemolyticus*

1. Identification of pandemic *V. parahaemolyticus*

All *V. parahaemolyticus* isolates were obtained from clinical samples in Hat Yai hospital, Songkhla during 2001-2012 as part of routine microbiological diagnostic. The species classification was confirmed by PCR targeting the *toxR* gene (Kim *et al.*, 1999). The identification of pandemic-clone specific characteristics (*toxRS* positive, *tdh* gene positive, and *trh* gene negative) was performed by PCR as described previously (Tada *et al.*, 1992, Matsumoto *et al.*, 2000) using primers listed in **Table 2.1**. The O (somatic) and K (capsular) serotypes were determined by the slide agglutination test using commercial anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan), according to the instructions of the manufacturer. A total of 46 pandemic *V. parahaemoltyicus* in various serotypes was identified as pandemic isolates and was included in this study **(Table 2.2)**. All strains were maintained at -80^{o}C using glycerol as cryoprotective agent for further analysis.

2. Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out based on the standard disk diffusion method on Mueller-Hilton agar plate according to the guidelines of the Clinical Laboratory and Standards Institute (CLSI) (CLSI, 2010, 2017). The antimicrobial disks (Oxoid, UK) were ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), norfloxacin (10 µg), and tetracyclin (30 µg). The data were interpreted as sensitive (S), intermediate (I), or resistant (R) following the methods of the CLSI.

Table 2.1 PCR Primers used in this study.

Target genes	Protein product	Primer name	Sequence (5'-3')	Product size (bp)	Reference
toxR	ToxR regulatory protein	tox R-F toxR-R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	(Kim *et al.*, 1999)
toxRS	ToxRS regulatory protein	GS-VP.1 GS-VP.2	TAATGAGGTAGAAACA ACGTAACGGGCCTACA	651	(Matsumoto *et al.*, 2000)
tdh	Thermostable direct hemolysin	tdh 1 tdh 2	GGTACTAAATGGCTGACATC CCACTACCACTCTCATATGC	251	(Tada *et al.*, 1992)
trh	TDH-related hemolysin	trh-F trh-R	GGCTCAAAATGGTTAAGCG CATTTCCGCTCTCATATGC	250	(Tada *et al.*, 1992)
*vcrD*1	T3SS1-structure VcrD1	vcrD1F vcrD1R	CTGCTGGTCTTGTTCGCTCT TCTGGTCGCTTCCTTCTGTG	493	(Yu *et al.*, 2003)
vopQ	T3SS1-effector VopQ	VP1680F VP1680R	GCCGAAGCGTATCATCATCAACTC CACAGAGCTTACACCAAACGTACC	183	(Makino *et al.*, 2003)
*vcrD*2	T3SS2-structure VcrD2	vcrD2(F) vcrD2(R)	GGTAACACTGCCTGGTGTGGTCATCG GTCTCTCAAAGTCTTCAAACTCACCTGC	196	(Okada *et al.*, 2009)
vopC	T3SS2-effector VopC	VPA1321F VPA1321R	GGTTAGTGAATCCAACCAAACCGC TTGCCGTGCATGTCATACAACCAG	485	(Makino *et al.*, 2003)
*vipA*1	T6SS1-structure VipA1	vipA1F vipA1R	CACGTGACGGCTCGGTGG CTCTTCTTTCGCGTCTTGGTCG	500	(Salomon *et al.*, 2013)
*vipA*2	T6SS2-structure VipA2	vipA2F vipA2R	CGAGTATCCACTCGAAACTTTC TTCTGCTCCCTCAGTACTTTCTG	524	(Salomon *et al.*, 2013)

Table 2.2 Pandemic (GS^+, *tdh*$^+$, *trh*$^-$) *V. parahaemolyticus* isolates used in this study.

Serotype	Year of isolation	No.	Strain	Presence of gene		GS-PCR
				tdh	***trh***	
Pandemic (46)						
O1:KUT	2001	1	PSU291	+	–	+
	2002	5	PSU732, PSU736, PSU748, PSU751, PSU752	+	–	+
	2003	2	PSU938, PSU974	+	–	+
	2004	4	PSU1297, PSU1352, PSU1601, PSU1918	+	–	+
	2005	3	PSU2030, PSU2453, PSU2487	+	–	+
	2006	2	PSU3031, PSU3460	+	–	+
	2008	2	PSU3916, PSU3972	+	–	+
	2009	7	PSU4792, PSU4793, PSU4915, PSU4918, PSU4994, PSU4998, PSU5009	+	–	+
	2011	5	PSU5126, PSU5139, PSU5140, PSU5150, PSU5221	+	–	+
	2012	1	PSU5291	+	–	+
O1:K25	2003	2	PSU1031, PSU1032	+	–	+
	2006	1	PSU2667	+	–	+
	2008	5	PSU3866, PSU3880, PSU3894, PSU3939, PSU3949	+	–	+
O3:K6	2008	6	PSU3868, PSU3872, PSU3887, PSU3892, PSU3896, PSU3921	+	–	+

3. Detection of secretion genes

Chromosomal DNA of pandemic *V. parahaemolyticus* was extracted by boiling method (Thaithongnum *et al.*, 2006). The detection of T3SSs and T6SSs genes (*vcrD1*, *vopQ*, *vcrD2*, *vopC*, *vipA1*, and *vipA2*) was performed by PCR method as previously described (Laohaprertthisan *et al.*, 2003, Makino *et al.*, 2003, Yu *et al.*, 2003, Okada *et al.*, 2009) using primers listed in **Table 2.1**.

4. Production of thermostable direct hemolysin (TDH)

Pandemic *V. parahaemolyticus* isolates were incubated on Tryptic soy agar (TSA) supplemented with 1% NaCl at 37°C for 18 h. Then, single colony was spotted on a special blood agar (Wagatsuma agar) and incubated at 37°C for 18-35 h. TDH production was detected by the presence of a clear zone around the colony (Kanagawa phenomenon) (Hara-Kudo *et al.*, 2003).

5. Swarming motility

The swarming assay was performed on swarming plate which is prepared by adding 6 g of Bacto agar to 1,000 ml of Luria Bertani (LB) medium. Briefly, pandemic *V. parahaemolyticus* isolates were cultured in LB broth at 37°C for 18 h. After that the bacteria were adjusted to 0.5 McFarland standards. Then 2 µl of bacteria were dropped on swarming plate. The diameters of swarm zones were determined after incubation at 37°C for 4 and 8 h.(Inoue *et al.*, 2007).

6. Twitching motility

The twitching motility assay was carried out on LB agar supplemented with 1.5% NaCl. Briefly, pandemic *V. parahaemolyticus* isolates were cultured on TSA supplemented with 1% NaCl at 37°C for 18 h. The single colony was stabbed through an LB agar supplemented with 1.5% NaCl and incubated at 37°C for 24 h. After incubation, the agar layers were removed and twitching zones were visualized at the agar plate interface by staining the spread zones with 0.1% of crystal violet (Antunes *et al.*, 2011).

7. Iron acquisition

The iron acquisition test was performed as previously described (Wong *et al.*, 1996, Almeida *et al.*, 2008) with some minor modifications. Briefly, 2 µl aliquots of hemoglobin (155 µM and 50 µM) were spotted on melted rich medium (1% peptone, 3% NaCl, 0.5% Na_2HPO_4, 0.5% glucose, 1.5% agar) supplemented with 150 µM of 2, 2'-dipyridyl to eliminate the iron. After leaving the plate for 1 h, 2 µl of *V. parahaemolyticus* suspension was dropped and incubated at 37°C, overnight. The growth of bacteria was investigated directly with the naked eye and under a microscope.

Part II: Molecular typing of pandemic *V. parahaemolyticus*

In order to distinguish among the isolates of pandemic *V. parahaemolyticus*, various molecular typing methods (AP-PCR, ERIC-PCR, and PFGE) were performed as shown in **Fig. 2.2**.

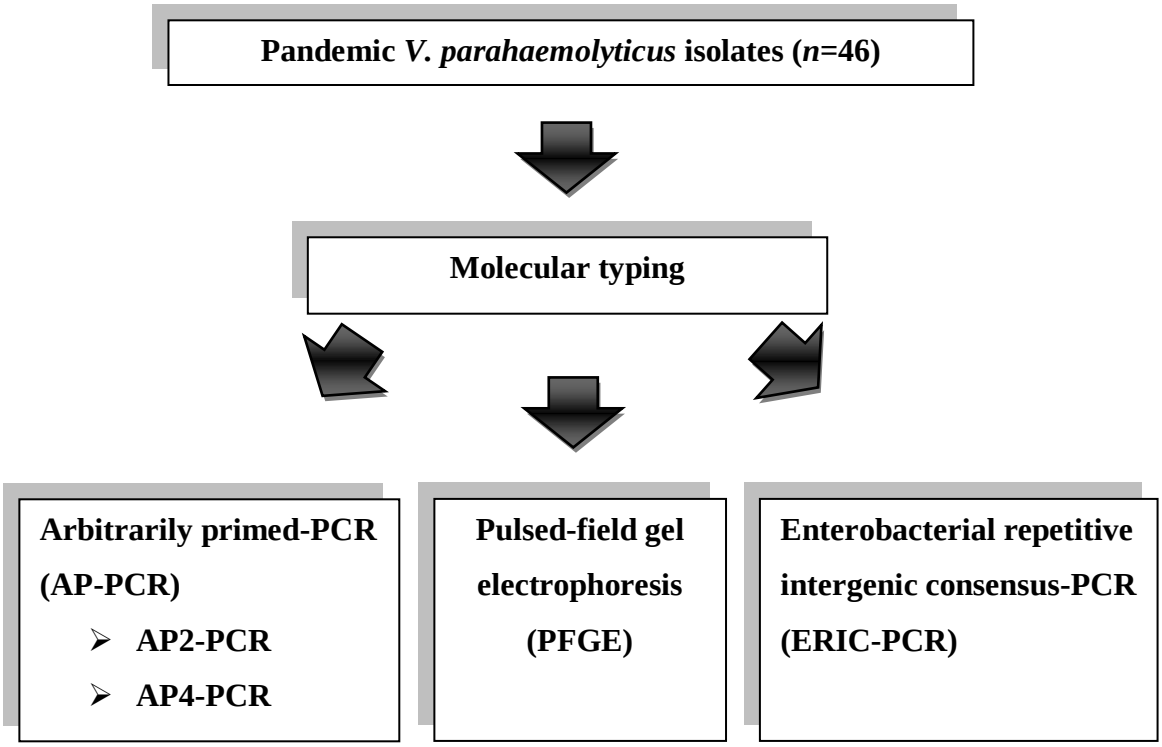

Fig. 2.2 Diagram represents the method of Part II: Molecular typing of pandemic *V. parahaemolyticus*

1. Arbitrarily primed-PCR (AP-PCR)

AP-PCR was carried out according to the method described previously using primer 2 (5'-GTTTCGCTCC-3') and primer 4 (5'-AAGAGCCCGT-3') with minor modifications. Amplification was conducted with 3µl of 10X buffer, 4 µl of 2.5 mM dNTP, 5 µl of 5 µM of a primer (primer 2 or 4), 0.5 µl of 5 units of *Takara Ex Taq*™ DNA polymerase, and 25 ng of purified DNA in a final volume of 30 µl.

PCR run was made in an automatic thermal cycler (Bio-Rad, USA). Conditions for AP-PCR were as follows: pre-denaturation at 95°C for 4 min, 45 cycles of a three-step cycling protocol: 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 7 min.

PCR products were visualized after electrophoresis in 1.5% agarose gel at a voltage of 100 V cm^{-1} for 5 min follow by 15 mA for 10 h. (Williams *et al.*, 1990). The dendrogram was constructed using a Bioprofile image analysis system (Vilber Lourmat, France).

2. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

ERIC-PCR was performed using primers ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGG TGAGCG-3'). Amplification was conducted with 2.5 µl 10X PCR buffer, 1.0 µl of each primer, 0.5 µl of 5 units of *Takara Ex Taq* DNA polymerase and 2 µl template DNA in a final volume of 25 µl.

PCRs runs were made in an automatic thermal cycle (Bio-Rad, USA). Conditions for AP-PCR were as follows: pre-denaturation at 95°C for 7 min, 30 cycles of a three-step cycling protocol: 90°C for 30 sec, 58°C for 1 min, and 68°C for 8 min, and a final extension at 68°C for 16 min.

PCR products were visualized after electrophoresis in 1.5% agarose gel at a voltage of 100 V cm^{-1} for 5 min follow by 50 V cm^{-1} for 6 h.(Zulkifli *et al.*, 2009). The dendrogram was constructed using a Bioprofile image analysis system (Vilber Lourmat, France).

3. Pulsed-field gel electrophoresis (PFGE)

PFGE of pandemic isolates was carried out as described previously with slight modification (Wang *et al.*, 2008). Briefly, agarose-embedded genomic DNA was digested with *Not*I restriction enzyme (NEB, Massachusetts, USA), and the digested DNA fragments were separated by electrophoresis using 1% Pulse-Field Certified agarose in 0.5X Tris–borate-ethylene diamine tetraacetic acid running buffer at 14°C for19 h with use of the CHEF-DRIII system (Bio-Rad Laboratories, California, USA). Electrophoresis was performed with pulse times of 2.2 to 54.2 s, a 120° angle, and a 6 V/cm gradient.

The gel was stained with ethidium bromide and the DNA patterns were analyzed both visually and with Bionumeric software (v.7.0) (Applied Maths, Sint-Martens-Latem, Belgium). Dendrogram based on the Dice coefficient was constructed by unweighted pairing group method with arithmetic averages (UPGMA).

Part III: Development of MLVA for strain typing

In order to differentiate pandemic O1:KUT *V. parahaemolyticus*, MLVA method was developed and evaluated as shown in **Fig. 2.3**.

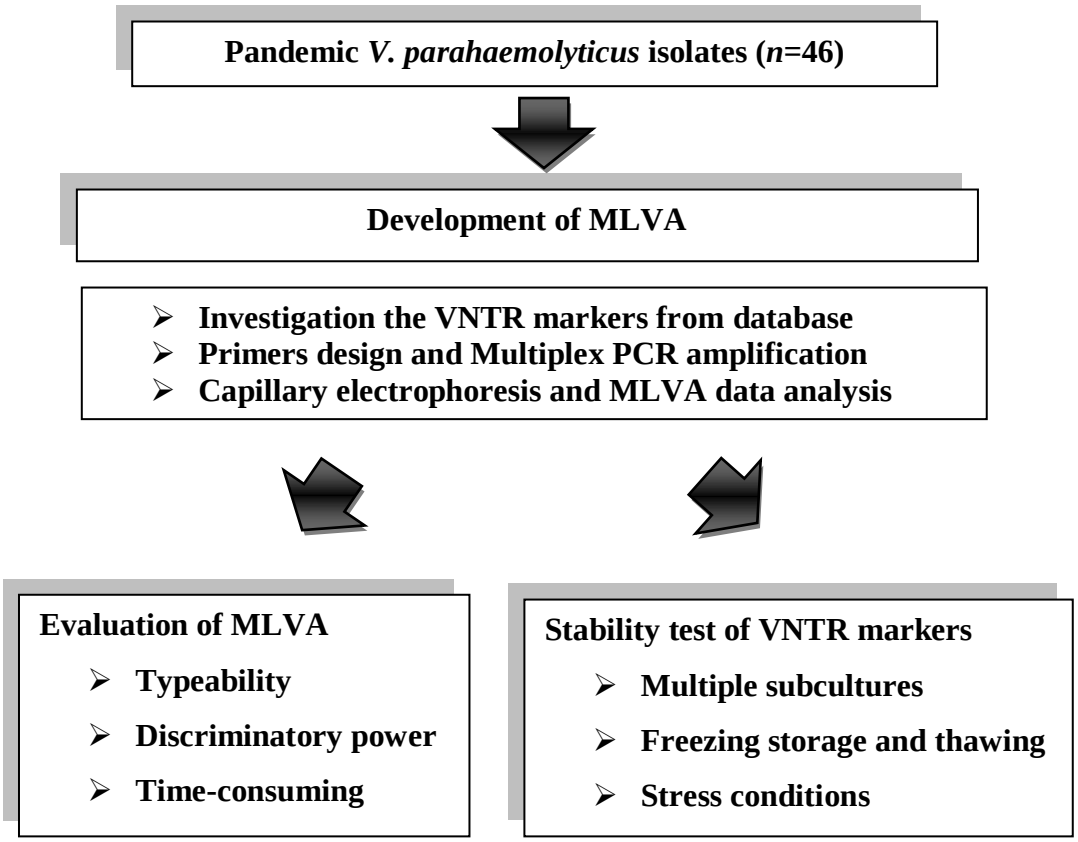

Fig. 2.3 Diagram represents the method of Part III: Development of MLVA for strain typing *V. parahaemolyticus*

1. Development of MLVA

1.1 Investigation the VNTR markers from database

For selection of VNTR markers, the genome sequence of *V. parahaemolyticus* RIMD2210633 was screened to locate the presence of VNTR sequences (http://minisatellites.u-psud.fr) (Denœud and Vergnaud, 2004) according to the following criteria: (i) a repeat unit range of 3 to 9 bp, (ii) a minimum copy numbers of 10, (iii) a minimum repeat size of 50 bp, and (iv) percent matches between 80 and 100. Three VNTR loci common to the previous studies (TR1, TR2, and TR4) (Kimura *et al.*, 2008, Harth-Chu *et al.*, 2009, Ansede-bermejo *et al.*, 2010, Lüdeke *et al.*, 2015, Jiang *et al.*, 2016) and one novel VNTR loci (TR3) were identified (Table 3.4).

1.2 Primers design and VNTR amplification

Primer sets specific for those 4 VNTR loci were designed with Primer3Plus software (http://primer3plus.com) (Rozen and Skaletsky, 1999). The 5'forward primers were labeled with distinctive fluorescent dyes (VIC, PET, NED, or FAM) to generate multiplex PCR products which were further separated by multi-colored capillary electrophoresis.

In VNTR amplification, bacterial DNA was extracted by boiling method (Kayali *et al.*, 2015) and subjected to a multiplex PCR amplification using primer mix to simultaneously amplify the VNTR loci. Each 30 μL multiplex PCR mixture contained 1× GoTaq® Reaction buffer, 0.5 U of GoTaq® DNA polymerase (Promega, Wisconsin, USA), 200 μM of each deoxynucleoside triphosphate (dNTP) (Promega, Wisconsin, USA), 0.2 μM of each primer, and 2 μL of template DNA. The multiplex amplification was carried out on a T100 thermal cycler (Bio-Rad Laboratories, California, USA) using a thermal profile of beginning at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min. Each run ended with a final elongation step of 68°C for 5 min.

1.3 Capillary electrophoresis and MLVA data analysis

The PCR products were analyzed by capillary electrophoresis using ABI 3130 Genetic Analyzer (Applied BioSystems, USA). Fragment sizes from each VNTR loci were determined by GeneMapper software (v.4.1) (Applied BioSystems, USA) and were converted into VNTR copy numbers using the following equation (Slack *et al.*, 2005):

Number of repeats (bp) = [fragment size (bp) – flanking regions (bp)] / repeat size (bp)

The combination of 4 numbers reflecting the number of repeats in 4 VNTR loci generated an MLVA profile. A dendrogram was constructed based on the categorical (mapping) with UPGMA method by Bionumeric software (v.7.0) (Applied Maths, Sint-Martens-Latem, Belgium).

The polymorphism information index or diversity index (DI) of each VNTR locus was calculated individually using the formula; $D = 1-\Sigma$ (allele frequency)^2 (Weir, 1990). The discriminatory power was calculated as previously described (Castañeda *et al.*, 2005). The congruence between typing methods was determined using the Wallace coefficients (W) (Fowlkes and Mallows, 1983).

2. Evaluation of MLVA

The typeability, discriminatory power, and time-consuming of MLVA and other typing methods (AP-PCR, ERIC-PCR, PFGE) was calculated by previously published formula (Castañeda *et al.*, 2005) as shown in **Table 2.3**.

Table 2.3 The formula for calculation the typeability, discriminatory power, and time-consuming of molecular typing techniques**.**

Properties	**Formula**
Typeability (T)	$T = N_t/N$
Discriminatory power (D)	$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j-1)$
Time-consuming (t)	DNA extraction + Time of PCR + Time of gel electrophoresis

N_t = number of isolates assigned a type

N = number of isolates tested

s = number of different types

n_j = number of isolates belonging to the *j*th type

3. Stability test of VNTR markers

A representative *V. parahaemolyticus* isolate was selected to investigate the stability of VNTR markers by multiple subcultures test, freezing storage and thawing test and stress conditions test.

3.1 Multiple subcultures (Boxrud *et al.*, 2007)

The single colony of *V. parahaemolyticus* was cultured on TSA supplemented with 1% NaCl at 37°C for 18 h. Five individual colonies were selected to culture on TSA supplemented with 1% NaCl as the initial plate, then the isolates were subcultured every day for 10 days and 10 colonies from each final subculture plate were selected to investigate the stability of VNTR markers.

3.2 Freezing storage and thawing (Malorny *et al.*, 2008)

V. parahaemolyticus was frozen in glycerol stock at -80°C overnight, and thawing was performed. Freezing and thawing were repeated for 9 cycles. Afterward, bacteria were grown on TSA supplemented with 1% NaCl at 37°C for 18 h and 5 colonies were picked again and repeated freezing and thawing for 10 cycles. Then, five subcultures were carried out and 10 colonies from each subculture were selected to investigate the stability of VNTR markers.

3.3 Stress conditions (Wong *et al.*, 2000, Chen *et al.*, 2009, Cooley *et al.*, 2010)

V. parahaemolyticus was grown in TSA supplemented with 1% NaCl at 37°C for 18 h, and 5 colonies were selected to investigate the stability of VNTR markers in stress conditions (TSB supplemented with 1% NaCl) including temperature, pH, and salt stress conditions as follow:

1. Temperature stress: 5 and 44°C.
2. pH stress: pH 4.8 and 11.
3. Salt stress: 0.5 and 8 % NaCl.

After incubation for 24 h, a loop of culture was streaked on TSA supplemented with 1% NaCl and 10 colonies from each subculture were picked to investigate the VNTR markers.

CHAPTER 3

RESULTS

Part I: Characterization of pandemic *V. parahaemolyticus*

1. Antimicrobial susceptibility profiles

The antimicrobial susceptibility rates of *V. parahaemolyticus* isolates are shown in **Table 3.1**. All of the isolates showed resistance to ampicillin. The resistance to ciprofloxacin (15%) and norfloxacin (2%) were found among the isolates obtained in 2001, 2003, and 2008. Intermediate susceptibility to choleramphenicol, ciprofloxacin, cotrimoxazole, or norfloxacin was observed in 2 (4%), 28 (61%), 1 (2%) and 10 (22%) isolates, respectively. None of the isolates showed resistance to chloramphenicol, cotrimoxazole, or tetracycline. In this study, thirty-nine isolates (85%) were found to be susceptible to all antimicrobials tested apart from ampicillin.

Table 3.1 Antimicrobial susceptibility profiles of *V. parahaemolyticus* isolates.

