Characterization of Human-and Shrimp-Pathogenic *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is a halophilic bacterium distributed in marine and estuarine environments worldwide. *V. parahaemolyticus* may causes gastroenteritis after consumption of contaminated seafood. The pathogenicity of this bacterium in humans is associated to the *tdh* and *trh* genes, which are considered to be the major virulence genes in the pathogenesis. The *trh* gene possesses a significantly broader nucleotide sequence variation and can be subdivided in two main subtypes (*trh*1 and *trh*2) which share 84% identical in sequence. In order to characterize *V. parahaemolyticus* carrying the *trh* gene, 73 clinical isolates of *trh*$^+$ *V. parahaemolyticus* obtained previously from various countries were investigated. No significant difference was observed in the urease production between *tdh*$^+$ *trh*1$^+$ and *tdh*$^+$ *trh*2$^+$ ($p = 0.063$) and between the *tdh*$^-$ *trh*1$^+$ and *tdh*$^-$ *trh*2$^+$isolates ($p = 0.788$). This implied that the *tdh* and *trh* genes of *V. parahaemolyticus* were not involved in the urease production. The isolates carrying only the *trh* gene showed variation in their haemolytic activity that might correlate to the sequence variation in *trh*1 and *trh*2 genes. The ratio of urease production and haemolytic activities between the *trh*1$^+$ and *trh*2$^+$ isolates and biofilm formation of *trh*$^+$ *V. parahaemolyticus* isolates were not significantly different. Out of 16 of 34 isolates (47.0%) of *trh*$^+$ *V. parahaemolyticus* gave positive for CRISPR detection. CRISPR-virulence typing of all CRISPR-positive isolates was constructed and compared to profiles obtained by CRISPR typing alone. The discriminatory power index (DI) of CRISPR-virulence typing was higher than the CRISPR typing alone.

V. parahaemolyticus causes acute hepatopancreatic necrosis disease (AHPND) in shrimp. To clarify the pathogenicity of AHPND *V. parahaemolyticus* in shrimp, all 33 AHPND *V. parahaemolyticus* isolates obtained in this study were characterized. All isolates were O1 serotype but possessed different K antigens. DNA

profiles of AHPND *V. parahaemolyticus* isolates were similar, but were distinct from those obtained from clinical and environmental isolates. This indicates the causative agent of AHPND might be originated from one clone and then subsequently different K antigens have been developed.

In this study, two sets of primers (LAMP-A2 and LAMP-A3) specific to the unique DNA sequences derived from the plasmid and toxin gene of AHPND *V. parahaemolyticus* were developed and evaluated for LAMP assay to identify AHPND *V. parahaemolyticus*. In pure culture and in spiked shrimp experiments, the LAMP assay was superior to PCR for the detection of AHPND *V. parahaemolyticus.* In pure cultures, the detection limit of LAMP-A3 was 53 cfu/ml or 0.1 cfu/reaction, whereas in spiked shrimp experiments, the detection limit was $4.4x10^5$ cfu/ml or $8.8x10^2$ cfu/reaction. This technique could also detect AHPND *V. parahaemolyticus* in shrimp, sediment and water samples collected from a shrimp farm. The results suggested that the LAMP-A3- based LAMP assay was suitable for the identification of AHPND *V. parahaemolyticus* in shrimp aquaculture.

To control AHPND *V. parahaemolyticus, Bdellovibrio* and like organisms (BALOs) were isolated and the capability to reduce AHPND *V. parahaemolyticus* was evaluated. Nine isolates of BALOs specific to AHPND *V. parahaemolyticus* were obtained and were identified as *Bacteriovorax* spp. The ability of BALOs designated as BV-A to reduce numbers of AHPND *V. parahaemolyticus in vitro* was observed in co-culture after incubation for 2 days and continued until 7 days of incubation period. *In vivo*, BV-A could reduce the mortality of shrimp post-larvae infected with AHPND *V. parahaemolyticus*. This study indicates that *Bacteriovorax* can be used as a biocontrol agent of AHPND *V. parahaemolyticus* in shrimp aquaculture.