Antimicrobial agent	*V. parahaemolyticus* (*n* = 46)		
	No. of Resistant (%)	No. of Intermidiate (%)	No. of Sensitive (%)
Ampicillin (AMP)	46 (100)	–	–
Chloramphenicol (CHL)	–	2 (4)	44 (96)
Ciprofloxacin (CIP)	7 (15)	28 (61)	11 (24)
Cotrimoxazole (CO)	–	1 (2)	45 (98)
Norfloxacin (NOR)	1 (2)	10 (22)	35 (76)
Tetracycline (TET)	–	–	46 (100)

2. Presence of secretion genes

The presence of T3SS and T6SS genes is presented in **Table 3.2**. *vopQ* gene encoding the effector protein of T3SS1 was identified in all isolates. However, 91% of the isolates were positive for the T3SS1 structural gene, *vcrD1*. For T3SS2, the effector and structural genes, *vopC* and *vcrD2*, were detected in 96 and 91% of the isolates, respectively. The majority of *V. parahaemolyticus* isolates contained all two T3SS1 (91%) and T3SS2 (89%) genes. The prevalence of T6SS genes was high but lower than that of T3SS. In this study, 37 (80%) and 31 (67%) isolates contained all four T3SS and two T6SS genes, respectively.

Table 3.2 Characteristics of *V. parahaemolyticus* isolates used in this study.

Year	Isolate	Serotype	Secretion genes					
			T3SS1		T3SS2		T6SS1	T6SS2
			vcrD1	*vopQ*	*vcrD2*	*vopC*	*vipA1*	*vipA2*
2001	PSU 291	O1:KUT	+	+	+	+	+	-
2002	PSU 732	O1:KUT	-	+	+	+	+	+
	PSU 736	O1:KUT	-	+	+	+	+	+
	PSU 748	O1:KUT	+	+	+	+	+	+
	PSU 751	O1:KUT	+	+	+	+	+	+
	PSU 752	O1:KUT	+	+	+	+	+	+
2003	PSU 938	O1:KUT	+	+	+	+	+	+
	PSU 974	O1:KUT	+	+	-	+	+	+
	PSU 1031	O1:K25	+	+	-	+	+	-
	PSU 1032	O1:K25	+	+	+	+	+	+
2004	PSU 1297	O1:KUT	+	+	+	+	+	+
	PSU 1352	O1:KUT	+	+	+	+	+	+
	PSU 1601	O1:KUT	+	+	+	+	-	-
	PSU 1918	O1:KUT	+	+	+	+	+	-
2005	PSU 2030	O1:KUT	+	+	+	+	+	+
	PSU 2453	O1:KUT	+	+	+	+	+	-
	PSU 2487	O1:KUT	+	+	+	+	+	-
2006	PSU 3031	O1:KUT	+	+	+	+	+	-
	PSU 3460	O1:KUT	+	+	+	+	+	-
	PSU 2667	O1:K25	+	+	+	+	+	-
2008	PSU 3916	O1:KUT	+	+	+	+	+	-
	PSU 3972	O1:KUT	+	+	+	+	+	+
	PSU 3866	O1:K25	+	+	-	+	-	-
	PSU 3880	O1:K25	+	+	+	+	+	+
	PSU 3894	O1:K25	+	+	+	+	+	+
	PSU 3939	O1:K25	+	+	+	+	+	+
	PSU 3949	O1:K25	+	+	+	+	+	+
	PSU 3868	O3:K6	+	+	+	+	+	+
	PSU 3872	O3:K6	+	+	+	+	+	+
	PSU 3887	O3:K6	+	+	+	+	+	+
	PSU 3892	O3:K6	+	+	+	+	+	+
	PSU 3896	O3:K6	+	+	+	+	+	+
	PSU 3921	O3:K6	+	+	+	+	+	+

Table 3.2 (continued).

Year	Isolate	Serotype	Secretion genes					
			T3SS1		T3SS2		T6SS1	T6SS2
			vcrD1	*vopQ*	*vcrD2*	*vopC*	*vipA1*	*vipA2*
2009	PSU 4792	O1:KUT	+	+	+	+	-	+
	PSU 4793	O1:KUT	+	+	+	-	+	+
	PSU 4915	O1:KUT	+	+	+	+	+	+
	PSU 4918	O1:KUT	+	+	+	+	+	+
	PSU 4994	O1:KUT	+	+	-	-	-	+
	PSU 4998	O1:KUT	+	+	+	+	+	+
	PSU 5009	O1:KUT	+	+	+	+	-	+
2011	PSU 5126	O1:KUT	-	+	+	+	+	+
	PSU 5139	O1:KUT	+	+	+	+	+	+
	PSU 5140	O1:KUT	+	+	+	+	+	+
	PSU 5150	O1:KUT	-	+	+	+	+	-
	PSU 5221	O1:KUT	+	+	+	+	+	+
2012	PSU 5291	O1:KUT	+	+	+	+	+	+
		Prevalence (%)	91	100	91	96	89	74

[a] +, present; –, absent.

3. Virulence-related characteristics

The virulence-related characteristics of *V. parahaemolyticus* were shown in **Table 3.3**. All *V. parahaemolyticus* isolates were positive for Kanagawa phenomenon which indicates the TDH production. The hemolytic zones observed on Wagatsuma agar ranged from 8.2 to 15.3 mm.

All *V. parahaemolyticus* isolates possessed twitching motility, while 91% possessed swarming motility with a migration zone of 8 to 49 mm. The twitching zones were ranged between 4 to 34 mm. Some isolates exhibited swarming motility with burst formation **(Fig. 3.1)**.

For iron acquisition, all except one isolate were able to grow on iron-depleted medium supplemented with 155 µM hemoglobin **(Fig. 3.2)**. It was found that more than 80% of the isolates were still able to grow well in the presence of 50 µM hemoglobin.

Table 3.3 Groups of the *V. parahaemolyticus* isolates according to the results of virulence-related phenotypes.

Groups	Number of isolate	Isolate	Serotype	Kanagawa phenomenon[a]	Motility phenotype		Growth[d]	
					Swarming[b]	Twitching[c]	155 μM hemoglobin	50 μM hemoglobin
1	2	PSU 3949, 3892	O3:K6	+	++	++	+	+
2	3	PSU 3896, 3921	O3:K6	+	++	++	+	–
		PSU 5139	O1:KUT					
3	16	PSU 3868, 3872	O3:K6	+	++	+	+	+
		PSU 1031, 1032, 3880, 3894	O1:K25					
		PSU 736, 751, 752, 938, 2487, 3460, 3916, 4918, 5009, 5150	O1:KUT					
4	6	PSU 3887	O3:K6	+	+	++	+	+
		PSU 748, 1352, 1601, 1918, 4915	O1:KUT					
5	1	PSU 4793	O1:KUT	+	+	++	–	–
6	12	PSU 3866, 3939	O1:K25	+	+	+	+	+
		PSU 974, 1297, 3031, 3972, 4792, 4998, 5126, 5140, 5221, 5291	O1:KUT					
7	2	PSU 2667	O1:K25	+	+	+	+	–
		PSU 291	O1:KUT					
8	2	PSU 732, 2453	O1:KUT	+	–	++	+	–
9	1	PSU 2030	O1:KUT	+	–	++	+	+
10	1	PSU 4994	O1:KUT	+	–	+	+	+

[a] +, positive; –, negative.

[b] ++, swarm zone was > 23 mm (above mean value); +, swarm zone was > 5 mm; –, no swarm zone.

[c] ++, twitch zone was > 13 mm (above mean value); +, twitch zone was > 4 mm; –, no twitch zone.

[d] +, growth; –, no growth.

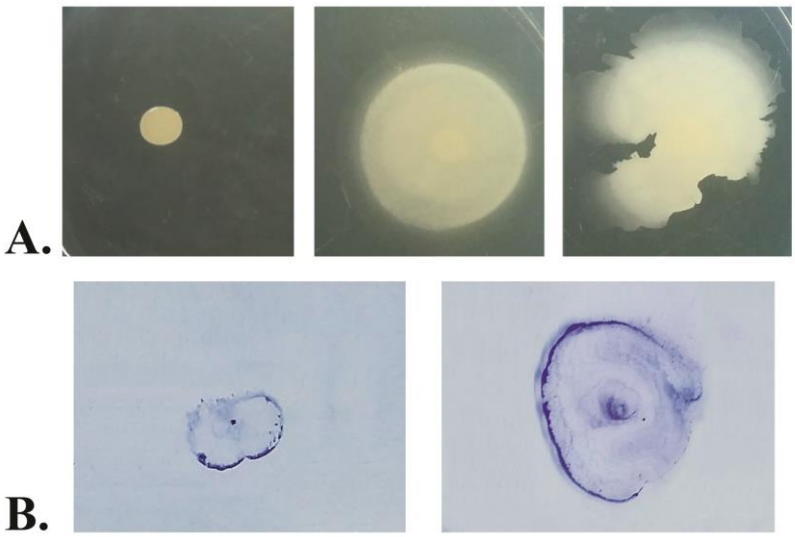

Fig. 3.1 Representative swarming and twitching motility of *V. parahaemolyticus*. (A) Swarming motility: non-swarming (left), swarming (middle) and burst formation (right). (B) Twitching motility: +, twitch zone was > 4 mm (left); ++, twitch zone was > 13 mm (above mean value) (right).

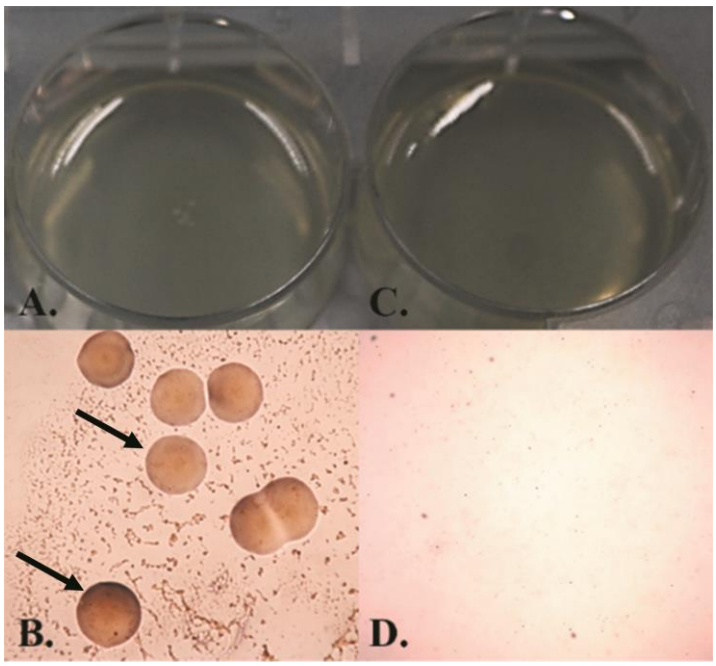

Fig. 3.2 Iron acquisition of representative *V. parahaemolyticus* isolates on melted rich medium supplemented with 2, 2'-dipyridyl and 155 µM of hemoglobin (A: 1X; B: 4X) and negative control (C: 1X; D: 4X) Black arrows indicate *V. parahaemolyticus* colonies observed under a microscope.

Part II: Molecular typing of pandemic *V. parahaemolyticus*

1. AP-PCR analysis

1.1 AP2-PCR

AP2-PCR generated 11 unique fingerptinting profiles with the number of DNA band ranged between 4-12. At 75% similarity of AP2-PCR profiles, *V. parahaemolyticus* isolates were differentiated into 2 clusters (A and B) **(Fig. 3.3)**. Interestingly, cluster A composed of 19 isolates of pandemic O1:K25 and O1:KUT *V. parahaemolyticus* isolated during 2001-2006 whereas other 25 isolates obtained after 2008 were grouped in cluster B together with all O3:K6 isolates and 2 remaining isolates cannot be grouped

1.2 AP4-PCR

AP4-PCR generated 5 unique fingerptinting profiles with the number of DNA band ranged between 7-11. At 75% similarity of AP4-PCR profiles, all forty-six *V. parahaemolyticus* isolates were classified into same cluster (cluster A) **(Fig. 3.4)**.

2. ERIC-PCR analysis

ERIC-PCR generated only 3 unique fingerptinting profiles with the number of DNA band ranged between 5-11. At 75% similarity of ERIC-PCR profiles, all forty-six *V. parahaemolyticus* isolates were classified into same cluster (cluster A) **(Fig. 3.5)**.

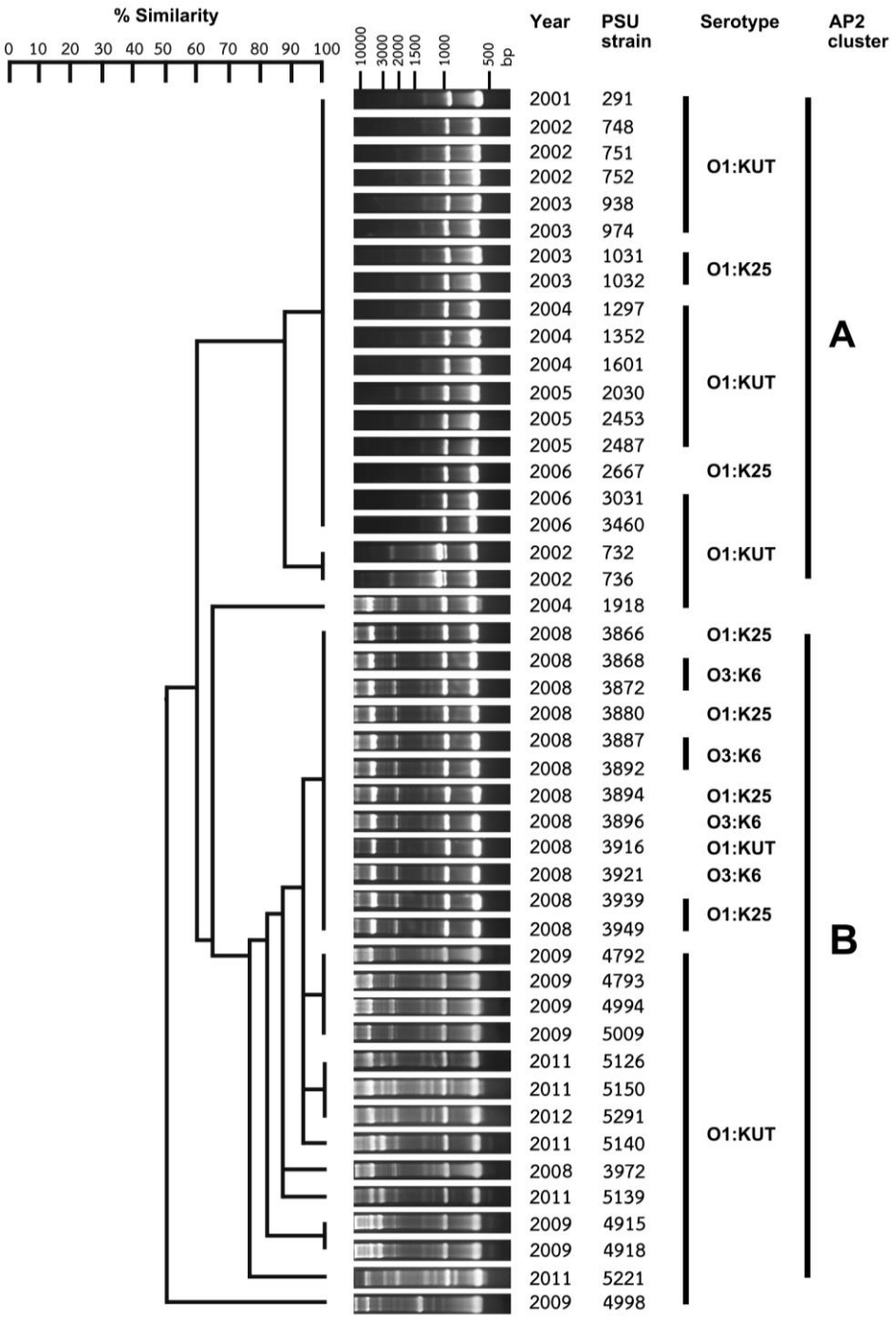

Fig. 3.3 Dendrogram generated with AP2-PCR patterns of the 46 pandemic *V. parahaemolyticus* isolates. Year of isolation and serotype of the isolate are included along each lane. A and B indicate AP2-PCR clusters at 75 % similarit

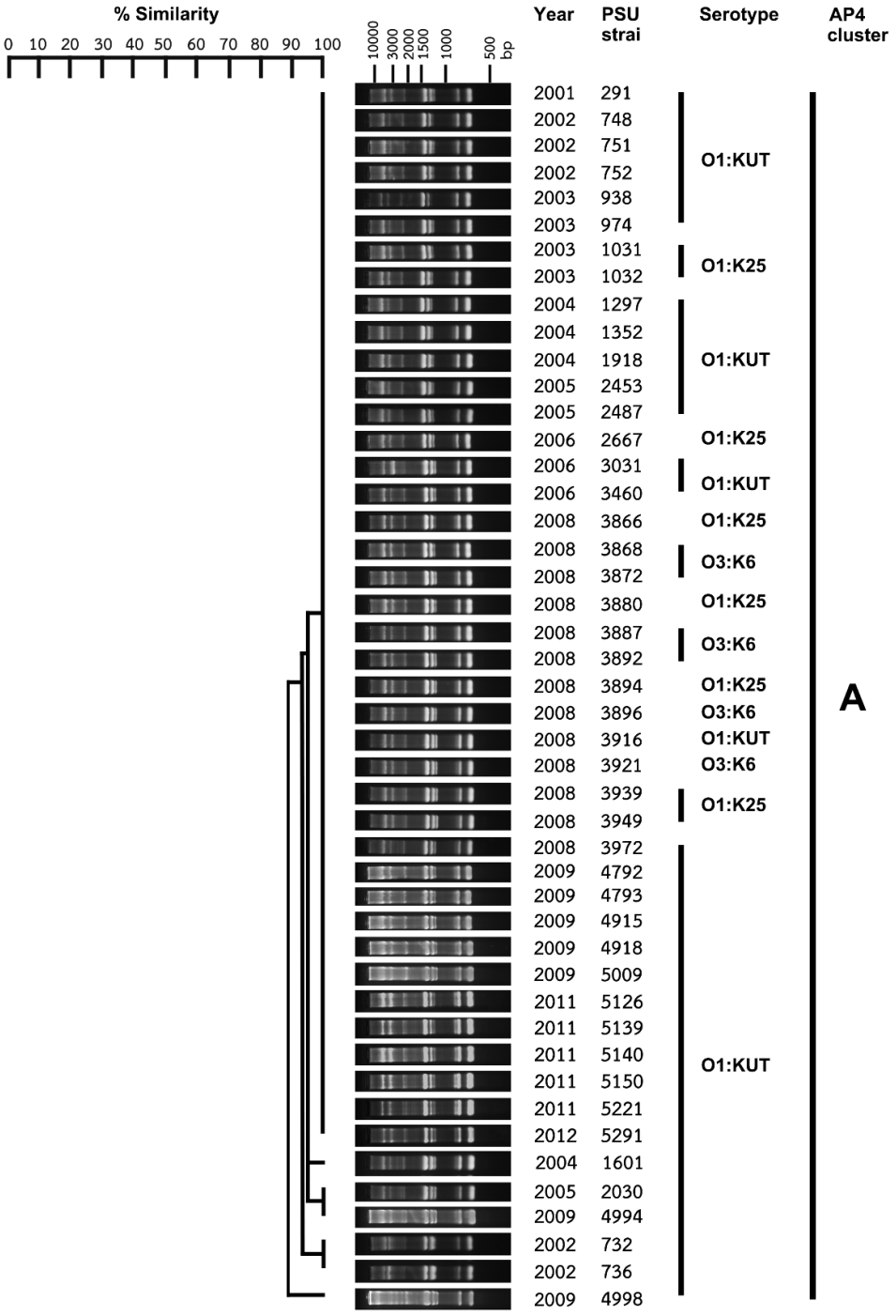

Fig. 3.4 Dendrogram generated with AP4-PCR patterns of the 46 pandemic *V. parahaemolyticus* isolates. Year of isolation and serotype of the isolate are included along each lane. Letter A indicates AP4-PCR cluster at 75 % similarity.

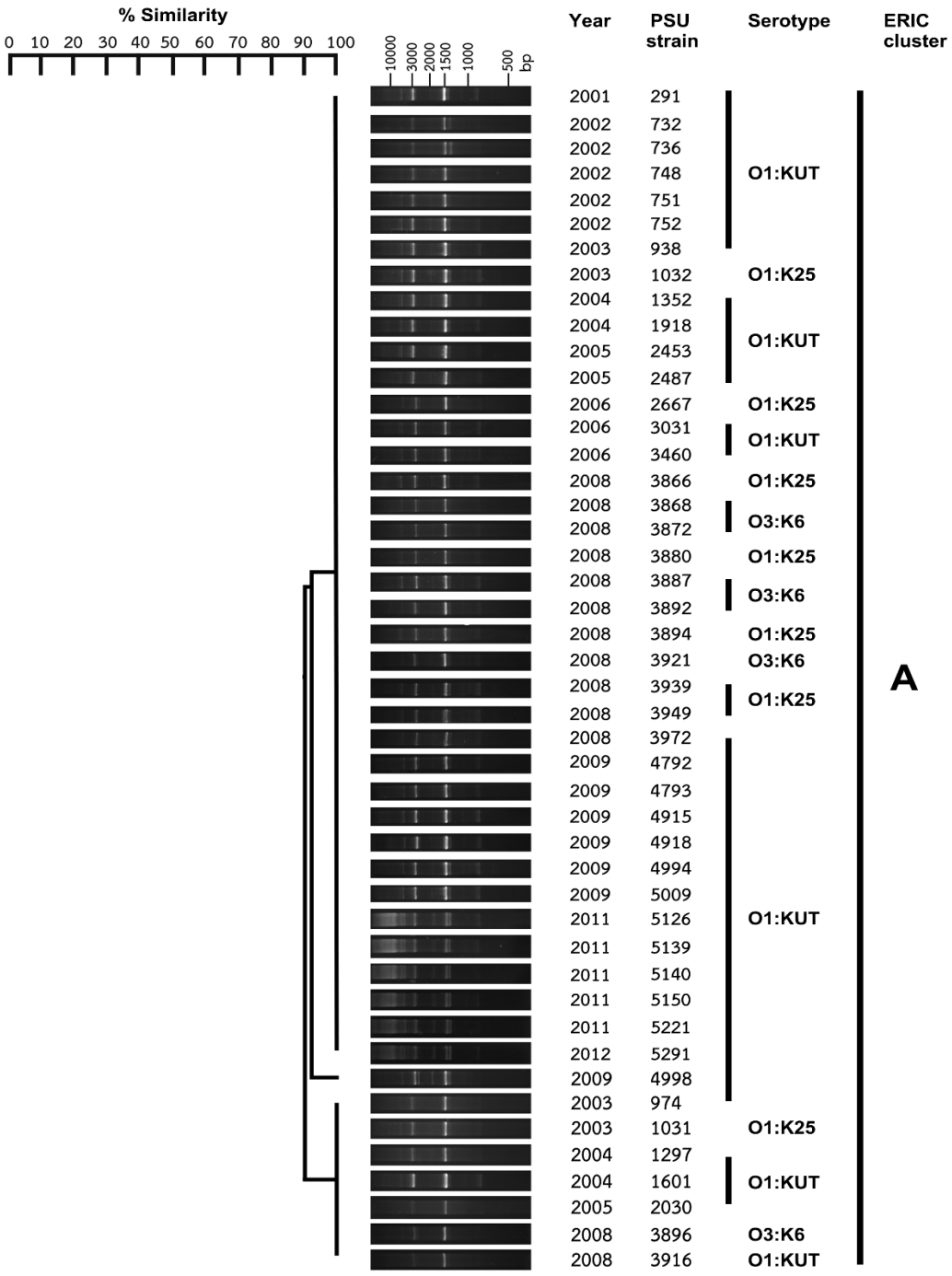

Fig. 3.5 Dendrogram generated with ERIC -PCR patterns of the 46 pandemic *V. parahaemolyticus* isolates. Year of isolation and serotype of the isolate are included along each lane. Letter A indicates ERIC-PCR cluster at 75 % similarity.

3. PFGE analysis

The *Not*I PFGE of 46 pandemic *V. parahaemolyticus* isolates was performed and 16 distinct PFGE types were generated **(Fig. 3.6)**. The discriminatory power of PFGE was 0.89. At 80% cutoff, all isolates obtained during 2001 to 2006 were grouped together regardless of serotypes.

It is of interest that 12 isolates of O1:KUT obtained during 2001 to 2005 exhibited indistinguishable PFGE patterns. All O3:K6 isolates had similar PFGE pattern and were clustered closely with four of five O1:K25 isolates obtained in the same year. New PFGE types were observed among the O:KUT isolates obtained during 2009-2012. Similarity values of more than 70% were observed among these isolates.

A minimum spanning tree (MST) based on PFGE illustrated that phylogenetic groupings of all pandemic *V. parahaemolyticus* isolates were well correlated with serotype and period of isolation **(Fig. 3.7A and B)**. However, one O1:K25 isolate were grouped together with seven O1:KUT isolates **(Fig. 3.7A)**.

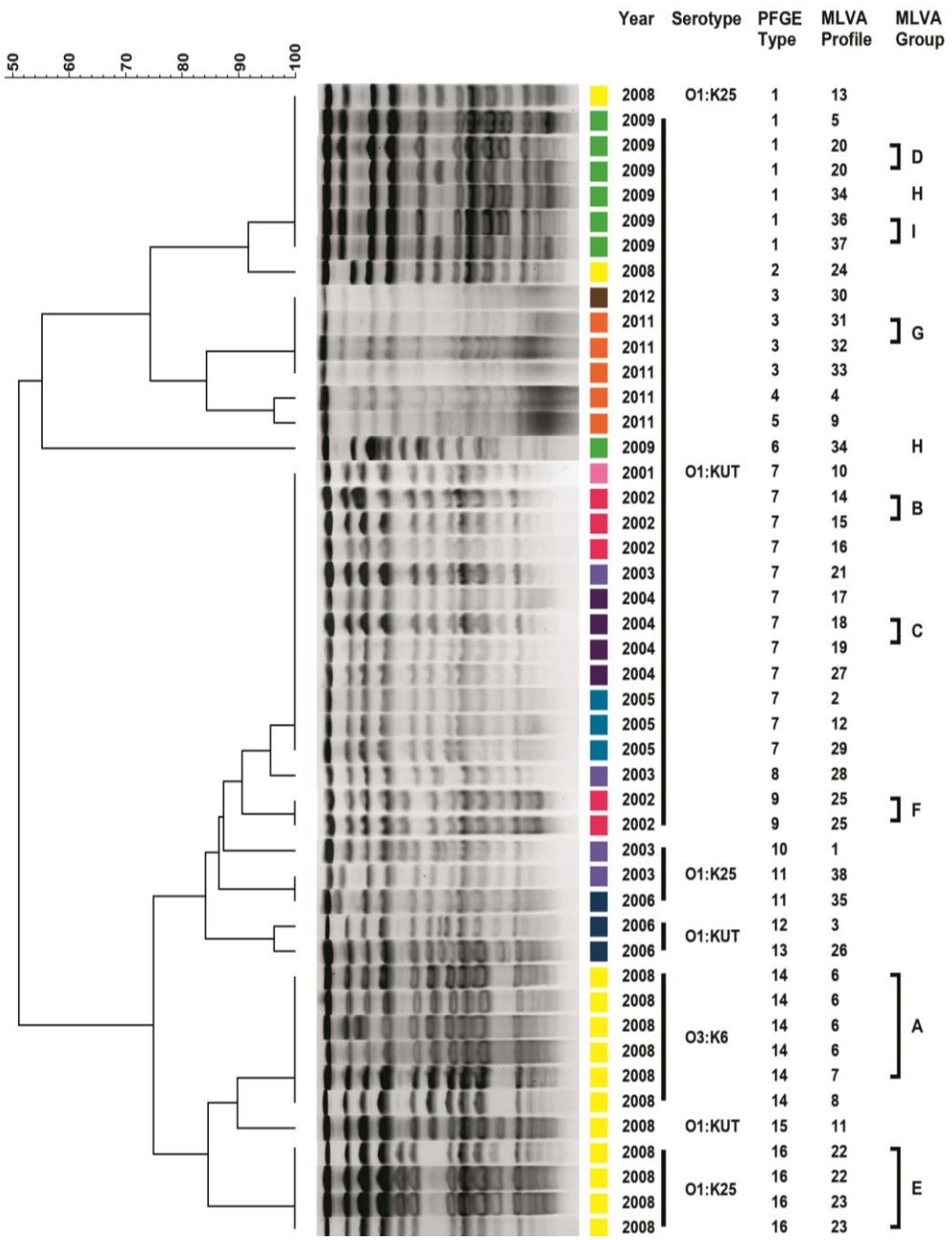

Fig. 3.6 Dendrogram generated with *Not*I-digested PFGE patterns of the 46 pandemic *V. parahaemolyticus* isolates. Colors within the squares correspond to year of isolation. Year of isolation and serotype of the isolate are included along each PFGE lane. MLVA profiles and MLVA group are provided for direct comparison.

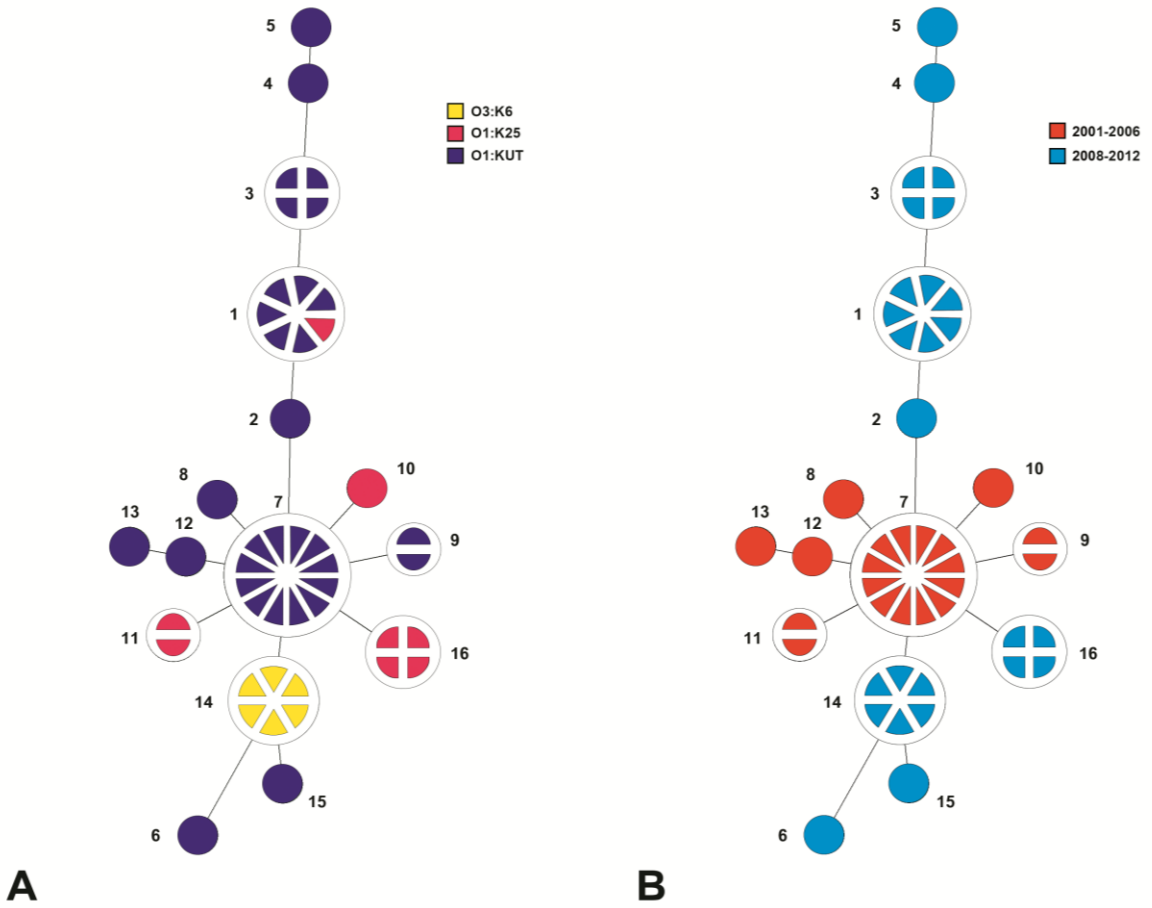

Fig. 3.7 Minimum spanning tree of the 46 pandemic *V. parahaemolyticus* isolates based on PFGE. Each circle represents a unique PFGE type. The colored pie chart sections inside circles correspond to serotypes (A) or period of isolation (B). The circle size indicates the number of isolates with that PFGE type. The numbers indicate PFGE Types.

Part III: Development of MLVA for strain typing

1. Analysis of VNTR loci

1.1 Characteristics of VNTR loci for MLVA typing

In this work, 4 VNTR loci (TR1 to TR4) with repeat consensus sequences between 6 and 8 were selected for MLVA typing **(Table 3.4)**. Analysis of these loci indicated that 3 (TR1, TR2 and TR4) and 1 VNTR (TR3) loci were located on chromosome I and II, respectively. TR1 and TR3 were localized in the non-coding regions; however, TR2 and TR4 were located in the open reading frame regions of hypothetical proteins, VP2226 and VP2892, respectively **(Fig. 3.8)**. TR3 primers could also amplify VPA1263 region which is located in the VPaI-6 and assigned as a pandemic marker region of *V. parahaemolyticus*. TR3 was not detected in all 20 non-pandemic isolates **(Table 3.5)**.

1.2 VNTR amplification

Specific primers targeted each VNTR locus were newly designed to support multiplex PCR method **(Table 3.4)**. Suitable multiplex VNTR amplification reaction and condition were investigated and are presented in **Table 3.6**-**3.7**, respectively. All VNTR amplification generated clearly distinguishes peaks **(Fig. 3.9)**.

The numbers of flanking nucleotides in TR1 to TR4 are 53, 88, 57 and 46 bp, respectively. A multiplex PCR simultaneously amplified 4 VNTR loci of *V. parahaemolyticus* and generated the copy number of each locus ranging from 8–17 (TR1), 7–24 (TR2), 5–44 (TR3), and 7–38 (TR4), respectively **(Fig. 3.10)**.

The DI based on genetic diversity for the 4 VNTR loci ranged from 0.83 to 0.92 **(Table 3.4)**. TR2 locus was the most polymorphic loci **(Table 3.4)**. No amplification product of TR1 and/or TR4 loci was observed in some isolates **(Fig. 3.10)**.

Table 3.4 Characteristics of the VNTR loci and list of primers used for MLVA.

VNTR locus name (chromosome)	Repeat consensus sequences	VNTR copy number[a]	Function	Diversity index (DI)	Primer	Expected amplicon size (bp)[b]
TR1 (I)	AGGTTCT	11	Non-coding region	0.83	F: 5'-VIC[c]-GTGACGGCTGGTCAGAAGAT-3'	130
					R: 5'-TTCACGCCAACTTCCTCAAC-3'	
TR2 (I)	TCTGGC	21	Hypothetical protein	0.92	F: 5'-PET[c]-AGTTGCGGTGCGTAGTTTTC-3'	190
					R: 5'-TCGCTCAGGAGCTATCCTCT-3'	
TR3 (II)	CAGCTAAA	22	Non-coding region	0.85	F: 5'-NED[c]-TTCACGGGTAAAAACACCAT-3'	233
					R: 5'-GCTGTGGTCTTTGTGTACGG-3'	
TR4 (I)	ATAGAG	28	Hypothetical protein	0.91	F: 5'-FAM[c]-TCAGCCAGACACTCCATACAA-3'	214
					R: 5'-GGAAGACATATCCGGTTTGC-3'	

[a] Repeat times were counted using the genome sequence of *V. parahaemolyticus* strain RIMD2210633.

[b] Amplicon size of each VNTR locus was estimated using the genome sequence of *V. parahaemolyticus* strain RIMD2210633.

[c] Fluorescent dyes

Table 3.5 Detection of TR3 (VPA1263 gene) in 20 non-pandemic isolates.

No.	Strain	GS-PCR	*tdh*	*trh*	VPA1263 gene
1	PSU255	-	+	-	-
2	PSU920	-	+	-	-
3	PSU3060	-	+	-	-
4	PSU3328	-	+	-	-
5	PSU3352	-	+	-	-
6	PSU3536	-	+	-	-
7	PSU3918	-	+	-	-
8	PSU3952	-	-	+	-
9	PSU5256	-	-	+	-
10	PSU5257	-	-	+	-
11	PSU4286	-	+	+	-
12	PSU4921	-	+	+	-
13	PSU5194	-	+	+	-
14	PSU3906	-	-	-	-
15	PSU3937	-	-	-	-
16	PSU4943	-	-	-	-
17	PSU4956	-	-	-	-
18	PSU5055	-	-	-	-
19	PSU5184	-	-	-	-
20	PSU5190	-	-	-	-

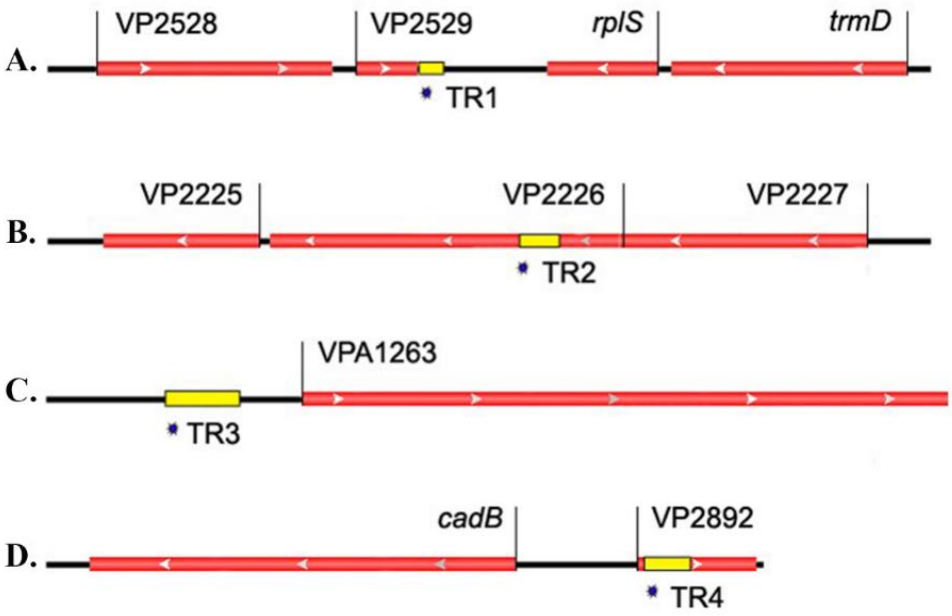

Fig. 3.8 *V. parahaemolyticus* RIMD2210633 genome regions containing the VNTR loci used for the MLVA. Each region corresponded to the VNTR locus TR1 (A), TR2 (B), TR3 (C), and TR4 (D), respectively. Arrows indicate the direction of transcription.

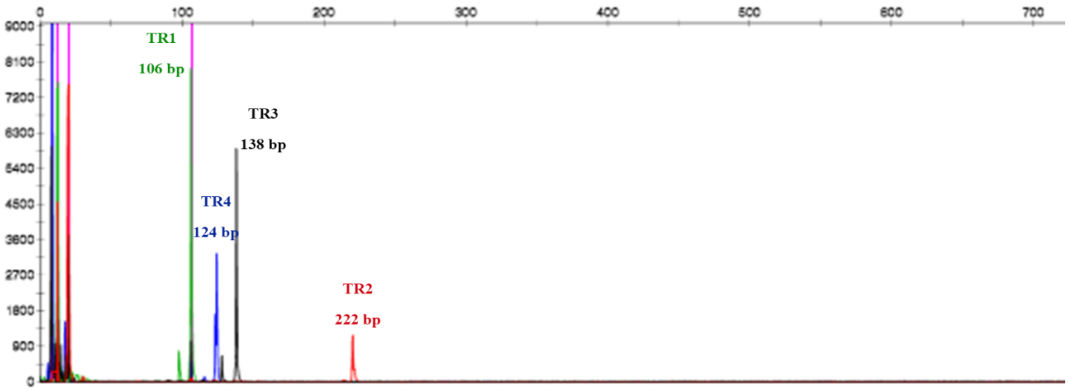

Fig. 3.9 Representative electropherograms generated by capillary electrophoresis. Four VNTR were amplified by multiplex PCR using fluorescent dye labeled-primers.

Table 3.6 Multiplex VNTR amplification reaction for MLVA.

Multiplex PCR reaction set up for MLVA		**µl per reaction**
	Final Conc.	
5x Buffer	1x	6
2.5 mM dNTP	0.2 mM	2.4
4 µM PrimerTR1_R	0.2 µM	1.5
4 µM PrimerTR1_F	0.2 µM	1.5
4 µM PrimerTR2_R	0.2 µM	1.5
4 µM PrimerTR2_F	0.2 µM	1.5
4 µM PrimerTR3_R	0.2 µM	1.5
4 µM PrimerTR3_F	0.2 µM	1.5
4 µM PrimerTR4_R	0.2 µM	1.5
4 µM PrimerTR4_F	0.2 µM	1.5
5 U *Taq* polymerase	0.5 U	0.1
DNA template		2
Water		10.5
Total volume		30

Table 3.7 Multiplex VNTR amplification condition for MLVA.

Step	**Temp. (°C)**	**Time (min)**	**cycle**
Hot start	95	3	1
Denature	95	1	30
Annealing	50	1	
Extension	68	1	
Final Extension	68	5	1

1.3 MLVA typing

Based on MLVA using 4 VNTR loci, thirty-eight distinct MLVA profiles revealing high genetic diversity were detected among 46 isolates of pandemic *V. parahaemolyticus* **(Fig. 3.10)**. Similar MLVA profiles were observed in some isolates of the same serotype obtained during the same periods. However, the results of this study showed that pandemic *V. parhaemolyticus* O1:KUT isolates were heterogeneous. It was of interest that all 4 loci were found to have a relatively high diversity among the tested isolates.

MST analysis revealed that all MLVA profiles were linked with other profiles regardless of the serotypes **(Fig. 3.11)**. MLVA profiles were grouped together if 2 neighboring profiles did not differ in more than 1 VNTR locus. Nine MLVA groups (A to I) were found in this study **(Fig. 3.10-3.11)**.

MLVA separated the 12 PFGE-indistinguishable O1:KUT isolates obtained in 2001-2005 (PFGE type 7) into unique profiles **(Fig. 3.10)**. Thirty two isolates of O1:KUT *V. parahaemolyticus* revealed 12 PFGE types and 29 MLVA profiles. MLVA offered a higher discriminatory power (0.99) than that of the PFGE (0.89).

The results showed that all isolates with the same MLVA profile had a high probability of having the same PFGE type. As calculated by using Wallace coefficient (W), MLVA predicted PFGE type (W=0.86). However, PFGE profile did not predict MLVA profile (W=0.33).

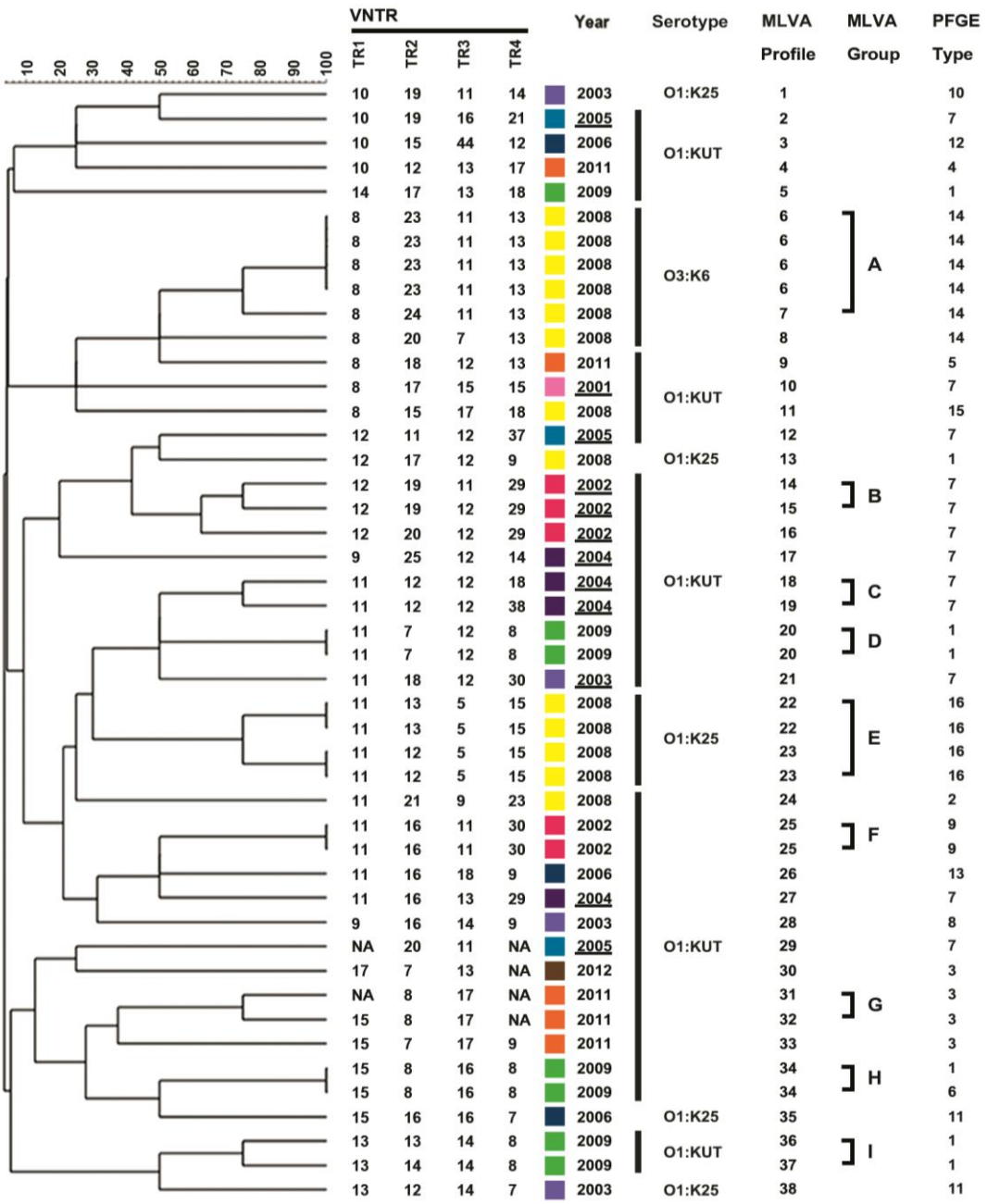

Fig. 3.10 Dendrogram of the 46 pandemic *V. parahaemolyticus* isolates based on MLVA profiles. Colors within the squares correspond to year of isolation. The number of tandem repeats is indicated. NA, not amplified, was given when no amplification was observed at a given locus. PFGE types are provided for direct comparison. Underline letter refer to the 2001-2005 isolates which are indistinguishable by PFGE.