ชื่อวิทยานิพนธ์ การศึกษาลักษณะของเชื้อ *Vibrio parahaemolyticus* ที่ก่อโรคในมนุษย์และกุ้ง

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บทคัดย่อ

Vibrio parahaemolyticus เป็นแบคทีเรียที่พบได้ในน้ำทะเลทั่วโลกเป็นสาเหตุของโรคทางเดินอาหารอักเสบจากการรับประทานอาหารทะเล เชื้อ *V. parahaemolyticus* สายพันธุ์ก่อโรคในคนมียีน *tdh* และ *trh* ซึ่งเป็นปัจจัยสำคัญ ยีน *trh* มี 2 กลุ่มย่อย คือ *trh*1 และ *trh*2 และมีลำดับนิวคลีโอไทด์เหมือนกัน ร้อยละ 84 จากการศึกษาลักษณะของเชื้อ *V. parahaemolyticus* ที่มียีน *trh* ซึ่งแยกได้จากผู้ป่วยในหลายประเทศ จำนวน 73 ไอโซเลท ไม่พบความสัมพันธ์กับสร้างเอนไซม์ยูรีเอสระหว่างเชื้อสายพันธุ์ tdh^+ $trh1^+$ และ tdh^+ $trh2^+$ (p = 0.063) รวมทั้งในสายพันธุ์ tdh^- $trh1^+$ และ tdh^- $trh2^+$ (p = 0.788) การศึกษาความสามารถในการย่อยสลายเม็ดเลือดแดงของเชื้อ *V. parahaemolyticus* สายพันธุ์ที่มียีน *trh* เพียงยีนเดียว พบว่าเกี่ยวข้องกับความแตกต่างของลำดับเบสนิวคลีโอไทด์ในยีน *trh*1 และ *trh*2 นอกจากนี้ไม่พบความแตกต่างอย่างมีนัยสำคัญของการสร้างไบโอฟิล์มของเชื้อ *V. parahaemolyticus* ในการศึกษานี้ได้ตรวจพบ CRISPR ในเชื้อ trh^+ *V. parahaemolyticus* จำนวน 16 ไอโซเลท จากทั้งหมด 34 ไอโซเลท (ร้อยละ 47.0) จากการศึกษาโดยการใช้ CRISPR เพื่อจำแนกสายพันธุ์ของเชื้อ trh^+ *V. parahaemolyticus* โดยเทคนิค CRISPR-virulence typing พบว่ามีอำนาจในการจำแนกที่สูงกว่าเมื่อเปรียบเทียบกับเทคนิค CRISPR typing เพียงอย่างเดียว

V. parahaemolyticus เป็นสาเหตุของโรคตับวายเฉียบพลัน (acute hepatopancreatic necrosis disease: AHPND) ส่งผลให้เกิดอัตราการตายจำนวนมากในกุ้งเพาะเลี้ยง ในการศึกษาครั้งนี้พบว่า AHPND *V. parahaemolyticus* จำนวน 33 ไอโซเลทที่แยกมาได้ มีซีโรไทป์ O1 แต่มี K แอนติเจนต่างกัน จีโนไทป์ของเชื้อ AHPND *V. parahaemolyticus* มีความคล้ายคลึงกัน แต่แตกต่างจากเชื้อ *V. parahaemolyticus* ที่แยกได้จากตัวอย่างผู้ป่วยและสิ่งแวดล้อม ผลการศึกษาชี้ให้เห็นว่า เชื้อ AHPND *V. parahaemolyticus* อาจจะมีที่มาจากแหล่งเดียวกันและเกิดการเปลี่ยนแปลงของ K ซีโรไทป์ในเวลาต่อมา

นอกจากนี้ได้พัฒนาวิธีการบ่งชี้ AHPND *V. parahaemolyticus* ด้วยเทคนิค LAMP โดยการออกแบบ ไพรเมอร์ 2 ชุด คือ LAMP-A2 และ LAMP-A3 ที่จำเพาะกับพลาสมิดและยีนก่อโรคของ AHPND *V. parahaemolyticus* จากการศึกษาพบว่า เทคนิค LAMP มีประสิทธิภาพในการตรวจหาเชื้อก่อโรคในกุ้ง AHPND *V. parahaemolyticus* ในการตรวจหาเชื้อที่เลี้ยงแบบบริสุทธิ์ พบว่าปริมาณของเชื้อน้อยที่สุดที่สามารถตรวจพบได้โดยเทคนิค LAMP-A3 คือ 53 cfu/ml หรือ 0.1 cfu/reaction ในขณะที่ปริมาณของเชื้อน้อยที่สุดที่สามารถตรวจพบได้จากการทดสอบแบบเติมเชื้อในตัวอย่างกุ้ง คือ 4.4×10^5 cfu/ml หรือ 8.8×10^2 cfu/reaction เทคนิคดังกล่าวยังสามารถตรวจหาเชื้อ AHPND *V. parahaemolyticus* ได้จากตัวอย่างกุ้ง น้ำ และตะกอนดิน ที่เก็บจากฟาร์มเพาะเลี้ยงกุ้ง เทคนิค LAMP โดยใช้ชุดไพรเมอร์ LAMP-A3 เหมาะสมสำหรับการนำมาใช้ในการตรวจหาเชื้อ AHPND *V. parahaemolyticus* ดีกว่าไพรเมอร์ LAMP-A2