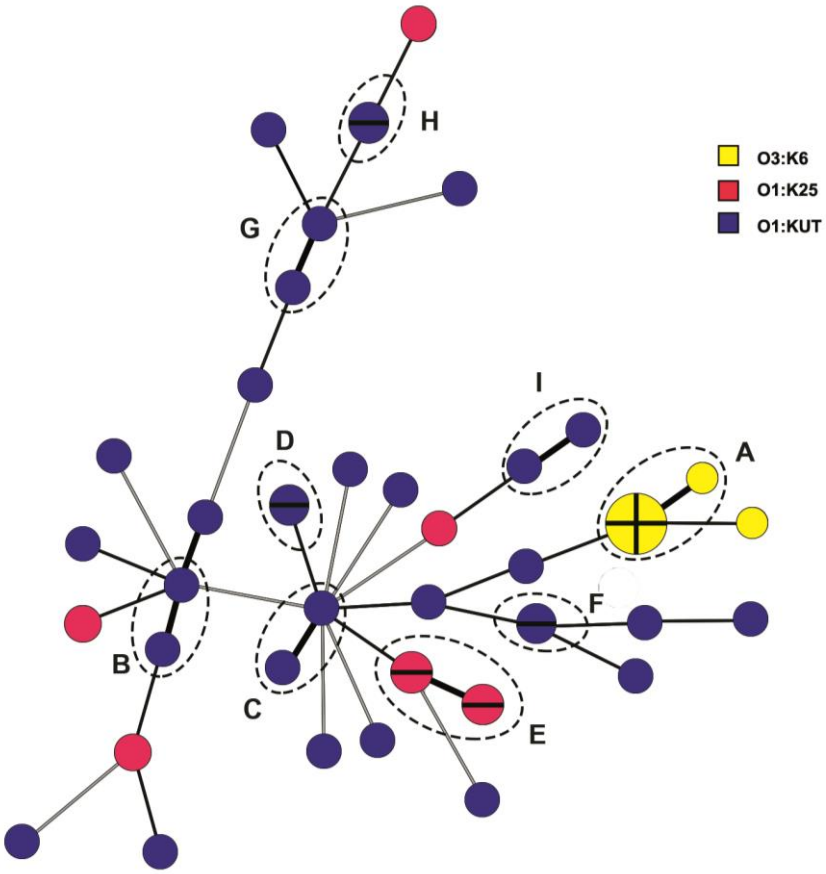

Fig. 3.11 Minimum spanning tree of the 46 pandemic *V. parahaemolyticus* isolates based on MLVA profiles. Each circle represents a unique MLVA profile. The color of the circles corresponds to serotypes. The circle size is proportional to the number of isolates. A distance of one locus between two MLVA profiles is indicated by a thick line, a distance of two loci is indicated by a thin line, and a distance of three loci is indicated by a grey line. Dotted circle indicated the nine MLVA groups A to H.

2. Evaluation of MLVA

All isolates were typeable by all fingerprinting methods used in this study (MLVA, AP2-PCR, AP4-PCR, ERIC-PCR, and PFGE). Thus, typeability of all methods was 1.00. The discriminatory power of MLVA was highest and followed by PFGE, AP2-PCR, ERIC-PCR, and AP4-PCR. MLVA was less time-consuming than ERIC-PCR, AP2-PCR, AP4-PCR, and PFGE for *V. parahaemolyticus* strain typing **(Table 3.8)**.

Table 3.8 Typeability and discriminatory power of AP2-PCR, AP4-PCR, ERIC-PCR, PFGE, and MLVA.

Properties	**AP2-PCR**	**AP4-PCR**	**ERIC-PCR**	**PFGE**	**MLVA**
Typeability	1.00	1.00	1.00	1.00	1.00
Discriminatory power	0.79	0.24	0.30	0.89	0.99
Time-consuming	17.30	17.30	12.05	26.42	4.40

3. Stability of VNTR

All VNTR markers except TR2 were highly stable after multiple subcultures, freezing and thawing, and growth in stress conditions. Changes in copy numbers were observed in TR2 locus after multiple subcultures, changes in temperature (5 and 44^{o}C), pH (4.8), and salt (0.5%) **(Table 3.9)**. A single or two repeat change in TR2 was observed in *V. parahaemolyticus* grown under stress conditions. In contrast, 10 repeats were deleted after repeated subcultured **(Table 3.9**)

Table 3.9 Copy number changing of TR2.

Conditions	**Copy number of TR2**	
	Wide type strain	**Mutant strain (n)**
Multiple subcultures	22	12 (1)
Freezing storage and thawing	No change	No change
Stress conditions		
Temperature		
5 °C	22	20 (1)
44°C	22	20 (5), 21(2)
pH stress		
pH 4.8	22	23 (8)
pH 11	No change	No change
Salt stress		
0.5 % NaCl	22	21 (6)
0.8 % NaCl	No change	No change

CHAPTER 4

DISCUSSIONS

Gastrointestinal tract infections caused by *V. parahaemolyticus* are commonly reported in several areas around the world (Ottaviani *et al.*, 2010, AESR, 2015, Li *et al.*, 2015). The three major pandemic serotypes including O3:K6, O1:K25, and O1:KUT are reported in several countries (Ottaviani *et al.*, 2010, Li *et al.*, 2015). In Thailand, these strains have been reported to continually detect among patients since 2000 (Wootipoom *et al.*, 2007, Thongjun *et al.*, 2013). This study investigated the characteristics and evaluated a novel tool for molecular typing of pandemic *V. parahaemolyticus*, especially O1:KUT serotypes. Other pandemic serotypes, O3:K6 and O1:K25, were also included in this study for comparison.

Treatment with antimicrobial could reduce disease severity and symptom duration. Our result showed that all *V. parahaemolyticus* isolates were resistant to ampicillin. This finding was consistent with the results reported in Indonesia, China, India, and Mexico where 100, 87, 98, and 94% of the isolates from clinical samples were resistant to ampicillin (Tjaniadi *et al.*, 2003, Pazhani *et al.*, 2014, de Jesús Hernández-Díaz *et al.*, 2015, Chen *et al.*, 2016). Since resistance to ampicillin has become widespread, fluoroquinolones, including ciprofloxacin and norfloxacin have been the first-line for initial treatment of acute diarrhea in adult patients (Riddle *et al.*, 2016). The increase in resistance among pathogens may be due to the overuse of antibiotics. The study of antibiotic prescription for treatment of acute diarrhea in Thailand indicates that fluoroquinolone was the most commonly prescribe antibiotic in adult (Supcharassaeng and Suankratay, 2011). Here, we report the fluoroquinolone resistant and intermediate susceptibility in 7 (15%) and 29 (63%) isolates of *V. parahaemolyticus*, respectively. The similar numbers of ciprofloxacin-resistant *V. parahaemolyticus* isolate has been reported in Indonesia (11%) since 2001(Tjaniadi *et al.*, 2003). The antibiotic resistance of *V. parahaemolyticus* is also associated to R plasmid transfer between bacteria (Hasegawa *et al.*, 1986, Li *et al.*, 1999, Wong *et al.*, 2000). Not only *V. parahaemolyticus*, the resistant to ciprofloxacin and other fluoroquinolones was also reported among *Campylobacter* species isolated

from patient with diarrhea in Thailand and Indonesia (Kuschner *et al.*, 1995, Tjaniadi *et al.*, 2003). Concernedly, careful surveillance and appropriate used of antimicrobial agents are required to preventing emergence of drug resistant bacteria.

T3SSs and T6SSs are well studied and found to be responsible for the pathogenicity of *V. parahaemolyticus*. All *V. parahaemolyticus* isolates in this study contained at least one gene encoding for T3SS component. Previous reported demonstrated that T3SS1 and T3SS2 genes were detected in all pandemic *V. parahaemolyticus* isolates by microarray hybridization (Meador *et al.*, 2007). In this study, the prevalence rates of T3SS genes were 91, 91, 96, and 100% for *vcrD1*, *vcrD2*, *vopC*, and *vopQ*, respectively. The lower prevalence observed in study may be due to the nucleotide variation in the PCR primer-binding site or lack of the targeted genes. In this study, T6SS genes were detected at lower rate than T3SS. This was correlated to the result from previous study which described that all clinical isolates possessed T3SS1, whereas 84 and 92% of clinical isolates were positive for T6SS1 and T6SS2, respectively (Kongrueng *et al.*, 2015). However, no information about pandemic background (GS-PCR, tdh^+, trh^-) was provided in that study. Further detection of another gene in T6SSs is needed to confirm the presence of T6SSs.

A production of TDH (Kanagawa phenomenon-positive) among clinical *V. parahaemolyticus* isolates and it relation with *V. parahaemolyticus* pathogenicity has been reported (Ceccarelli *et al.*, 2013). In addition, this phenomenon was previously shown to be strongly associated with tdh^+ trh^- *V. parahaemolyticus* isolates (Suzuki *et al.*, 1997). In our study we have demonstrated that all tdh^+ trh^- isolates were capable to produce TDH at similar level. The presence of TDH could benefit bacteria to produce significant disease in human.

In this study, *V. parahaemolyticus* isolates demonstrated high variation in swarming and twitching motilities. All isolates possessed at least one type of swarming or twitching motility. A previous report demonstrated that *V. parahaemolyticus* is able to motile by several ways depends on their living condition (McCarter, 1999, Kim and McCarter, 2000, Broberg *et al.*, 2011). The ability to exhibit different types of motility in *V. parahaemolyticus* depends on the numbers of genetic regulations, motility protein, and surface sensing (Kim and McCarter, 2000, Mattick, 2002, Wang *et al.*, 2013a). The inability to swarm

observed in 4 isolates in this study may be due to the defect in cell division during the swarm cell cycle, lateral flagella formation, or ability to produce autoinducers (McCarter, 1999).

Iron is essential for almost all bacteria including *V. parahaemolyticus*. During infection, *V. parahaemolyticus* is able to utilize siderophores for uptake iron during growth in iron-limited environment (León-Sicairos *et al.*, 2015). Hemoglobin is one of the iron-rich proteins in human red blood cells. Thus, all isolates were examined for the utilization of hemoglobin on iron-limited agar plate. In this study, all except one *V. parahaemolyticus* isolates grow well in the presence of 155 µM hemoglobin, whereas 5 isolates were unable to grow when tested at 50 µM which is correlated with the previous study (Wong *et al.*, 1996). Thus, these isolates may possess low virulence in human where free iron is limited.

Most serotypes of O3:K6, O1:K25, O4:K28 and O1:KUT pandemic *V. parahaemolyticus* have been continually detected among patients worldwide (Nair *et al.*, 2007, Wootipoom *et al.*, 2007, Thongjun *et al.*, 2013). The molecular typing is necessary tool for epidemiology study of pandemic strains. AP-PCR and ERIC-PCR have been reported to use in the genetic diversity study of *V. parahaemolyticus* isolated form cockles in Indonesia and these methods revealed the high diversity among environmental *V. parahaemolyticus* isolates (Zulkifli *et al*., 2009). However, in this study demonstrated that both AP-PCR and ERIC-PCR are low resolution methods for typing pandemic *V. parahaemolyticus*, especially O1:KUT. They provided only 3-11 individual profiles of 46 pandemic *V. parahaemolyticus* isolates. Moreover, in this study, PFGE showed the similar profile of twelve O1:KUT *V. parahaemolyticus* isolates. This was correlated to the previous reports which described that pandemic O3:K6, O1:K25, and O1:KUT serotypes are the closely strains originated form O3:K6 clone (Chowdhury *et al*., 2000, Matsumoto *et al*., 2000, Bhuiyan *et al*., 2002). Therefore, the high discriminatory power method is required to typing these strains. MLVA technique has been demonstrated for typing pandemic *V. parahaemolyticus* isolates, however, no direct evidence of this technique for differentiation among O1:KUT. In this work, MLVA technique has been constructed to discriminate pandemic O1:KUT and other pandemic serogroups of *V. parahaemolyticus*

Previous MLVA protocols have been associated with a number of VNTR loci and primer sets (Kimura *et al.*, 2008, Harth-Chu *et al.*, 2009, Ansede-bermejo *et al.*, 2010, Lüdeke *et al.*, 2015, Jiang *et al.*, 2016). In this study, we optimized the criteria for the selection of VNTR loci and redesigned a set of fluorescence-labeled specific primers for single-tube multiplex VNTR amplification. A novel VNTR locus (TR3) was used in combination with three of the previously described loci (TR1, TR2, and TR4) (Kimura *et al.*, 2008, Harth-Chu *et al.*, 2009, Ansede-bermejo *et al.*, 2010, Lüdeke *et al.*, 2015, Jiang *et al.*, 2016). Thus, the MLVA developed in this study differs considerably from the MLVA previously described. In this study, MLVA scheme yielded a higher discriminatory power than that of the standard PFGE, AP-PCR, and ERIC-PCR. Moreover, using this multiplex MLVA, 96 isolates can be analyzed in a single run.

TR1 locus was identified in non-coding region that located between the VP2529 and *rplS* gene, encoded for a zinc-binding protein and the 50S ribosomal protein L19, respectively (Makino *et al.*, 2003). TR2 was located on the VP2226 gene which encoded a hypothetical protein ortholog to chemotaxis protein CheW that was necessary for the control of bacterial motility patterns (Miller *et al.*, 2009). Amplified DNA derived from TR2 primers revealed the highest variability in copy numbers. Therefore, this is one of important regions to generate diversity among *V. parahaemolyticus* isolates. TR3, a VNTR loci that were not used previously, was detected on chromosome II and amplification product included VPA1263 pandemic region found within *V. parahaemolyticus* genomic island VPaI-6 (Hurley *et al.*, 2006). Thus, TR3 positive isolates confirmed the pandemic strains. In this study, all pandemic isolates were positive with the TR3 primers whereas amplified DNA was not detected in all 20 non-pandemic isolates. TR4 was located on the gene encoding for a putative protein VP2892, and four isolates belonging to the O1:KUT were negative for the TR1 and/or TR4 amplification. This might be due to either sequence variation in the PCR binding regions or lack of those VNTR loci. However, 4 locus combination of MLVA in this work could generate distinguishable profiles of those isolates.

Identical PFGE type and MLVA profile of the isolates obtained from patients indicated that the infection might be associated with the same clones because the isolates were taken from the patients in the same period and geographic region.

MST analysis based on MLVA revealed the linked between pandemic O1:KUT isolates with other pandemic serotypes. Previous study suggested that the O1:KUT and O1:K25 isolates are originated from the pandemic O3:K6 stains by alteration of their O:K antigens in response to the host's immunological pressure (Chowdhury *et al.*, 2004, Nair *et al.*, 2007). The results obtained in this study confirmed the genetic relatedness of pandemic O3:K6, O1:KUT, and O1:K25 serotypes.

Two isolates defined as unrelated by PFGE (PFGE type 1 and 6) were not distinguished by MLVA (MLVA profile 34). This may be due to the reason that tandem repeats in this isolates evolved slower than others (Noller *et al.*, 2006). MLVA and PFGE detect different genetic variation in different regions of the chromosome. Variations in MLVA profiles are caused by the mutation in tandem repeats; however mutations in enzyme restriction sites have been shown to affect PFGE type (Tenover *et al.*, 1995). Although, the high variation of VNTR can indicate the differentiation of bacteria strains, it can cause the limitation in the correlated investigation between bacteria strains isolated from different period (Lam *et al.*, 2012). The discriminatory values greater than 0.90 are acceptable for epidemiological typing of bacterial strains (Hunter and Gaston, 1988).

The variation of VNTR makers is an importance key of MLVA typing for differentiation the strain of bacteria (Okada *et al.*, 2012), and has been reported to involve DNA repair systems which occurred when bacteria are under pressure (van Belkum *et al.*, 1998, Cooley *et al.*, 2010). In addition, genetic diversity in many bacteria, including *Salmonella* Typhimurium, *Pseudomonas* sp., and *E. coli* has been reported to associate with stress conditions (Martinez and Baquero, 2000, Van Den Broek *et al.*, 2005, Cooley *et al.*, 2010). In *E.coli*, irradiation, high temperature, and starvation condition, affect the change in tandem repeats (Cooley *et al.*, 2010). In this study, all VNTR loci, except TR2, remained stable in all conditions of the stability test. However, change in TR2 repeat numbers was detected when bacterium was subcultured for multiple times (10 times) or growth under some stress conditions.

This study indicates that some stresses may increase phylogenetic diversity of *V. parahaemolyticus* strain due to the change in VNTR loci. In addition, it is importance to work with freshly isolates as repeated subcultured may induce mutation to bacterial repeated sequences. However, routine cultivation did not produce detectable repeat variation.

CHAPTER 5

CONCLUSIONS

1. A total of 46 pandemic *V. parahaemolyticus* isolates (*tdh* gene positive, *trh* gene negative, GS-PCR positive) obtained from diarrhea patients in Hat Yai hospital, Songkhla during 2001-2012 were examined for their antimicrobial resistance profiles and virulence-associated characteristics. All isolates were resistant to ampicilin, and some isolates showed resistant to ciprofloxacin and norfloxacin which are the first-line antimicrobial agents used to treat infections. All isolates possessed virulence characteristics including hemolytic activity, genes required for type III and/or type VI secretion systems, twitching and/or swarming motility, and iron acquisition phenotypes. The pattern of antimicrobial resistant among these isolates is important for both epidemiological and clinical purposes. Moreover, the results obtained from these investigations may inform the virulence potential of this organism and may be useful to the study of their virulence mechanisms in the future.
2. Four VNTR loci used in this study are suitable genetic markers for differentiation of pandemic *V. parahaemolyticus*, especially among various O1:KUT isolates and can be applied for molecular epidemiological investigations of KUT *V. parahaemolyticus* strains possessed other O serogroups. The multiplex MLVA developed in this study using these 4 VNTR loci has higher discriminatory power (D=0.99) than PFGE (0.89). In addition, MLVA technique is considerably more rapid than PFGE technique. Large-scale samples can be typed by this MLVA method in a single working day with high reproducibility.

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APPENDIX

APPENDIX A. Media

1. Tryptic soy agar (TSA) + 1% NaCl

a.	Pancreatic digest of casein	17.0	g
b.	Papaic digest of soybean	3.0	g
c.	Dextrose	2.5	g
d.	Sodium chloride (NaCl)	5.0	g
e.	Agar	15.0	g
f.	Distilled water (dH_2O)	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

2. Tryptic soy broth (TSB) 1% NaCl

a.	Pancreatic digest of casein	17.0	g
b.	Papaic digest of soybean	3.0	g
c.	Dextrose	2.5	g
d.	NaCl	5.0	g
e.	dH_2O	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

3. Thiosulfate citrate bile salts sucrose (TCBS) agar

a.	Yeast extract	5.0	g
b.	Proteose peptone	10.0	g
c.	Sodium citrate	10.0	g
d.	Sodium Thiosulfate	10.0	g
e.	Oxgall	8.0	g
f.	Saccharose	20.0	g
g.	NaCl	10.0	g
h.	Ferric ammonium citrate	1.0	g

3. Thiosulfate citrate bile salts sucrose (TCBS) agar (continued)

i. Bromthymol blue	0.04	g
j. Thymol Blue	0.04	g
k. Agar	15.0	g
l. Distilled water (dH_2O)	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

4. Wagatsuma agar

a. Yeast extract	3.0	g
b. Peptone	10.0	g
c. NaCl	70.0	g
d. Dipotassium hydrogen phosphate (K_2HPO_4)	5.0	g
e. Mannitol	10.0	g
f. Crystal violet	0.001	g
g. Agar	15.0	g
h. dH_2O	1000.0	ml
i. Rabbit red blood cells, fresh (24h), with anticoagulant	50	ml

Mix fresh rabbit blood with same volume of physiological saline. Centrifuge cells at about 4000 x g at 4 ºC for 15 min. Pour off saline and wash 2 more times. Then, pour off saline and resuspend cells to original volume with saline.

Dissolve ingredients, except blood, in dH_2O and boil to dissolve agar. Adjust to pH 8.0 $\pm$ 0.2. Then, Stem 30 min. After that, cool to 45-50 ºC. Add 50 ml of wash red blood cells to the cooled medium.

5. Luria Bertani (LB) Agar

a. Tryptone	10.0	g
b. Yeast extract	5.0	g
c. NaCl	10.0	g
d. Bacto agar	15.0	g
e. dH_2O	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

6. Swarming plate

a. Luria Bertani (LB)	25.0	g
b. Bacto agar	6.0	g
c. dH_2O	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

7. Melted rich medium

a. Peptone	10.0	g
b. NaCl	30.0	g
c. Disodium hydrogen phosphate heptahydrate (Na_2HPO_4)	5.0	g
d. Glucose	5.0	g
e. Agar	15.0	g
f. 50 µM and 155 µM of 2, 2'-dipyridyl to eliminate the iron		
g. dH_2O	1000.0	ml

Dissolve ingredients in dH_2O and autoclave at 121 ℃ for 15 min.

APPENDIX B. Reagents and buffers

1. 0.85% Sodium chloride

a. NaCl	8.5 g
b. dH_2O	1000.0 ml

Dissolve NaCl in dH_2O and autoclave at 121 ℃ for 15 min.

2. 0.5 M EDTA (pH 8.0)

a. EDTA.$2H_2O$	186.1 g
b. dH_2O	800.0 ml
c. NaOH (Solid)	~20.0 g

Dissolve EDTA.$2H_2O$ in dH_2O. Adjust pH to 8.0 with NaOH and autoclave at 121 ℃ for 15 min.

3. Tris Borate (TBE) buffer (5x)

a. Tris base	54.0 g
b. Boric acid	27.5 g
c. 0.5 M EDTA (pH 8.0)	20.0 ml
d. dH_2O	1000.0 ml

Dissolve Tris base, Boric acid, and 0.5 in dH_2O. After that sterile by autoclaving at 121 ℃ for 15 min.

4. 1M Tris pH8.0

a. Tris base	121.14 g
b. dH_2O	1000.0 ml

Dissolve Tris base in dH_2O and autoclave at 121 ℃ for 15 min.

5. 1M NaCl

a. NaCl	58.44 g
b. dH_2O	1000.0 ml

Dissolve NaCl in dH_2O and sterile by autoclaving at 121 ℃ for 15 min.

6. Cell suspension buffer (100ml)

a. 1M Tris pH 8.0	10.0 ml
b. 0.5M EDTA pH 8.0	20.0 ml
c. 1M NaCl	2.0 ml
d. dH_2O	68.0 ml

Mix 1M Tris, 0.5M EDTA, 1M NaCl with dH_2O and sterile by autoclaving at 121 ℃ for 15 min.

7. Cell lysis buffer

a. 1M Tris pH 8.0	50.0 ml
b. 0.5M EDTA pH 8.0	100.0 ml
c. 10% Sacosyl (N-lauroyl sarcosine salt)	100.0 ml
d. dH_2O	68.0 ml

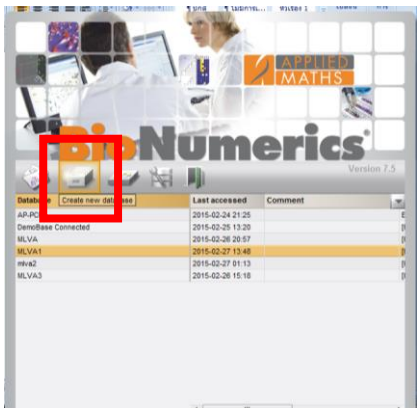
Mix 1M Tris, 0.5M EDTA, 10% Sacosyl with dH_2O and sterile by autoclaving at 121 ℃ for 15 min.

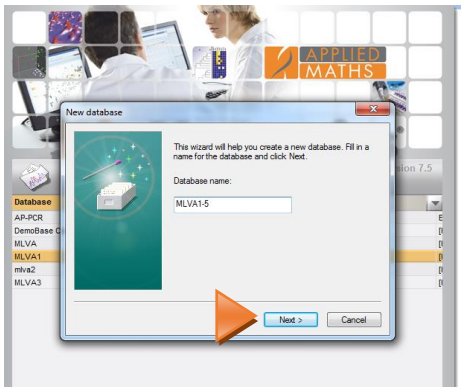
APPENDIX C. Guideline for using BioNumeric software

1. Install BioNumeric software

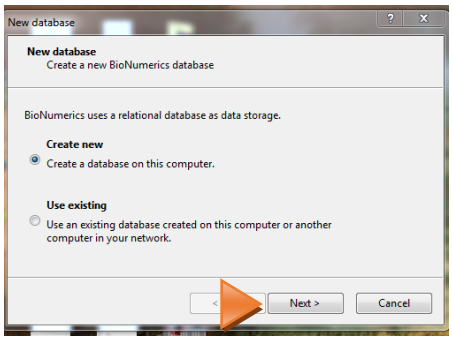
2. Create new database:

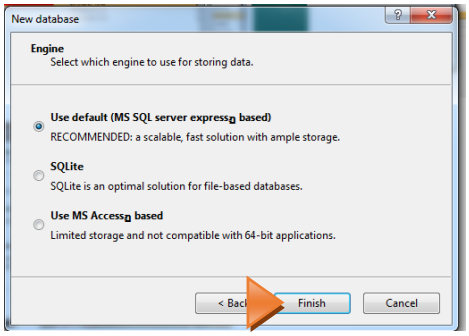
2.1 Press  > Press key database name



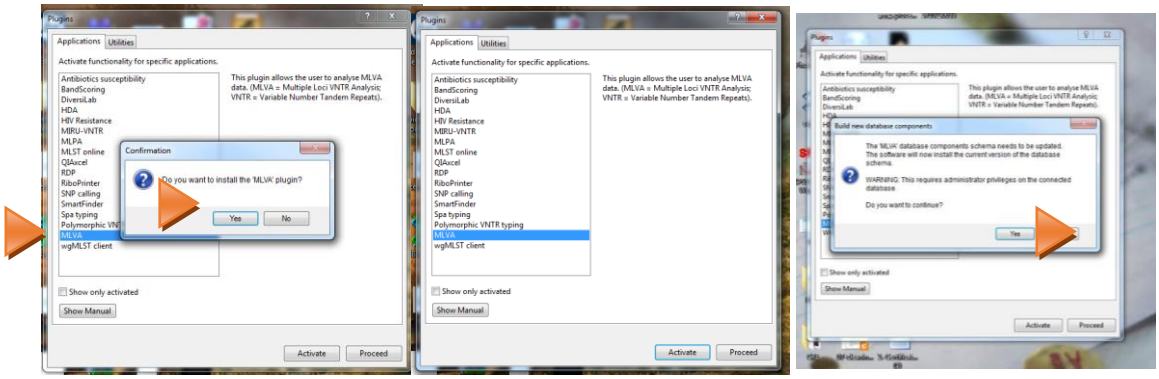


2.2 Press Create new > Press Next > Press Use default > Press Finish.



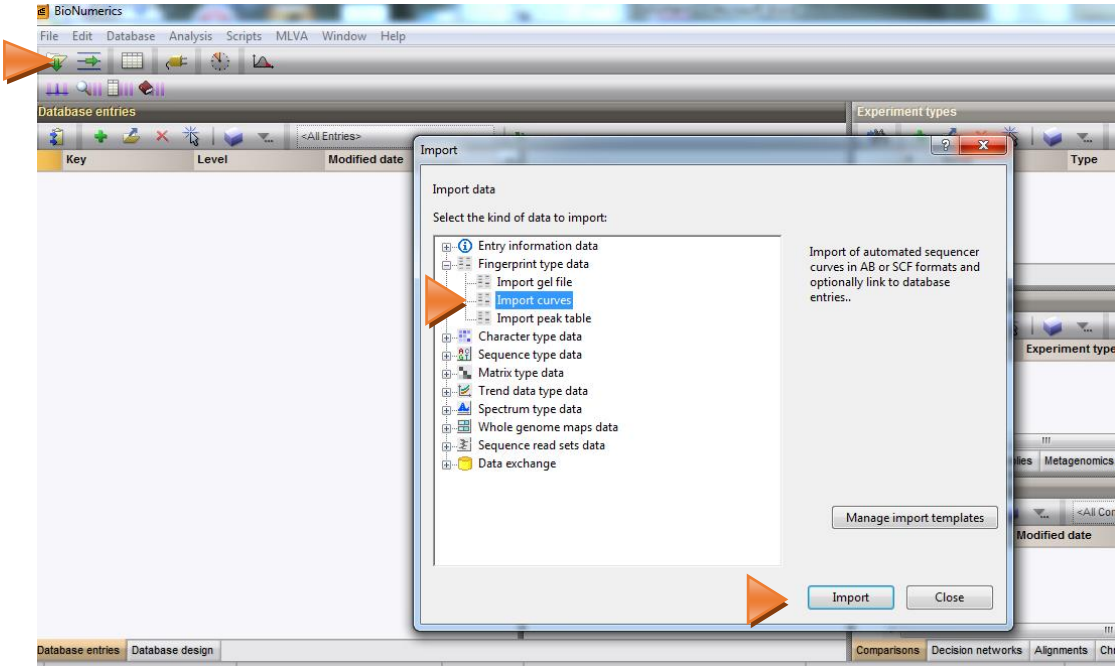


3. Install plugin of MLVA: Press MLVA > Press Yes > Press Activate > Press Yes

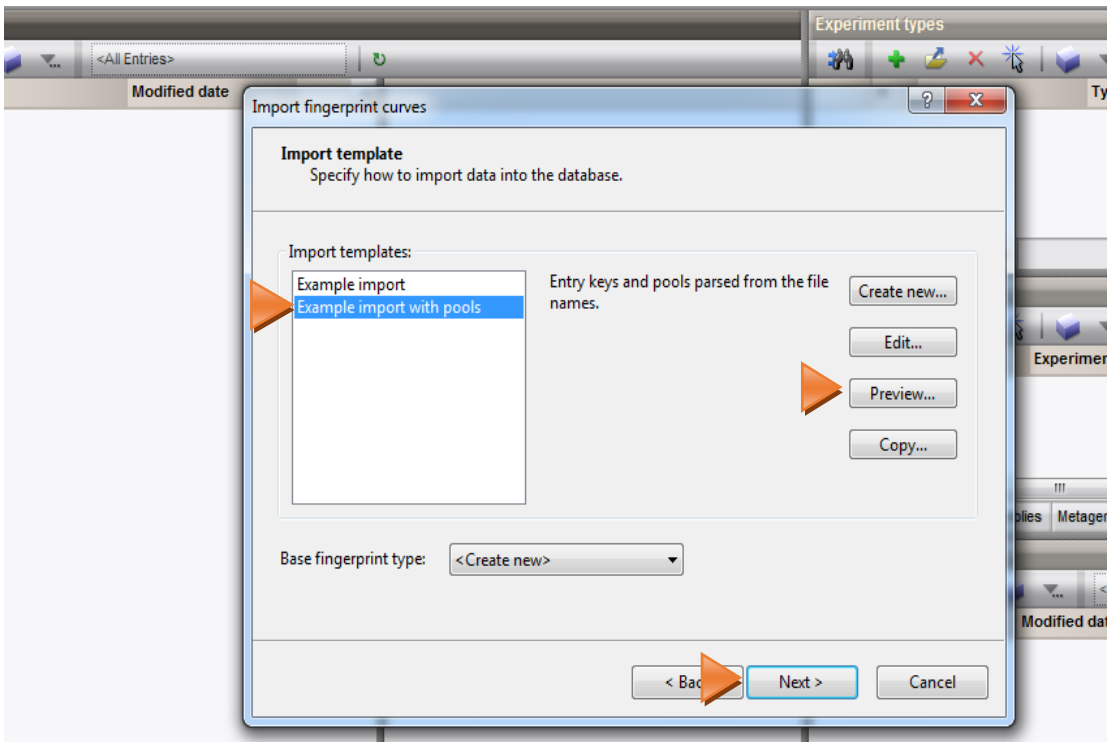


4. Import curve:

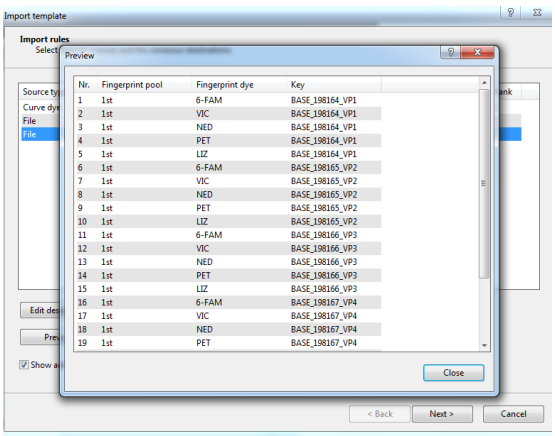
4.1 Press file > Press import > Press import cures > Press import > choose files for analysis (.fas) > Press next

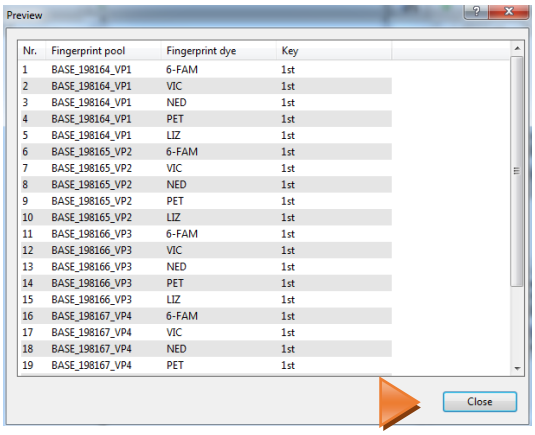


4.2 Example import with pools > Press Preview > Press Next

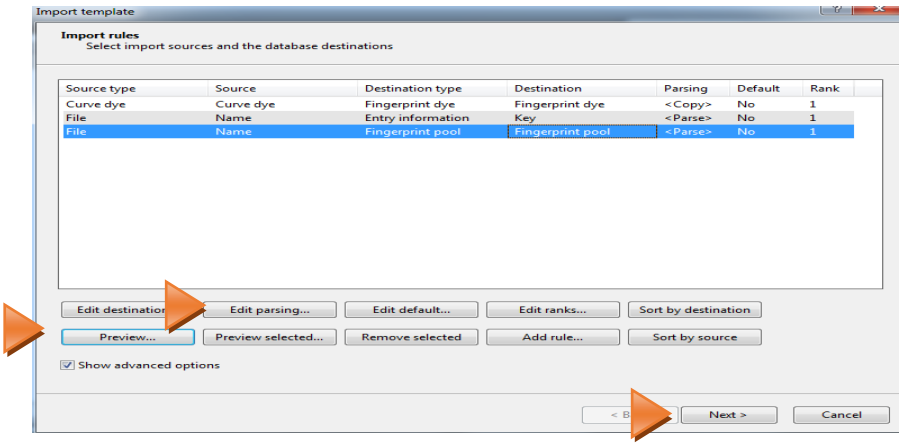


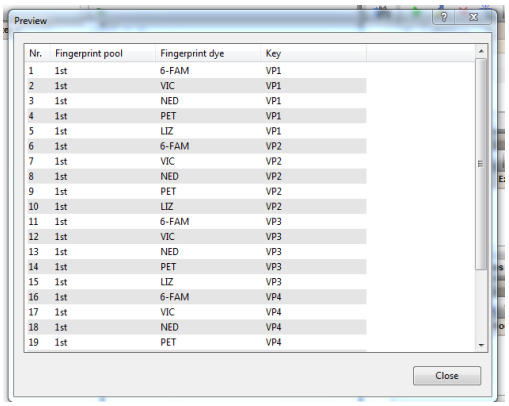
4.3 Press edit to edit the information of sample: Fingerprint pool is company name; Fingerprint dye is fluorescent dye; Key is sample name; > Press close



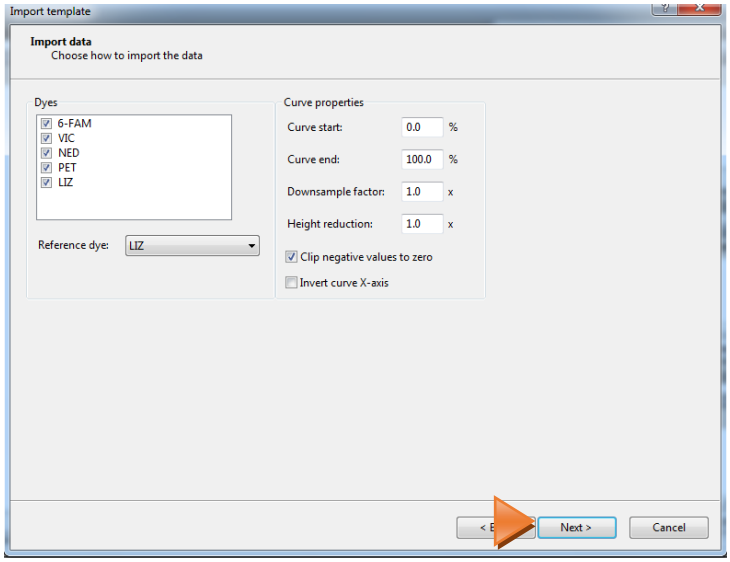


4.4 Press √ in Show advanced options box > Press Preview to check information of sample or Press Edit parsing to edit showing information > Press Next> Press Next > Press finish



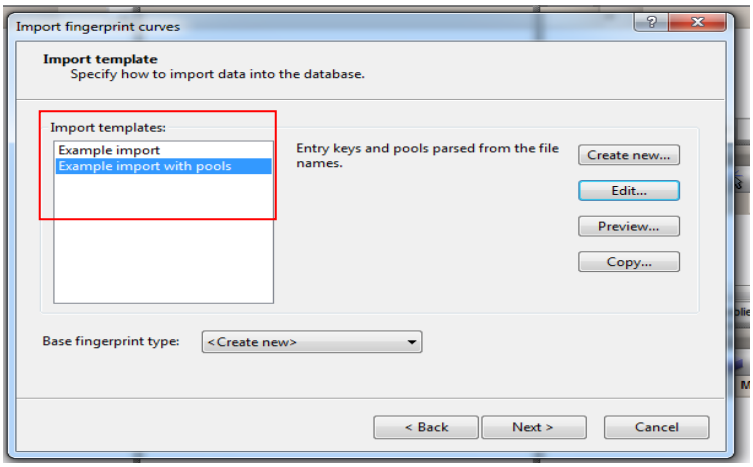


5. Import data: Press √ in dye box which are labeled dyes on 5'primer and choose reference dye which is label dye of marker.

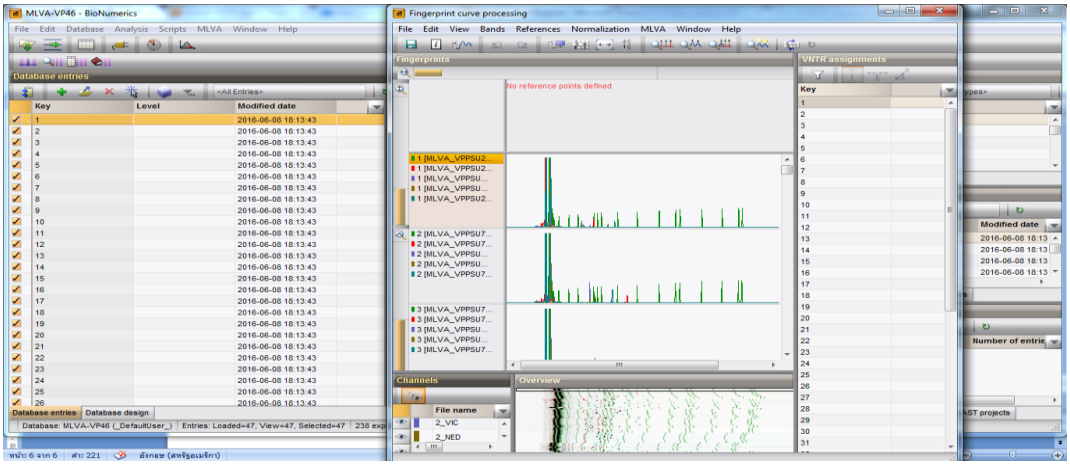


6. Create template for fingerprint type:

6.1. Press Example import with pool > Press Next

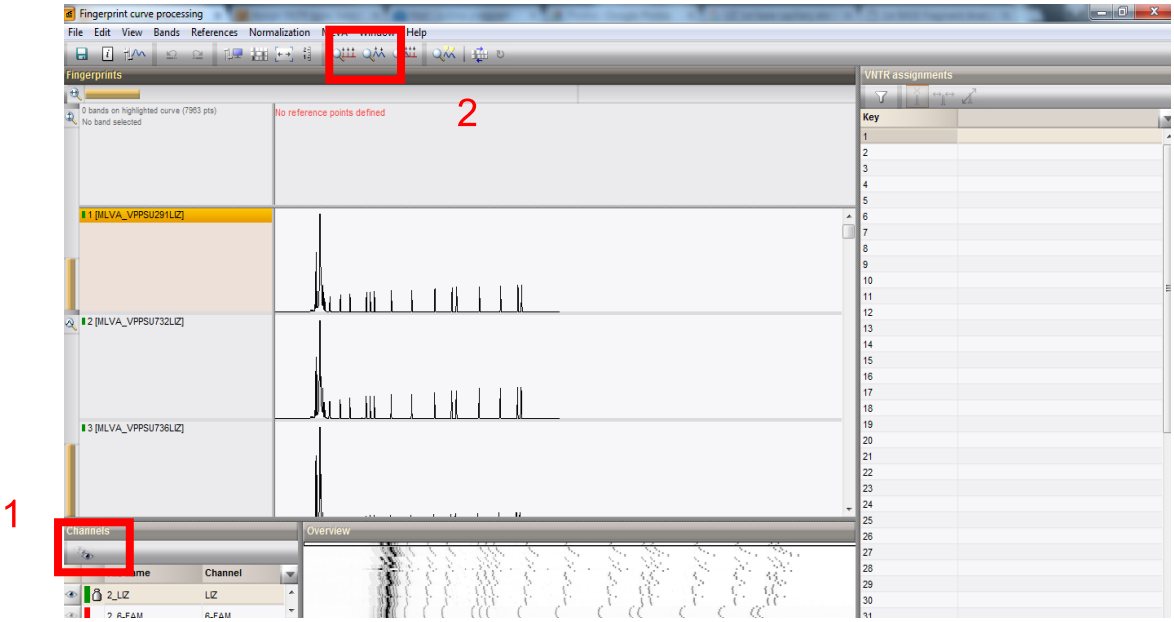


6.2. Main window when finish the creation template for fingerprint type

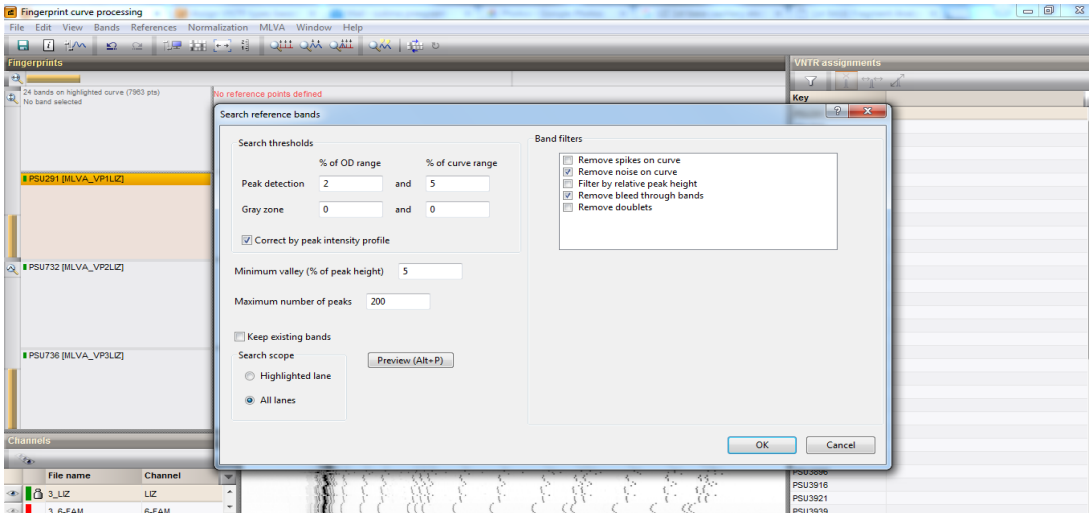


7. Set size of maker:

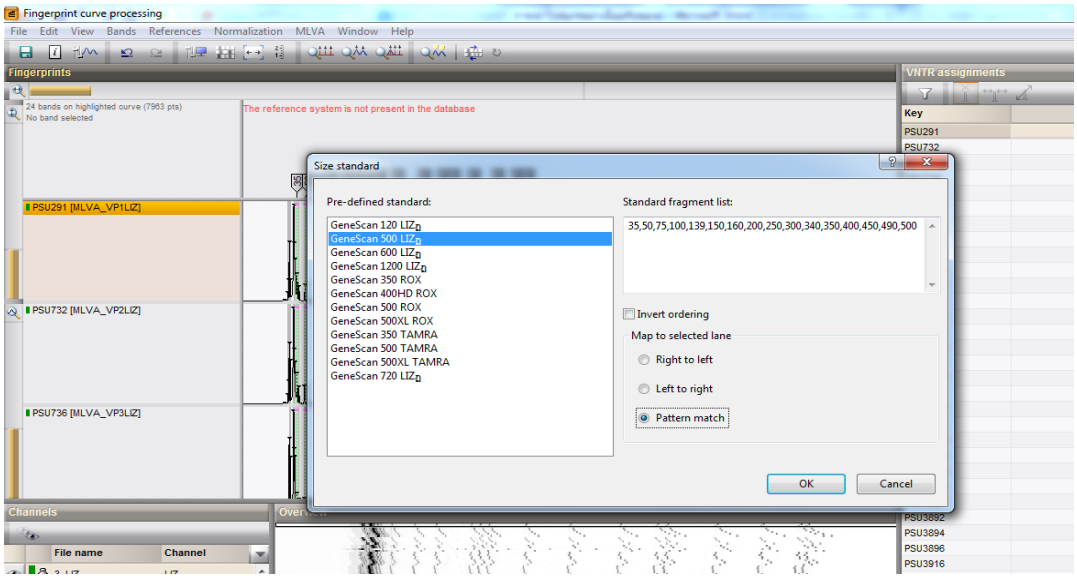
7.1 Press icon No. 1 and then Press icon No.2 for showing the peak