เพื่อควบคุมปริมาณของเชื้อ AHPND *V. parahaemolyticus* แบคทีเรียในกลุ่ม *Bdellovibrio* and like organisms (BALOs) ได้ถูกคัดแยกและทดสอบประสิทธิภาพในการกำจัดเชื้อก่อโรคดังกล่าว จากการศึกษา BALOs ที่แยกได้ทั้งหมด 9 ไอโซเลท เป็นเชื้อในจีนัส *Bacteriovorax* ในการทดสอบแบบ *in vitro* เชื้อ *Bacteriovorax* BV-A สามารถลดปริมาณของเชื้อ AHPND *V. parahaemolyticus* ได้ ภายใน 2 วันของการทดลอง และลดลงเรื่อยๆจนกระทั้งสิ้นสุดการทดลอง (วันที่ 7) นอกจากนี้การทดสอบแบบ *in vivo* ยังพบว่า BV-A สามารถลดอัตราการตายของกุ้งระยะโพสต์ลาร์วาที่มีการติดเชื้อ AHPND *V. parahaemolyticus* ผลการทดลองแสดงให้เห็นว่า *Bacteriovorax* สามารถใช้เป็นตัวควบคุมทางชีวภาพเพื่อลดปริมาณ *V. parahaemolyticus* ที่เป็นสาเหตุของโรค AHPND ในฟาร์มเพาะเลี้ยงกุ้ง

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Jetnapang Kongrueng

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS AND SYMBOLS

16S rRNA	=	16S subunit of ribosomal ribonucleic acid
µg	=	Microgram(s)
µl	=	Microliter(s)
µM	=	Micromolar(s)
°C	=	Degree Celsius
AHPND	=	Acute hepatopancreatic necrosis disease
Amp	=	Ampicillin
APW	=	Alkaline peptone water
ASW	=	Artificial sea water
BALOs	=	*Bdellovibrio* and like organisms
BLAST	=	Basic local alignment search tool
bp	=	Base pair(s)
BSA	=	Bovine serum albumin
C	=	Chloramphenicol
$CaCl_2$	=	Calcium chloride
CDRs	=	Consensus direct repeat sequences
cfu	=	Colony forming unit
cm	=	Centimeter(s)
CN	=	Gentamycin
CRISPR	=	Clustered regularly interspaced short palindromic repeat
$CuSO_4 \cdot 5H_2O$	=	Copper (II) sulfate pentahydrate
CV	=	CHROMagar Vibrio
dH_2O	=	Distilled water
DNA	=	Deoxyribonucleic acid
DNAg	=	Diluted nutrient agar
DNB	=	Diluted nutrient broth
dNTPs	=	Deoxyribonucleotide triphosphates
DRs	=	Direct repeats
E	=	Erythromycin

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

EDTA	=	Ethylenediaminetetraacetic acid
g	=	Gram(s)
GS-PCR	=	Group specific polymerase chain reaction
h	=	Hour(s)
HCl	=	Hydrochloric acid
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KCl	=	Potassium chloride
LAM	=	Low-melted-point-agarose
LAMP	=	Loop-mediated isothermal amplification
M	=	Molarity
mg	=	Milligram(s)
$MgSO_4$	=	Magnesium sulfate
min	=	Minute(s)
MLVA	=	Multilocus variable number of tandem repeat analysis
mM	=	Millimolar(s)
mm	=	Millimeter(s)
MOI	=	Multiplicity of infection
N	=	Normality
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate
NaN_3	=	Sodium azide
NaOCl	=	Sodium hypochlorite
NaOH	=	Sodium hydroxide
NCBI	=	National center for biotechnology information
ND	=	Not determined
NH_3	=	Ammonia
NH_4Cl	=	Ammonium chloride
$(NH_4)_2SO_4$	=	Ammonium sulfate
nm	=	Nanometer(s)