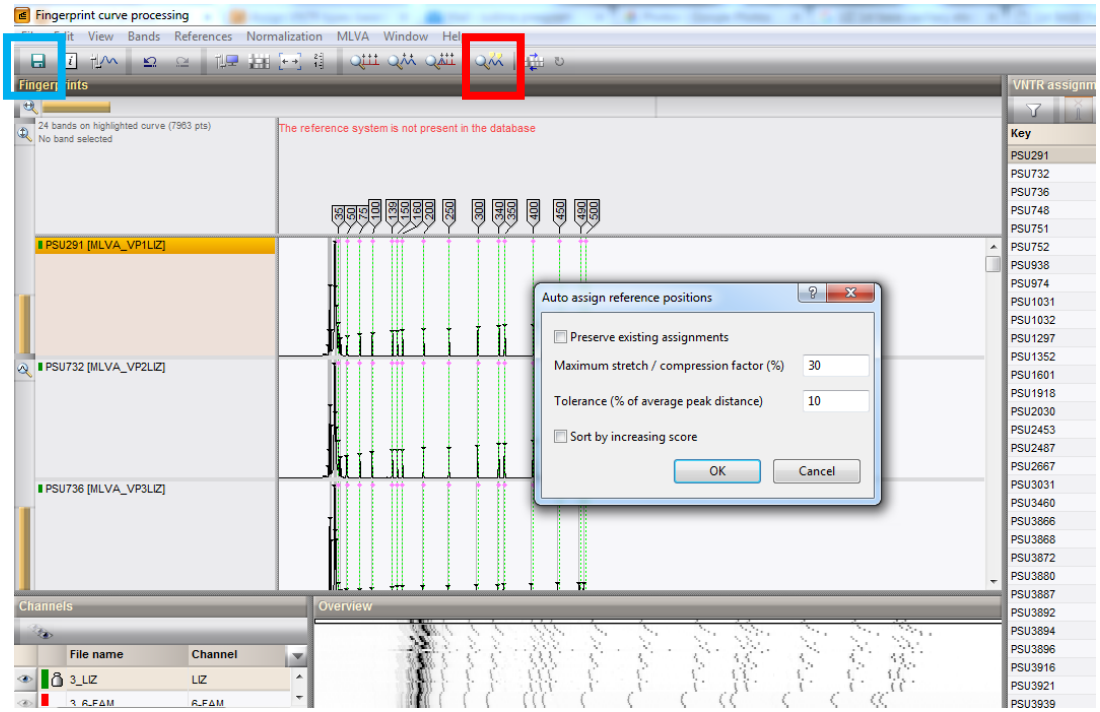
7.2 Press OK and reference icon



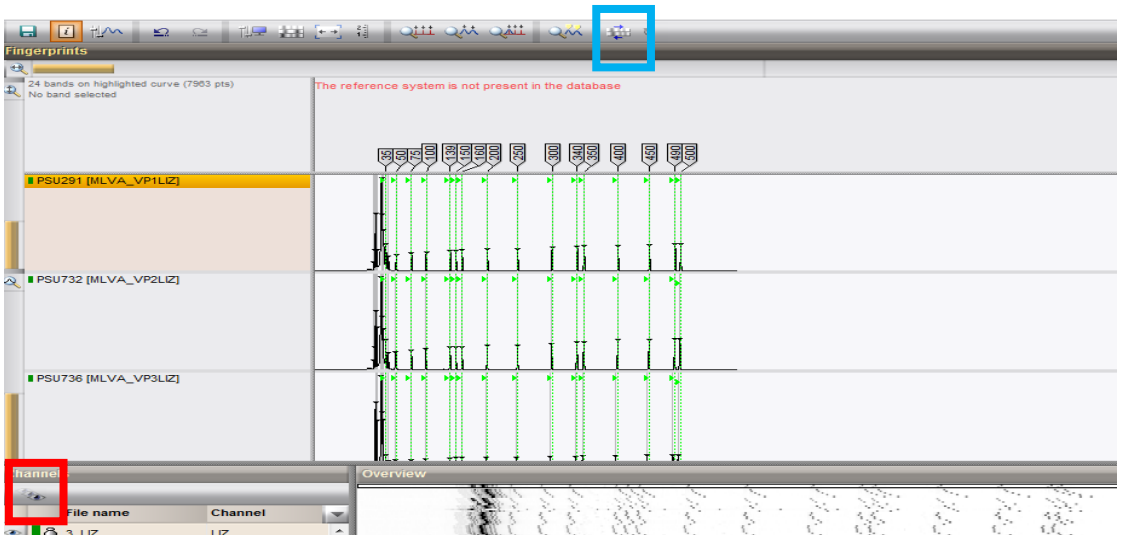
7.3 Press define size standard (500LIZ) > Press Pattern match > Press OK> Press OK



7.4 Press save icon > Press Auto assign reference > Press OK

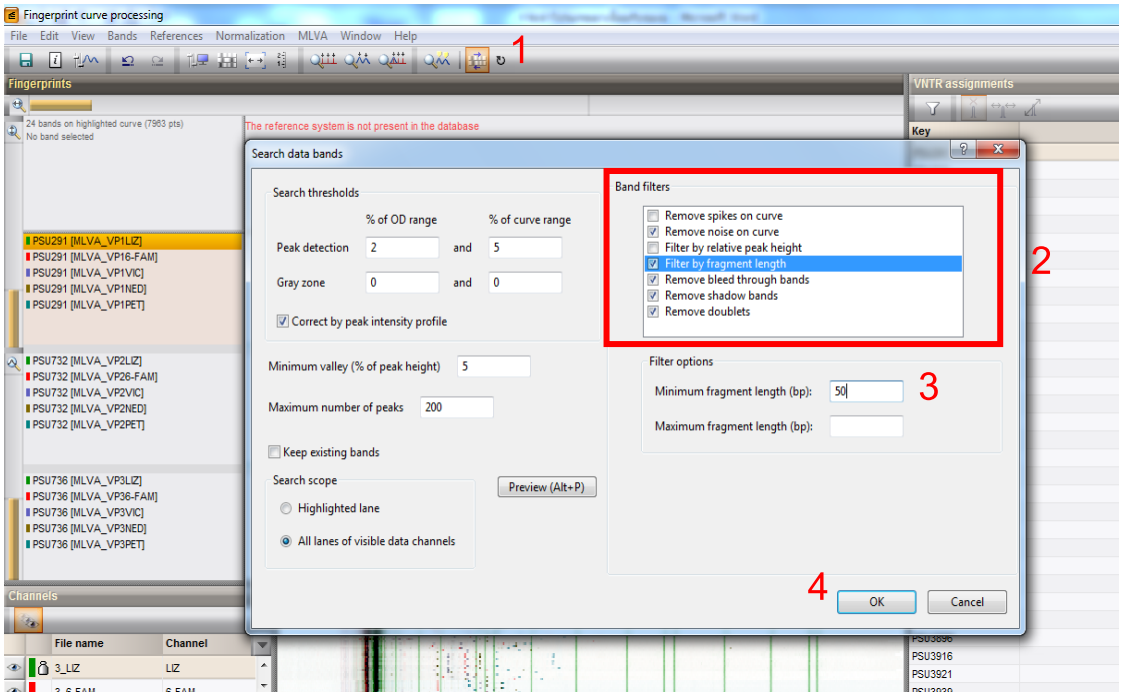


7.5 Click show normalized > Click view

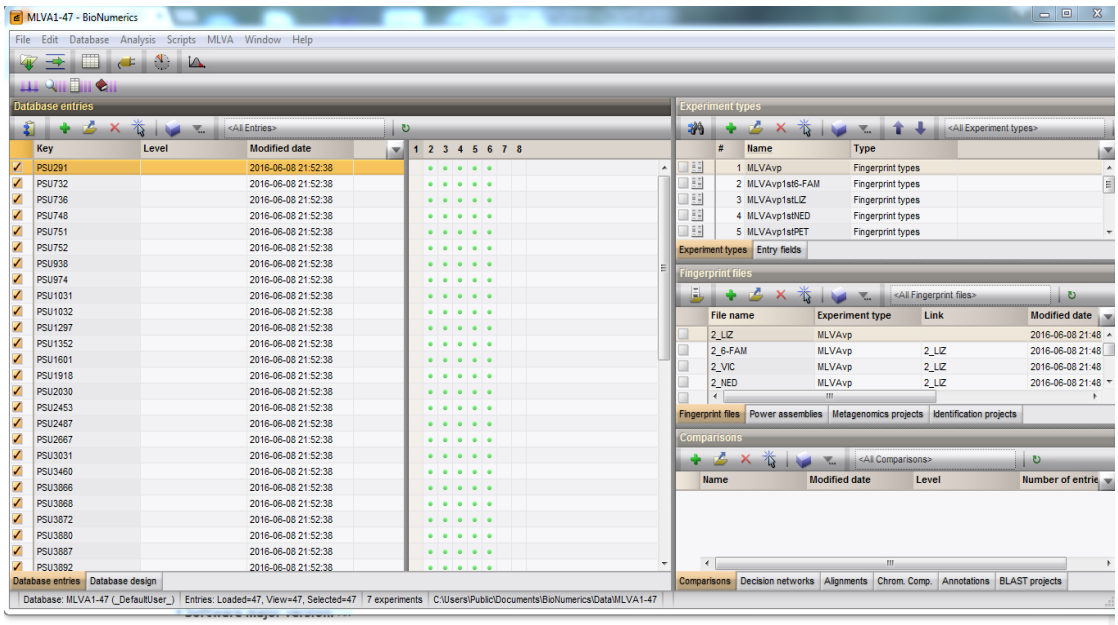


8. Search data band

8.1 Press search data band> Press √ Remove noise on curve, filter fragment length, Remove bleed through bands, Remove shadow bands, Remove doublet > Check remove shadow band >Add minimum length 50 > Press OK



8.2 Press cross> Press conformation note and press yes> Press yes

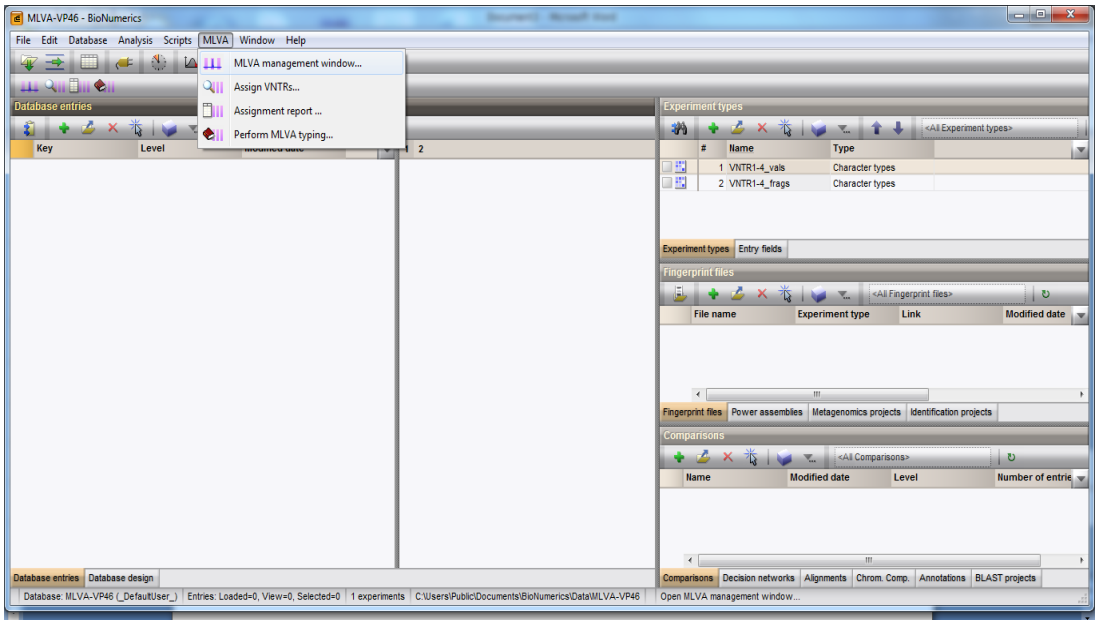


9. Creation the template for calculation the copy number of VNTR

> Training Video for the creation the template for calculation the copy number of VNTR

: **http://www.applied-maths.com/video/7x/assign-vntr-types-based-vntr-copy-numbers**

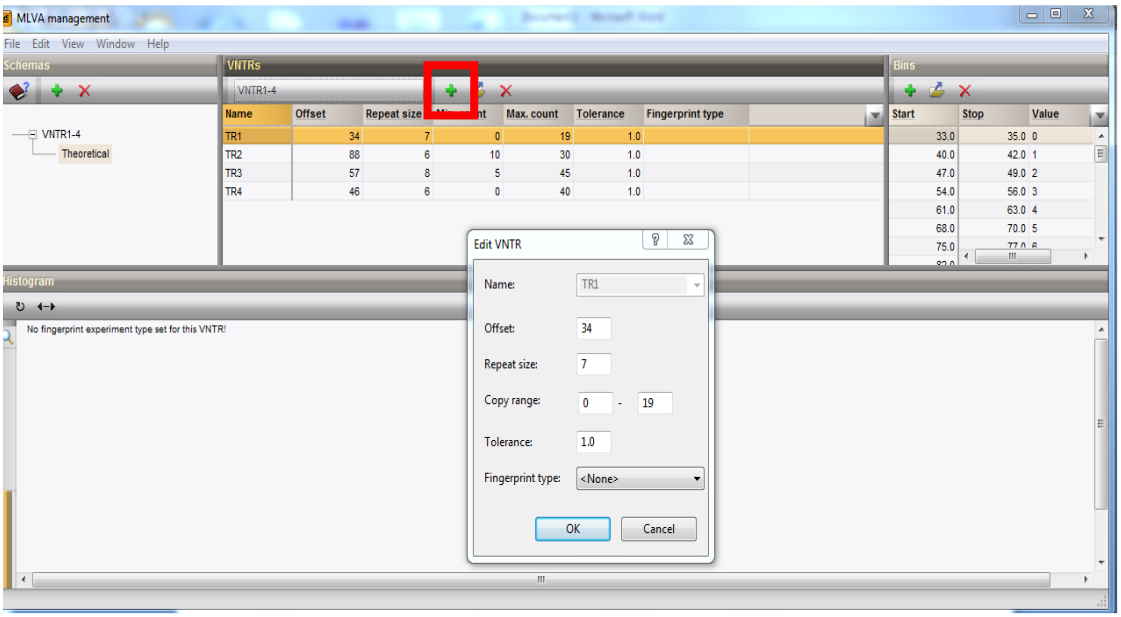
9.1 Press icon of MLVA > Press MLVA management….



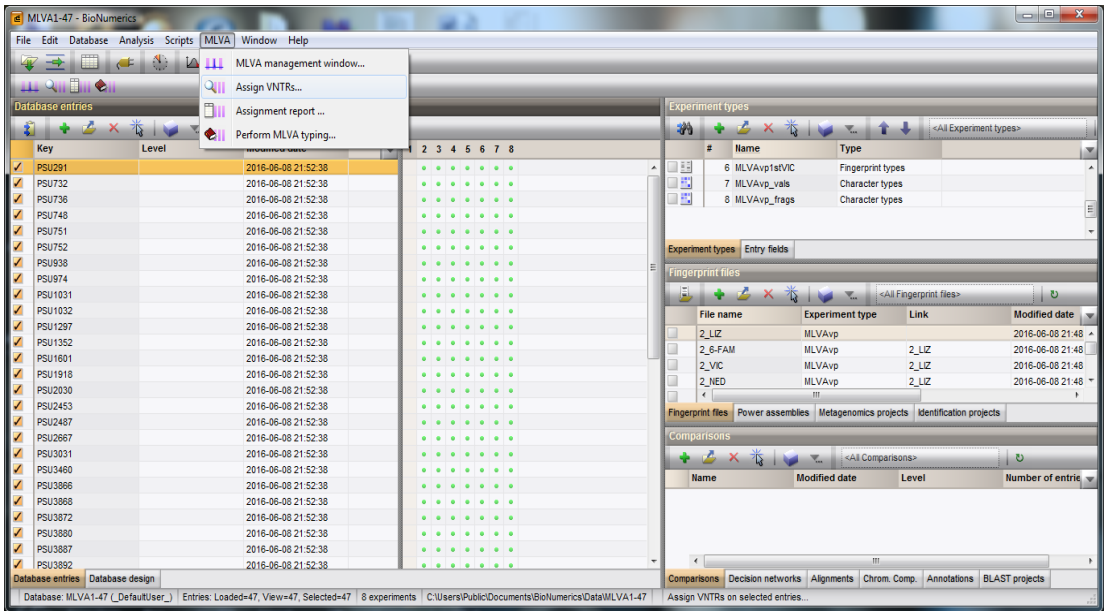
9.2 Press + icon > Key information in each boxes, following this link:

http://www.applied-maths.com/video/7x/assign-vntr-copy-numbers

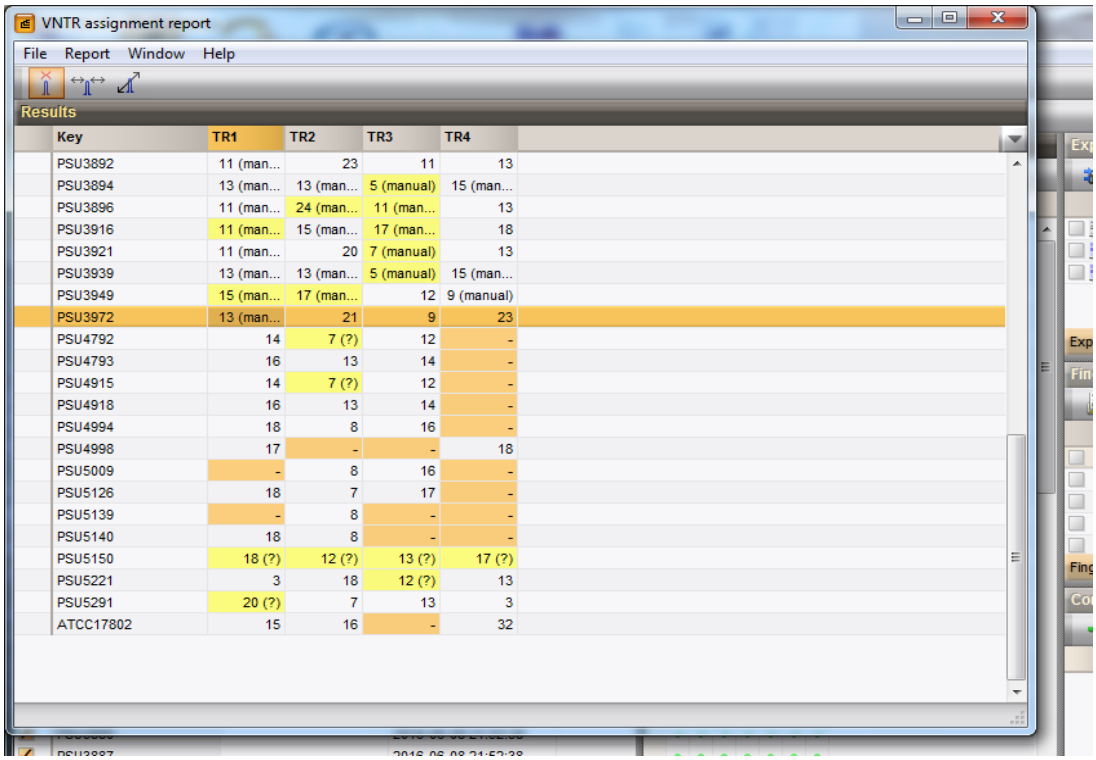
(TR1 Hex= VIC, TR2 Rox= PET, TR3 Tamra= NED, TR4 FAM=FAM)



10. VNTR calculation: Press MLVA > Press VNTR



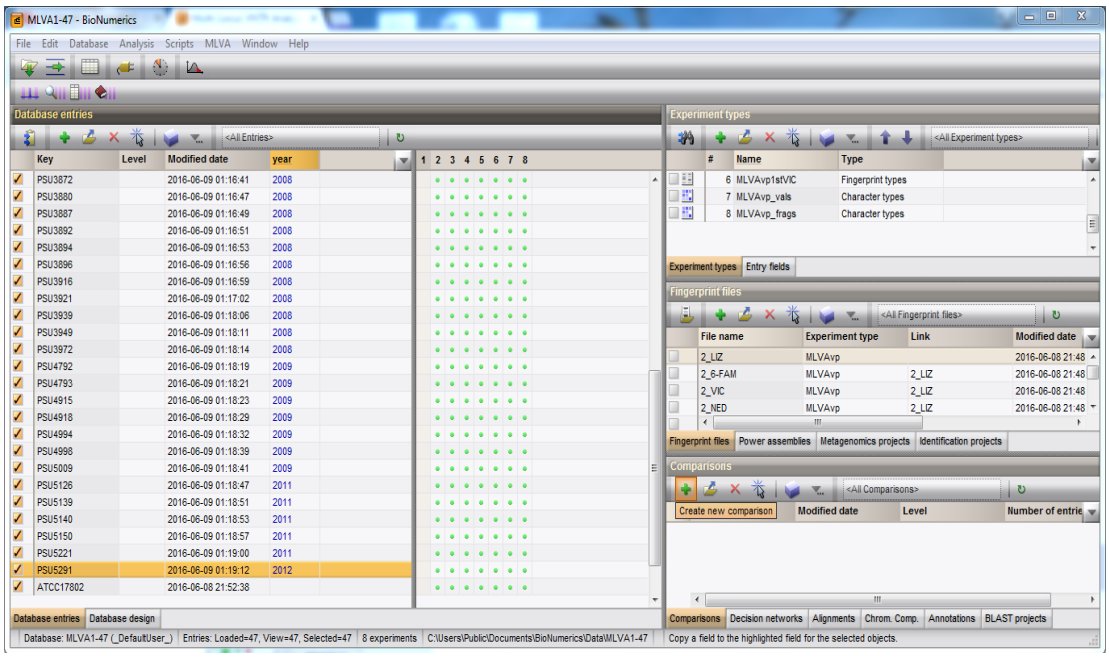
11. VNTR report: Press MLVA> Press VNTR assignment report



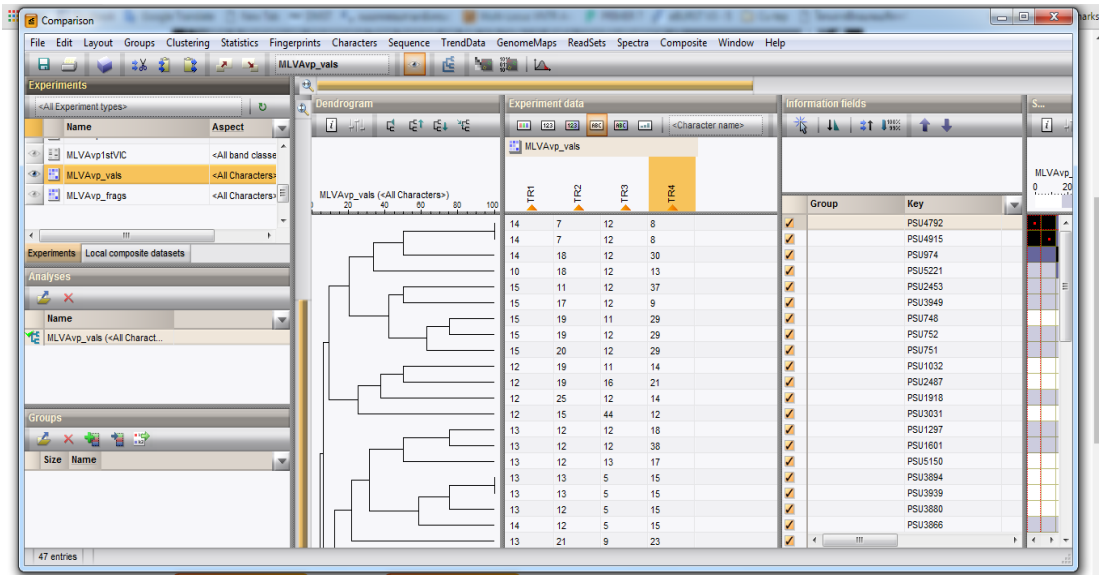
Key	TR1	TR2	TR3	TR4
PSU3892	11 (man...	23	11	13
PSU3894	13 (man...	13 (man...	5 (manual)	15 (man...
PSU3896	11 (man...	24 (man...	11 (man...	13
PSU3916	11 (man...	15 (man...	17 (man...	18
PSU3921	11 (man...	20	7 (manual)	13
PSU3939	13 (man...	13 (man...	5 (manual)	15 (man...
PSU3949	15 (man...	17 (man...	12	9 (manual)
PSU3972	13 (man...	21	9	23
PSU4792	14	7 (?)	12	-
PSU4793	16	13	14	-
PSU4915	14	7 (?)	12	-
PSU4918	16	13	14	-
PSU4994	18	8	16	-
PSU4998	17	-	-	18
PSU5009	-	8	16	-
PSU5126	18	7	17	-
PSU5139	-	8	-	-
PSU5140	18	8	-	-
PSU5150	18 (?)	12 (?)	13 (?)	17 (?)
PSU5221	3	18	12 (?)	13
PSU5291	20 (?)	7	13	3
ATCC17802	15	16	-	32

12. Dendrogram creation:

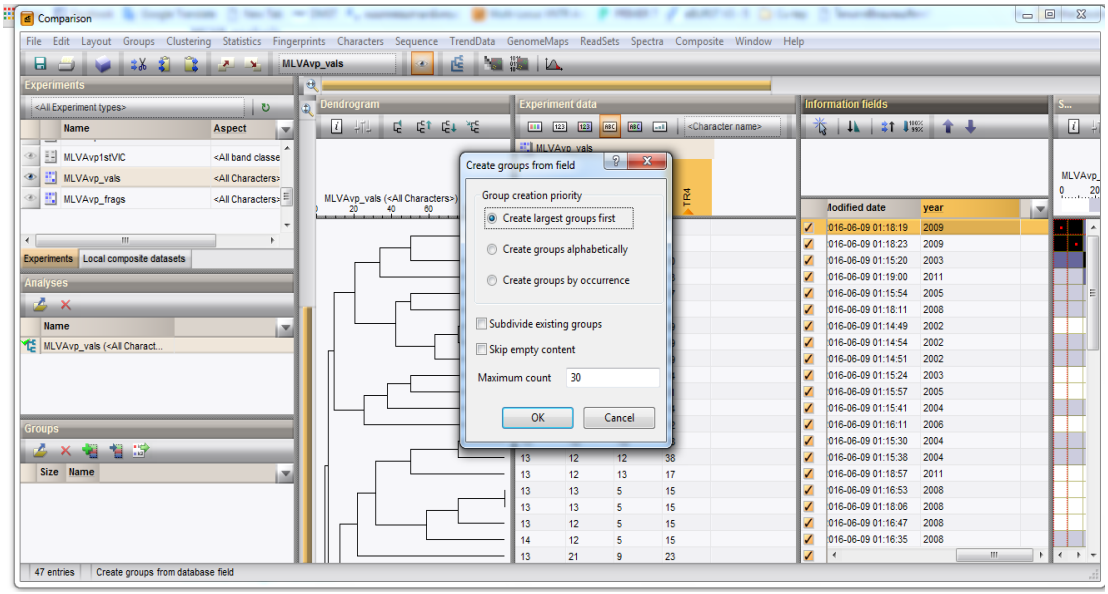
12.1. Press create new comparison



12.2. Press MLVA_Vale> Press All charater> Press pic tree> Press UPGMA> Press finish



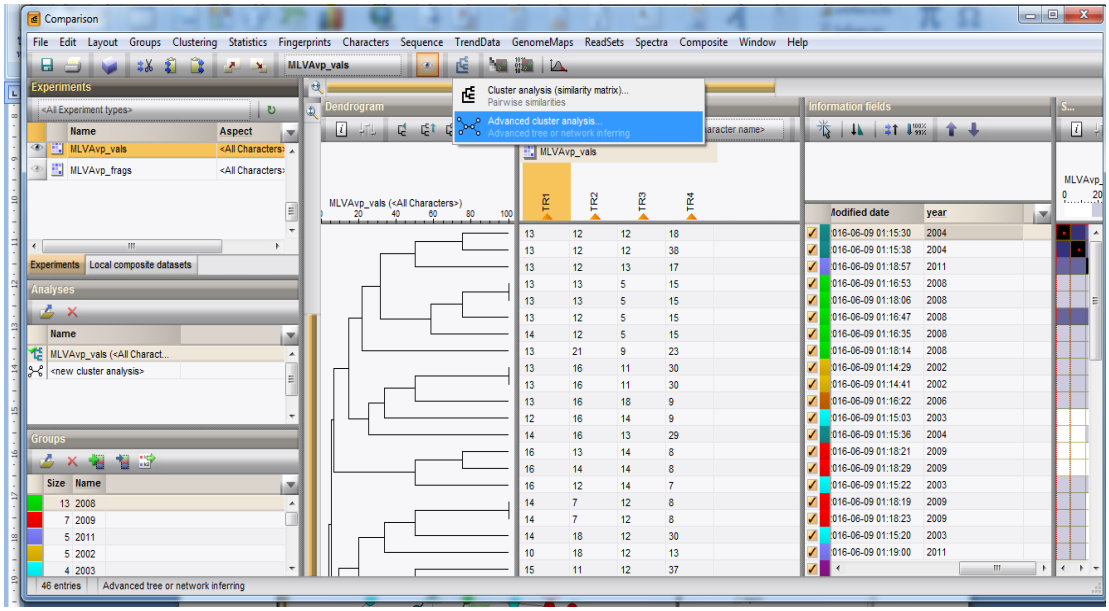
12.3. Create group: Press year> cick right> create group >OK



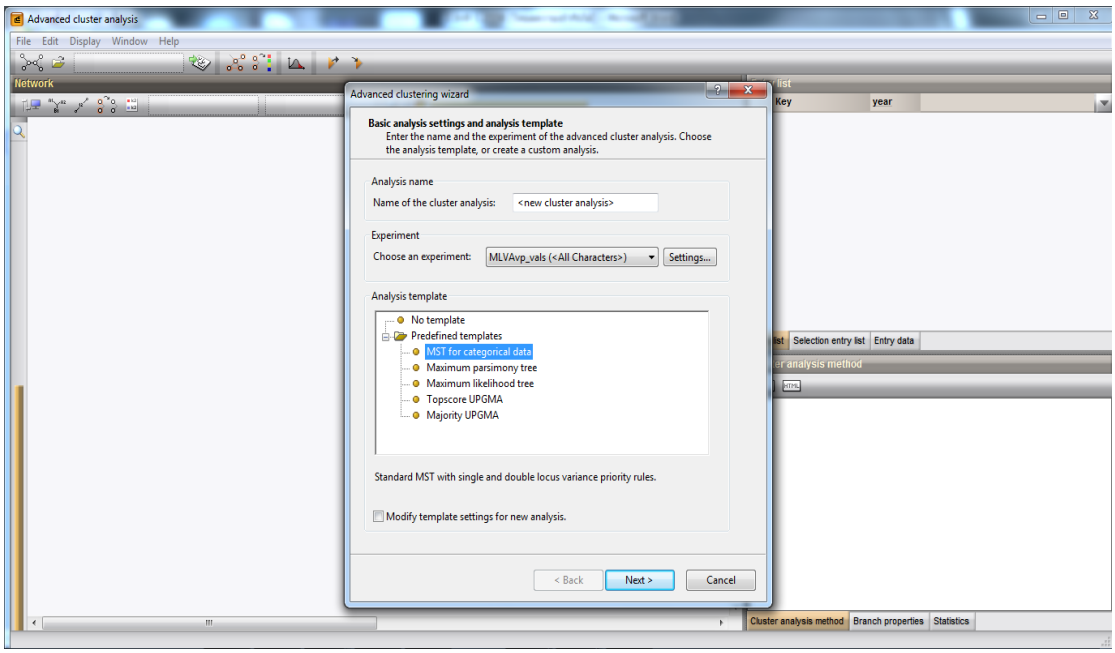
12.4. Export pic: Press print preview> Press adjeust pic > Press Export pic.tif

13. Minimum spanning tree (MTS) creation:

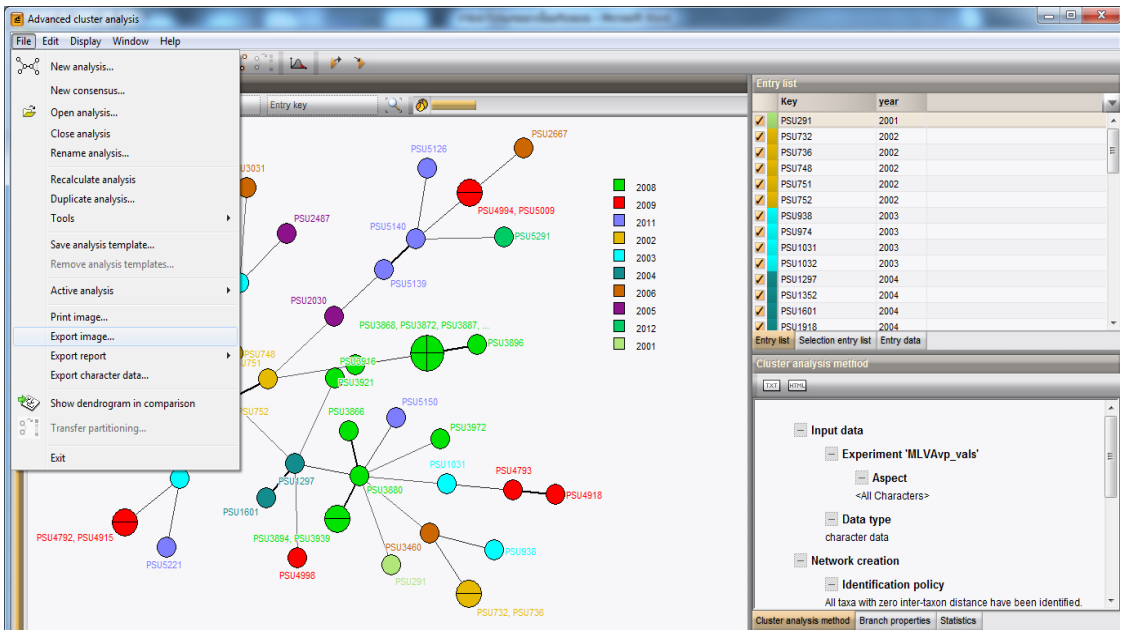
13.1. Press Tree



13.2. Press MST > Press Next



13.3. Export file: Press File> Press export image .tif



APPENDIX D. Publication

Preeprem, S., Singkhamanan, K., Nishibuchi, M., Vuddhakul, V., and Mittraparp-arthorn, P. 2018. Multiplex Multilocus Variable-Number Tandem-Repeat Analysis for Typing of Pandemic *Vibrio parahaemolyticus* O1: KUT Isolates. Foodborne pathogens and disease. 16.

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Original Article

Multiplex Multilocus Variable-Number Tandem-Repeat Analysis for Typing of Pandemic *Vibrio parahaemolyticus* O1:KUT Isolates

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Abstract

Pandemic O3:K6 *Vibrio parahaemolyticus* emerged in 1996. Since then, this strain of pathogen and its serovariants (predominantly O1:KUT [untypable], O1:K25 and O4:K68) have caused gastroenteritis worldwide. Owing to the limitation in established K antisera, tracking the sources of KUT for epidemiological investigation is difficult. Therefore, the effective molecular typing is required to discriminate the strains. The aim of this study was to develop a multiplex multilocus variable-number tandem-repeat analysis (MLVA) assay for typing pandemic *V. parahaemolyticus*, including various O1:KUT isolates. The assay was based on the analysis of four variable number tandem repeat loci. Forty-six pandemic isolates, including O1:KUT, O1:K25, and O3:K6, were investigated. MLVA generated 38 distinct MLVA profiles, whereas only 16 types were obtained from pulsed-field gel electrophoresis (PFGE). In this work, MLVA resolved the 12 isolates of O1:KUT obtained in 2001–2005 with identical PFGE patterns into unique profiles. Our data indicated that multiplex MLVA developed in this study has high discriminatory power ($D = 0.99$), and is superior to PFGE for distinct pandemic *V. parahaemolyticus*, including O1:KUT isolates.

Keywords: molecular epidemiology, MLVA, PFGE, VNTR, *V. parahaemolyticus*

Introduction

VIBRIO PARAHAEMOLYTICUS is a marine bacterium that causes seafoodborne gastroenteritis outbreaks worldwide (CDC, 2016). In 1996, infection by pandemic O3:K6 serotype that possesses the thermostable direct hemolysin (*tdh*) gene but not the *tdh*-related (*trh*) gene appeared suddenly and was responsible for diarrhea outbreaks in various parts of the world (Okuda *et al.*, 1997; Daniels *et al.*, 2000; Martinez-Urtaza *et al.*, 2004; Ansaruzzaman *et al.*, 2005; AESR, 2014). Group-specific polymerase chain reaction (GS-PCR) was established to detect nucleotide variations within the *toxRS/new* region that are unique to the pandemic group of *V. parahaemolyticus* (Matsumoto *et al.*, 2000). In addition, four genomic islands, VPaI-1, VPaI-4, VPaI-5, and VPaI-6, were proposed to be pandemic markers for *V. parahaemolyticus* (Hurley *et al.*, 2006).

In recent years, O3:K6 and other distinct serotypes, such as O1:KUT, O1:K25 and O4:K68, have been demonstrated in Asia, America, Africa, and Europe (Nair *et al.*, 2007; Serichantalergs *et al.*, 2007). In Thailand, those serotypes are predominant and have been reported in clinical specimens (Wootipoom *et al.*, 2007). Molecular analysis of those serovariant strains reveals that they are nearly identical and may originate from the pandemic O3:K6 clone by alteration of the O:K antigens and a genetic recombination (Chowdhury *et al.*, 2000; Matsumoto *et al.*, 2000; Bhuiyan *et al.*, 2002; Chen *et al.*, 2011). However, for epidemiological investigations, some isolates could not be typed to the existing K serogroups (KUT). Therefore, tracking the origins of those various KUT isolates in epidemiological investigations may be difficult. Thus, a high-sensitivity technique is required for differentiating those strains.

Many molecular typing techniques have been developed to gain a better understanding of genetic relatedness among strains of *V. parahaemolyticus*. Several PCR-based typing methods, such as arbitrarily primed PCR and enterobacterial repetitive intergenic consensus PCR sequence, have been demonstrated for *V. parahaemolyticus* strain typing. Although these techniques are affordable, rapid, and easy-to-perform, they are occasionally associated with low discriminatory

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power and low reproducibility (Wong and Lin, 2001; Sabat *et al.*, 2013; Oyarzabal and Kathariou, 2014). A standard method for epidemiological investigation of this pathogen is pulsed-field gel electrophoresis (PFGE). However, this technique requires an expensive specialized equipment, it is time-consuming, and PFGE banding patterns are sometimes difficult to interpret.

Multilocus variable-number tandem-repeat analysis (MLVA) has been developed for typing of bacterial pathogens based on the detection of variable number tandem repeats (VNTRs) at multiple VNTR loci (Lindstedt, 2005). MLVA results can be easily and unambiguously interpreted allowing inter-laboratory comparisons. This method has been demonstrated to provide high-resolution information and reproducibility for epidemiological studies of *V. parahaemolyticus*, especially for pandemic O3:K3 serotype. The five previously reported MLVA typing protocols differ in the number of VNTR loci that were used, including 7 (Ansede-Bermejo *et al.*, 2010), 8 (Kimura *et al.*, 2008; Jiang *et al.*, 2016), 10 (Harth-Chu *et al.*, 2009), and even 12 (Lüdeke *et al.*, 2015). So far, 19 VNTRs has been used for typing of *V. parahaemolyticus* (Kimura *et al.*, 2008; Harth-Chu *et al.*, 2009; Ansede-Bermejo *et al.*, 2010; Lüdeke *et al.*, 2015; Jiang *et al.*, 2016).

The aims of this study were to identify novel potential VNTR loci and develop a new multiplex MLVA scheme using fewer VNTR loci than previous protocols for typing pandemic *V. parahaemolyticus*, especially O1:KUT serotype. The results were compared with those obtained by PFGE. This technique may be useful for molecular epidemiological investigations of other O serogroups *V. parahaemolyticus* that possess KUT.

Materials and Methods

Bacterial strains included in the study

All *V. parahaemolyticus* isolates used in this study were obtained from clinical specimens in southern Thailand and were confirmed by PCR targeting the *toxR* gene (Kim *et al.*, 1999). A total of 46 pandemic isolates, including 32, 8, and 6 isolates of O1:KUT, O1:K25, and O3:K6, respectively were used (Table 1). Nonpandemic *V. parahaemolyticus* isolates ($n=20$) were included for evaluation of pandemic-specific VNTR marker. The pandemic-clone specific characteristics (*tdh*$^+$, *trh*$^-$, and GS-PCR$^+$) were determined by PCR as described previously (Tada *et al.*, 1992; Matsumoto *et al.*, 2000). The O and K serotypes were evaluated by the slide agglutination test using commercial anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan), according to the manufacturer's instructions.

Pulsed-field gel electrophoresis

PFGE of pandemic isolates was carried out as described previously with slight modification (Wang *et al.*, 2008). In brief, agarose-embedded genomic DNA was digested with *Not*I restriction enzyme (NEB, Ipswich MA), and the digested DNA fragments were separated by electrophoresis using 1% pulse-field certified agarose in 0.5×Tris-borate-ethylene diamine tetraacetic acid running buffer at 14°C for19 h with use of the CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed with pulse times of 2.2–54.2 s, a 120° angle, and a 6 V/cm gradient. The gel was stained with ethidium bromide and the DNA patterns were analyzed both visually and with Bionumeric software (v.7.0) (Applied Maths, Sint-Martens-Latem, Belgium). Dendrogram based on the Dice coefficient was constructed by unweighted-pair group method with arithmetic mean (UPGMA).

Table 1. Characteristics and Group-Specific Polymerase Chain Reaction Results of *Vibrio parahaemolyticus* Isolates Used in This Study

Serotype (no. of isolates)	*Year of isolation*	*No. of isolates*	*Presence of gene*		*GS-PCR result*
			tdh	trh	
Pandemic (46)					
O1:KUT (32)	2001	1	+	−	+
	2002	5	+	−	+
	2003	2	+	−	+
	2004	4	+	−	+
	2005	3	+	−	+
	2006	2	+	−	+
	2008	2	+	−	+
	2009	7	+	−	+
	2011	5	+	−	+
	2012	1	+	−	+
O1:K25 (8)	2003	2	+	−	+
	2006	1	+	−	+
	2008	5	+	−	+
O3:K6 (6)	2008	6	+	−	+
Nonpandemic (20)					
	2000–2007	7	+	−	−
	2009–2011	3	+	+	−
	2007–2011	3	−	+	−
	2008–2011	7	−	−	−

GS-PCR, group-specific polymerase chain reaction; *tdh*, thermostable direct hemolysin; *trh*, *tdh*-related.

VNTR analyses

For the selection of VNTR markers, the genome sequence of *Vibrio parahaemolyticus* RIMD2210633 was screened to locate the presence of VNTR sequences (http://minisatellites.u-psud.fr) (Denœud and Vergnaud, 2004) according to the following criteria: (1) a repeat unit range of 3–9 bp, (2) a minimum copy numbers of 10, (3) a minimum repeat size of 50 bp, and (4) percentage matches between 80 and 100. Three VNTR loci common to the previous studies (TR1, TR2, and TR4) (Kimura *et al.*, 2008; Harth-Chu *et al.*, 2009; Ansede-Bermejo *et al.*, 2010; Lüdeke *et al.*, 2015; Jiang *et al.*, 2016) and one novel VNTR loci (TR3) were identified (Table 2). Primer sets specific for those four VNTR loci were designed with Primer3Plus software (http://primer3plus.com) (Rozen and Skaletsky, 1999). The 5′ forward primers were labeled with distinctive fluorescent dyes (VIC, PET, NED, or FAM) to generate multiplex PCR products, which were further separated by multicolored capillary electrophoresis. In VNTR amplification, bacterial DNA was extracted by boiling method (Kayali *et al.*, 2015) and subjected to a multiplex PCR amplification using primer mix to simultaneously amplify the VNTR loci. Each 30 μL multiplex PCR mixture contained 1×GoTaq® Reaction buffer, 0.5 U of GoTaq® DNA polymerase (Promega, Madison, WI), 200 μM of each deoxynucleoside triphosphate (Promega), 0.2 μM of each primer, and 2 μL of

TABLE 2. CHARACTERISTICS OF THE VARIABLE NUMBER TANDEM REPEAT LOCI AND LIST OF PRIMERS USED FOR MULTILOCUS VARIABLE-NUMBER TANDEM-REPEAT ANALYSIS

VNTR locus name (chromosome)	*Repeat consensus sequences*	*VNTR copy number*[a]	*Function*	*DI*	*Primer*	*Expected amplicon size (bp)*[b]
TR1 (I)	AGGTTCT	11	Noncoding region	0.83	F: 5′-VIC[c]-GTGACGGCTGG TCAGAAGAT-3′ R: 5′-TTCACGCCAACTTC CTCAAC-3′	130
TR2 (I)	TCTGGC	21	Hypothetical protein	0.92	F: 5′-PET[c]-AGTTGCGGTG CGTAGTTTTC-3′ R: 5′-TCGCTCAGGAGCTA TCCTCT-3′	190
TR3 (II)	CAGCTAAA	22	Noncoding region	0.85	F: 5′-NED[c]-TTCACGGGTA AAAACACCAT-3′ R: 5′-GCTGTGGTCTTTGT GTACGG-3′	233
TR4 (I)	ATAGAG	28	Hypothetical protein	0.91	F: 5′-FAM[c]-TCAGCCAGAC ACTCCATACAA-3′ R: 5′-GGAAGACATATCCG GTTTGC-3′	214

[a]Repeat times were counted using the genome sequence of *Vibrio parahaemolyticus* strain RIMD2210633.
[b]Amplicon size of each VNTR locus was estimated using the genome sequence of *V. parahaemolyticus* strain RIMD2210633.
[c]Fluorescent dyes.
DI, diversity index; F, forward; R, reverse; VNTR, variable number tandem repeat.

template DNA. The multiplex amplification was carried out on a T100 thermal cycler (Bio-Rad Laboratories) using a thermal profile of beginning at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min. Each run ended with a final elongation step at 68°C for 5 min. The PCR products were analyzed by capillary electrophoresis using ABI 3130 Genetic Analyzer (Applied BioSystems, Foster City, CA). Fragment sizes from each VNTR loci were determined by GeneMapper software (v.4.1) (Applied BioSystems) and were converted into VNTR copy numbers using the following equation (Slack *et al.*, 2005):

$$\text{Number of repeats (bp)} = [\text{fragment size (bp)} - \text{flanking regions (bp)}]/\text{repeat size (bp)}.$$

The combination of four numbers reflecting the number of repeats in four VNTR loci generated an MLVA profile. A dendrogram was constructed based on the categorical (mapping) with UPGMA method by Bionumeric software (v.7.0) (Applied Maths).

Statistical analysis

The polymorphism information index or diversity index (DI) of each VNTR locus was calculated individually using the formula; $D = 1\text{-}\Sigma\ (\text{allele frequency})^2$ (Weir, 1990). The discriminatory power was calculated as previously described (Castañeda *et al.*, 2005). The congruence between typing methods was determined using the Wallace coefficients (W) (Fowlkes and Mallows, 1983).