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

NOR	=	Norfloxacin
OD	=	Optical density
PBA	=	Prawn blood agar
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PFGE	=	Pulse field gel electrophoresis
pfu	=	Plaque forming unit
pH	=	Hydrogen ion concentration
PL	=	Post larvae
rpm	=	Revolutions per minute
s	=	Second(s)
SBA	=	Sheep blood agar
spp.	=	Species
SXT	=	Sulphamethoxazole/trimethoprim
T	=	Tetracycline
TBE	=	Tris-borate-EDTA
TCBS	=	Thiosulfate citrate bile salt sucrose
TDH	=	Thermostable direct hemolysin
tdh	=	Thermostable direct hemolysin gene
TE	=	Tris-EDTA
tlh	=	Thermolabile hemolysin gene
TRH	=	Thermostable direct hemolysin-related hemolysin
trh	=	Thermostable direct hemolysin-related hemolysin gene
TSA	=	Trypticas soy agar
TTSS	=	Type III secretion system
U	=	Unit
UPGMA	=	Unweighted pair group method using arithmetic mean
UT	=	Untypeable
V	=	Volt(s)

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

V/cm	=	Volt per centimeter
VNTRs	=	Variable number of tandem repeats
Vp	=	*V. parahaemolyticus*
w/v	=	Weight per volume

CHAPTER 1

INTRODUCTION

BACKGROUD AND RATIONALE

Vibrio parahaemolyticus, a member of the family *Vibrionaceae*, is a non-sucrose fermenting halophilic Gram-negative, rod shaped, motile and non-spore-forming bacterium that occurs naturally in aquatic environments worldwide (Chiou *et al*., 2000). This organism can exist in temperature ranging from 5 to 44ºC, at pH ranging from 4.8-11.0 and in the presence of NaCl 1-8%. However, optimal growth of *V. parahaemolyticus* is usually at 30-35ºC and pH 7.6-8.6 with the concentration of 2-3% NaCl. It can be isolated from a variety of seafood and diarrheal patients (Wong *et al*., 1999; Hara-Kudo *et al*., 2001; DePaola *et al*., 1990). *V. parahaemolyticus* was first identified as a foodborne pathogen in Japan in the 1950 (Fujino *et al*., 1953). The organism causes gastroenteritis in several countries due to consumption of raw or undercooked seafood (Chiou *et al*., 2000). *V. parahaemolyticus* infection is characterized with vomiting, acute abdominal pain, watery or bloody diarrhea and gastroenteritis (Alam *et al*., 2002; Wagley *et al*., 2009). The incubation period is around 4-96 h (Levin, 2006).

Most clinical isolates of *V. parahaemolyticus* produce either the thermostable direct hemolysin (TDH) encoded by *tdh* gene or the TDH-related hemolysin (TRH) encoded by *trh* gene, or both, which are considered to be the major virulence factors in the pathogenesis, however, not many isolates from the environment possess these genes (Vuddhakul *et al*., 2000). TDH and TRH are approximately 67% identity in their amino acid sequences and possess common biological activities including hemolytic activity, enterotoxicity, cytotoxicity, and cardiotoxicity (Nishibuchi *et al*., 1992). Five variants of the *tdh* gene share identity of 96 to 98% homology (Nishibuchi and Kaper, 1990). The *trh* gene possesses a significantly broader nucleotide sequence variation and can be subdivided in two main subtypes (*trh*1 and *trh*2) which share 84% identical in sequences (Kishishita *et al*.,

1992). Normally, *V. parahaemolyticus* is urease negative, but some clinical isolates of *V. parahaemolyticus* possess this enzyme (Abbott *et al*., 1989; Cai and Ni, 1996; Huq *et al*., 1979). Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia, raising the pH of environment inside the host or habitat (Mobley *et al*., 1989). Urease-positive phenotype of *V. parahaemolyticus* is always associated with the *trh* gene, making urease production as a marker of *trh*$^{+}$ *V. parahaemolyticus* (Suthienkul *et al*., 1995).