Results

Genotyping by PFGE

The *Not*I PFGE of 46 pandemic *V. parahaemolyticus* isolates was performed and 16 distinct PFGE types were generated. The discriminatory power of PFGE was 0.89. At 80% cutoff, all isolates obtained during 2001–2006 were grouped together regardless of serotypes (Fig. 1). It is of interest that 12 isolates of O1:KUT obtained during 2001–2005 exhibited indistinguishable PFGE patterns (Fig. 1). All O3:K6 isolates had similar PFGE pattern and were clustered closely with four of five O1:K25 isolates obtained in the same year (Fig. 1). New PFGE types were observed among the O1:KUT isolates obtained during 2009–2012. Similarity values of >70% were observed among these isolates (Fig. 1). A minimum spanning tree (MST) based on PFGE illustrated that phylogenetic groupings of all pandemic *V. parahaemolyticus* isolates were well correlated with serotype (Fig. 2A), except for one O1:K25 isolate, and period of isolation (Fig. 2B).

Analysis of VNTR loci and MLVA analysis

In this work, four VNTR loci (TR1–TR4) with repeat consensus sequences between six and eight were selected. Analysis of these loci indicated that three (TR1, TR2, and TR4) and one VNTR (TR3) loci were located on chromosome I and II, respectively (Table 2). TR1 and TR3 were localized in the noncoding regions; however, TR2 and TR4 were located in the open reading frame regions of hypothetical proteins, VP2226 and VP2892, respectively. TR3 primers could also amplify VPA1263 region, which is located in the VPaI-6 (Fig. 3). In this study, all pandemic isolates were positive with the TR3 primers, whereas amplified DNA was not detected in all 20 nonpandemic isolates.

Specific primers that targeted each VNTR locus were newly designed to support multiplex PCR method (Table 2). The numbers of flanking nucleotides in TR1 to TR4 are 53, 88, 57, and 46 bp, respectively. A multiplex PCR simultaneously amplified four VNTR loci of *V. parahaemolyticus* and generated the copy number of each locus ranging from 8 to 17 (TR1), 7 to 24 (TR2), 5 to 44 (TR3), and 7 to 38 (TR4),

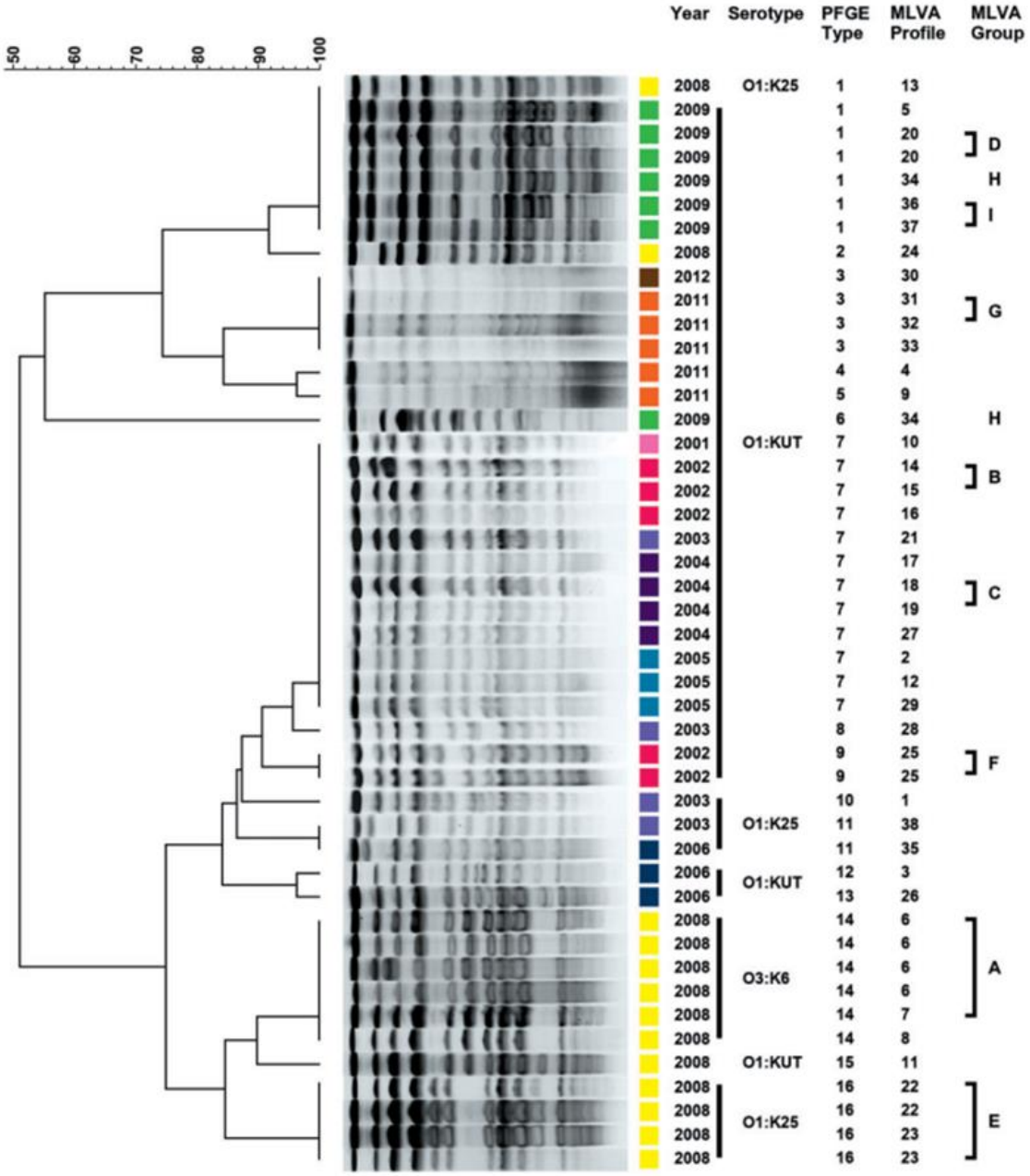

FIG. 1. Dendrogram was generated with *Not*I-digested PFGE patterns of the 46 pandemic *Vibrio parahaemolyticus* isolates. Colors within the squares correspond to year of isolation. Year of isolation and serotype of the isolate are included along each PFGE lane. MLVA profiles and MLVA groups **(A–I)** are provided for direct comparison. MLVA, multilocus variable-number tandem-repeat analysis; PFGE, pulsed-field gel electrophoresis. Color images available online at www.liebertpub.com/fpd

respectively (Fig. 4). The DI based on genetic diversity for the four VNTR loci ranged from 0.83 to 0.92 (Table 2). TR2 locus was the most polymorphic loci (Table 2). No amplification product of TR1 and/or TR4 loci was observed in some isolates (Fig. 4).

Based on MLVA using these four VNTR loci, 38 distinct MLVA profiles revealing high genetic diversity were detected among 46 isolates of pandemic *V. parahaemolyticus* (Fig. 4). Similar MLVA profiles were observed in some isolates of the same serotype obtained during the same periods

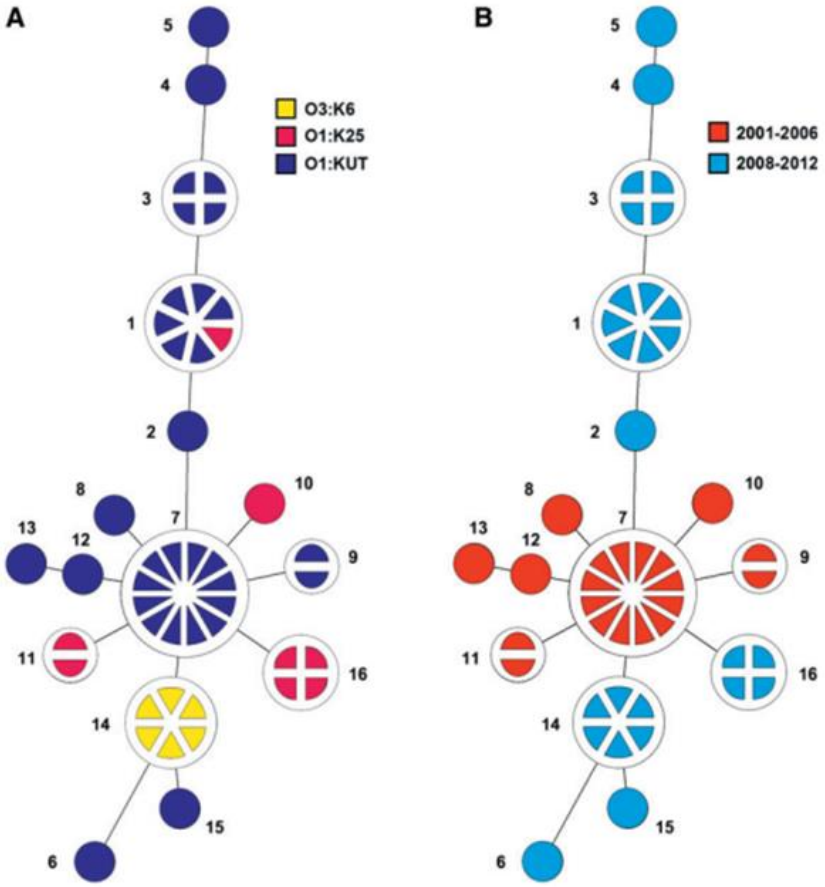

FIG. 2. Minimum spanning tree of the 46 pandemic *Vibrio parahaemolyticus* isolates based on PFGE. Each circle represents a unique PFGE type. The colored pie chart sections inside circles correspond to serotypes **(A)** or period of isolation **(B)**. The circle size indicates the number of isolates with that PFGE type. The numbers indicate PFGE types. PFGE, pulsed-field gel electrophoresis. Color images available online at www.liebertpub.com/fpd

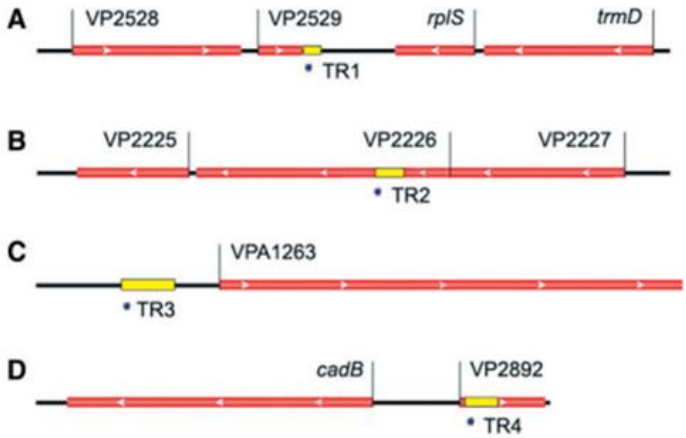

FIG. 3. *Vibrio parahaemolyticus* RIMD2210633 genome regions containing the VNTR loci used for the MLVA. Each region corresponded to the VNTR locus TR1 **(A)**, TR2 **(B)**, TR3 **(C)**, and TR4 **(D)**, respectively. Arrows indicate the direction of transcription. MLVA, multilocus variable-number tandem-repeat analysis; VNTR, variable number tandem repeat. Color images available online at www.liebertpub.com/fpd

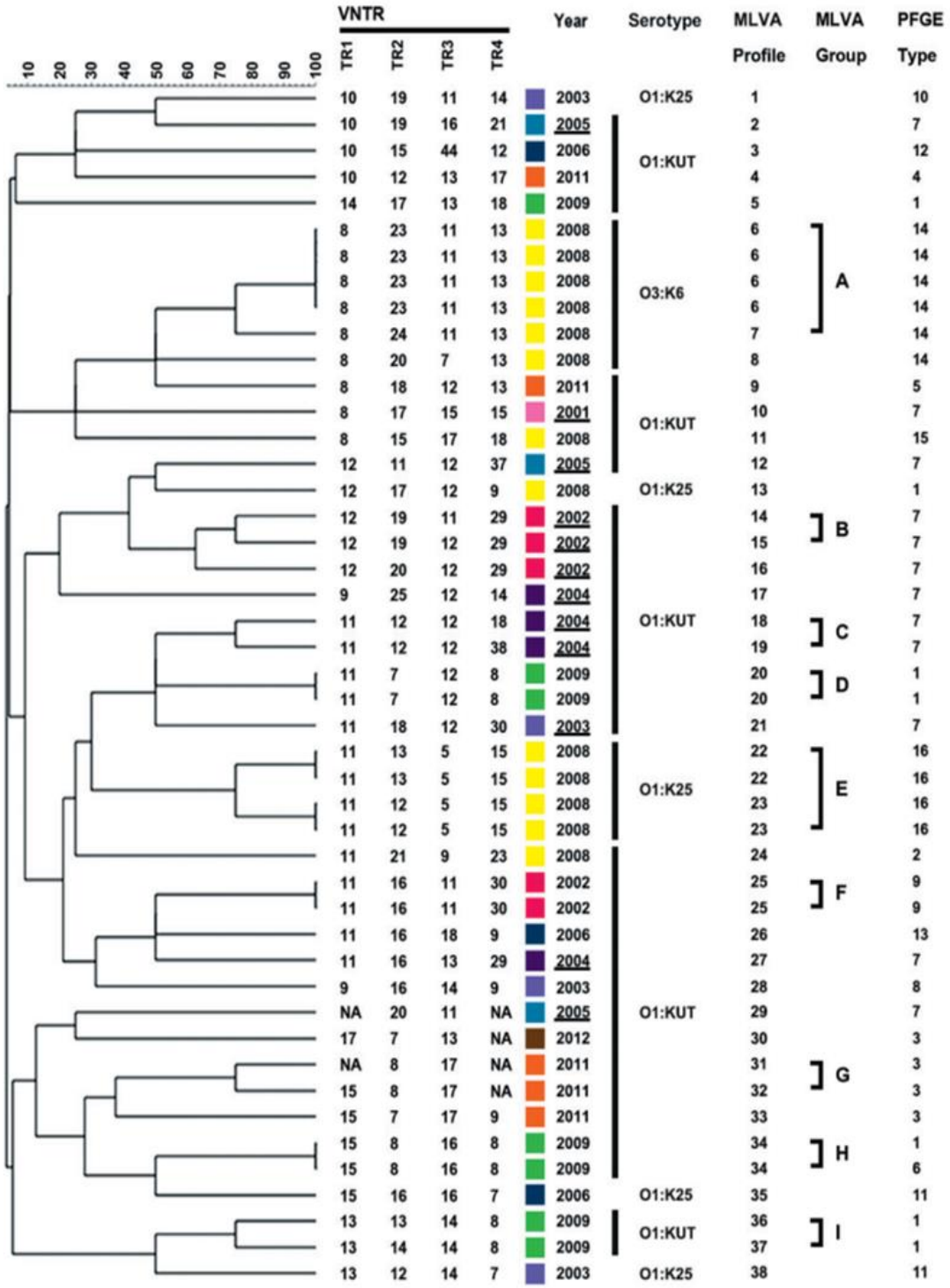

FIG. 4. Dendrogram of the 46 pandemic *Vibrio parahaemolyticus* isolates based on MLVA profiles. Colors within the squares correspond to year of isolation. The number of tandem repeats is indicated. The MLVA groups are indicated (**A–I**). NA was given when no amplification was observed at a given locus. PFGE types are provided for direct comparison. Underlined letters refer to the 2001–2005 isolates, which are indistinguishable by PFGE. MLVA, multilocus variable-number tandem-repeat analysis; NA, not amplified; PFGE, pulsed-field gel electrophoresis; VNTR, variable number tandem repeat. Color images available online at www.liebertpub.com/fpd

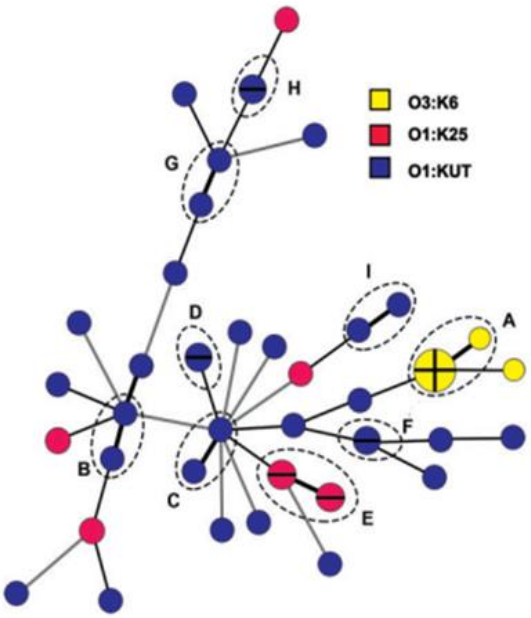

FIG. 5. Minimum spanning tree of the 46 pandemic *Vibrio parahaemolyticus* isolates based on MLVA profiles. Each circle represents a unique MLVA profile. The color of the circles corresponds to serotypes. The circle size is proportional to the number of isolates. A distance of one locus between two MLVA profiles is indicated by a thick line, a distance of two loci is indicated by a thin line, and a distance of three loci is indicated by a gray line. Dotted circle indicated the nine MLVA groups **A–I**. MLVA, multilocus variable-number tandem-repeat analysis. Color images available online at www.liebertpub.com/fpd

(Fig. 4). However, the results of this study showed that pandemic *Vibrio parhaemolyticus* O1:KUT isolates were heterogeneous. The discriminatory power of MLVA was 0.99.

MST analysis revealed that all MLVA profiles were linked with other profiles regardless of the serotypes (Fig. 5). MLVA profiles were grouped together if two neighboring profiles did not differ in more than one VNTR locus. Nine MLVA groups (**A–I**) were found in this study (Figs. 4 and 5).

Comparison of MLVA with PFGE

MLVA separated the 12 PFGE-indistinguishable O1:KUT isolates obtained in 2001–2005 (PFGE type 7) into unique profiles (Fig. 1). Thirty-two isolates of O1:KUT *Vibrio parahaemolyticus* revealed 12 PFGE types and 29 MLVA profiles (Fig. 4). MLVA offered a higher discriminatory power (0.99) than that of the PFGE (0.89).

The results showed that all isolates with the same MLVA profile had a high probability of having the same PFGE type. As calculated by using W, MLVA predicted PFGE type ($W=0.86$). However, PFGE profile did not predict MLVA profile ($W=0.33$).

Discussion

Most serotypes of O3:K6, O1:K25, O4:K28, and O1:KUT pandemic *V. parahaemolyticus* have been continually detected among patients worldwide (Nair *et al.*, 2007; Wootipoom *et al.*, 2007; Thongjun *et al.*, 2013). MLVA technique has been demonstrated for typing pandemic *V. parahaemolyticus* isolates; however, there is no direct evidence of this technique for differentiation among O1:KUT. In this work, MLVA technique has been constructed to discriminate pandemic O1:KUT and other pandemic serogroups *V. parahaemolyticus* strains.

Previous MLVA protocols have been associated with a number of VNTR loci and primer sets (Kimura *et al.*, 2008; Harth-Chu *et al.*, 2009; Ansede-Bermejo *et al.*, 2010; Lüdeke *et al.*, 2015; Jiang *et al.*, 2016). In this study, we optimized the criteria for the selection of VNTR loci and redesigned a set of fluorescence-labeled specific primers for single-tube multiplex VNTR amplification. A novel VNTR locus (TR3) was used in combination with three of the previously described loci (TR1, TR2, and TR4) (Kimura *et al.*, 2008; Harth-Chu *et al.*, 2009; Ansede-Bermejo *et al.*, 2010; Lüdeke *et al.*, 2015; Jiang *et al.*, 2016). Thus, the MLVA developed in this study differs considerably from the MLVA previously described. In this study, MLVA scheme yielded a higher discriminatory power than that of the standard PFGE.

VNTR can be found in both noncoding and coding regions of bacterial genomes. TR1 locus was located in noncoding region downstream of the gene encoded for a zinc-binding protein (Makino *et al.*, 2003). Thus, copy number variation in this region may have no effect on the downstream gene. However, TR2 was located within the gene encoded for a hypothetical protein ortholog to chemotaxis protein CheW that was necessary for the control of bacterial motility patterns (Miller *et al.*, 2009). Variations in the repeat numbers could affect protein synthesis and structure. Effects of changing the repeat length on virulence and phenotypes have been demonstrated in many pathogens, including *Haemophilus influenzae*, *Enterococcus* sp., and *Streptococcus* sp. (Schouls *et al.*, 2005; Wałecka *et al.*, 2009; Naseer *et al.*, 2012). VNTRs were also shown to be involved in the variation of promoter activity (Tantivitayakul *et al.*, 2010). Amplified DNA derived from TR2 primers revealed the highest variability in copy numbers. Therefore, this is one of the important regions to generate diversity among *V. parahaemolyticus* isolates. For TR3 locus, primers were designed to target the pandemic region found within *V. parahaemolyticus* genomic island VPaI-6 (Hurley *et al.*, 2006), which was located next to the TR3 locus. In this study, TR3 was specific to all 46 pandemic strains. In this study, four isolates belonging to the O1:KUT were negative for the TR1 and/or TR4 amplification. This might be due to either sequence variation in the PCR binding regions or lack of those VNTR loci. This also can indicate the variation among isolates. In addition, four-loci combination of MLVA in this work could generate distinguishable profiles of those isolates.

Identical PFGE type and MLVA profile of the isolates obtained from patients indicated that the infection might be associated with the same clones because the isolates were taken from the patients in the same period and geographic region.

MST analysis based on MLVA revealed the link between pandemic O1:KUT isolates with other pandemic serotypes. Previous study suggested that the O1:KUT and O1:K25 isolates are originated from the pandemic O3:K6 stains by

alteration of their O:K antigens in response to the host's immunological pressure (Chowdhury *et al.*, 2004; Nair *et al.*, 2007). The results obtained in this study confirmed the genetic relatedness of pandemic O3:K6, O1:KUT, and O1:K25 serotypes.

Two isolates defined as unrelated by PFGE (PFGE types 1 and 6) were not distinguished by MLVA (MLVA profile 34). This may be due to the reason that tandem repeats in these isolates evolved slower than others (Noller *et al.*, 2006). MLVA and PFGE detect different genetic variation in different regions of the chromosome. Variations in MLVA profiles are caused by the mutation in tandem repeats; however, mutations in enzyme restriction sites have been shown to affect PFGE type (Tenover *et al.*, 1995). Therefore, MLVA may be capable to detect the fast evolution among bacterial strains, which other methods such as PFGE are unable to detect, and beneficial for fast surveillance and tracking the source of foodborne disease outbreaks (Lüdeke *et al.*, 2015; Jiang *et al.*, 2016). Although, the high variation of VNTR can indicate the differentiation of bacteria strains, it can cause the limitation in the correlated investigation between bacteria strains isolated from different periods (Lam *et al.*, 2012). The discriminatory values >0.90 are acceptable for epidemiological typing of bacterial strains (Hunter and Gaston, 1988). Thus, this indicates that four VNTR loci used in this study are suitable genetic markers for differentiation of pandemic *V. parahaemolyticus*, especially among various O1:KUT isolates, and can be applied for molecular epidemiological investigations of other O serogroups *V. parahaemolyticus* with KUT serotype. In addition, MLVA technique is considerably more rapid than PFGE technique. It is of interest for future studies to evaluate relationship between the difference in the VNTR copy numbers among the various loci and virulence or phenotypic variation of *V. parahaemolyticus*.

Conclusions

This study demonstrated a reliable multiplex MLVA assay for typing pandemic *V. parahaemolyticus*, including various O1:KUT isolates based on four VNTR loci (TR1–TR4). It is highly reproducible with high discriminatory power and would significantly contribute to surveillance and outbreak investigation of pandemic *V. parahaemolyticus* with untypable serotypes.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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