V. parahaemolyticus has been demonstrated as the causative agent of a new emerging shrimp disease known as "early mortality syndrome" (EMS) or "acute hepatopancreatic necrosis disease" (AHPND) (Tran *et al*., 2013). The disease was first reported in China in 2009 and later spread to Vietnam, Malaysia and Thailand. It affects both black tiger shrimp (*Penaeus monodon*) and white leg shrimp (*P. vannamei*) within 20–35 days after post larvae stocking and could lead to 100% mortality (FAO, 2013). AHPND causes serious production losses in affected areas and influences employment, social welfare and shrimp trade (Bondad-Reantaso *et al*., 2012; Mooney, 2012). In Thailand, Department of Fisheries (DOF) reported that total shrimp production in the first quarter of 2013 was 63,500 tons while it was 94,400 tons for the same period in 2012, indicating that the production was reduced approximately 30,900 tons due to AHPND outbreaks (DOF, 2013). Although *V. parahaemolyticus* is identified as a causative agent of AHPND, both *tdh* and *trh* genes have not been detected in *V. parahaemolyticus* causing AHPND (AHPND *V. parahaemolyticus*) (Tran *et al*., 2013). Furthermore, AHPND *V. parahaemolyticus* has not been reported to cause any diseases in humans. Therefore, characterization of AHPND *V. parahaemolyticus* strains including investigation the rapid detection technique is critically required.

Shrimp cultivation is the most important aquaculture in many countries in Southeast Asia (FAO, 2012). Most of bacteria commonly cause disease in shrimp aquaculture are *Vibrio* spp. Thus, biocontrol of those bacteria is urgently needed. The use of bioagents in aquaculture is recently considered as environmental friendly. *Carnobacterium* sp. (Robertson *et al.,* 2000), lactic acid bacteria (Villamil *et al*., 2003), *Pseudomonas* sp. (Vijayan *et al*., 2006), *Roseobacter* sp. (Planas *et al*., 2006), *Pseudoalteromonas* sp., *Flavobacterium* sp., *Alteromonas* sp., *Phaeobacter* sp.,

Bacillus sp. have been demonstrated to be able to inhibit *Vibrio* spp. (Rengpipat *et al*., 2000; Wang *et al*., 2008). *Bdellovibrio* and like organisms (BALOs) are a group of Gram-negative predatory bacteria that attack and invade prey to multiply. Mechanism of BALOs against their prey is nonspecific (Snyder *et al*., 2002). BALOs hunt specific bacteria in the environments and naturally control those bacteria populations. However, the effect of BALOs in aquatic microbial communities such as in shrimp pathogenic bacteria is not clearly understood. Thus, BALOs is interesting to be investigated as a candidate of biocontrol agents for shrimp cultivation.

LITERATURE REVIEWS

Family Vibrionaceae

The family Vibrionaceae has been classified under the order γ-proteobacteria (Veron, 1965). Members of this family are widespread in the marine environment including estuaries, coastal waters and sediment (Thompson *et al.*, 2004). Previously, this family consisted of eight genera including *Vibrio*, *Allivibrio*, *Enterovibrio*, *Salinivibrio*, *Catenococcus*, *Grimontia*, *Listonella* and *Photobacterium* (Garrity *et al.*, 2002). However, at present, it includes only genus *Vibrio* (Thompson *et al*., 2001). Members of this genus are characterized as rod or curved rod-shaped Gram-negative, non-spore forming, and are facultative anaerobes that highly capable of using both fermentative and respiratory metabolism (Ripabelli *et al.*, 1999). They are 0.5-0.8 μm in diameter with 1.4 to 2.6 μm in length and are motile (except *V. gallicus* and *V. halioticoli*) by means of a single polar flagellum (except *V. fischeri* possesses multiple polar flagella). Most of the *Vibrio* species produce oxidase and catalase, ferment glucose without producing gas, and are sensitive to acidic but tolerant to alkaline environments. All *Vibrio* species, except for *V. cholerae* and *V. mimicus*, require 2-3% NaCl for optimal growth and are referred as "halophilic" bacteria (BAM, 1998). However, 9 species have been implicated in marine animals and human pathogens, including *V. alginolyticus, V. cholerae, V. cincinnatiensis, V. fluvialis, V. furnissii, V. metschnikovii, V. mimicus, V. parahaemolyticus* and *V. vulnificus* (Janda *et al*., 1988 with slightly modification).

Genus *Vibrio*

A large number of species in this genus are associated with marine animals such as mollusks and crustaceans. In the last decade, more than 50 new species have been described in the genus *Vibrio*. Most species produce oxidase, catalase and ferment glucose without gas production (McLaughlin, 1995). Previously, taxonomic identification of vibrios was performed by phenotypic characteristics including flagellation, morphology of the cells and cultural aspects which led to

poorly characterized species (Baumann *et al*., 1984). Recently, a new technique for bacterial systematic to help understanding the vibrios evolution has been developed using multilocus sequence analysis (MLSA) of eight housekeeping gene loci including *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* (Figure 1) (Sawabe *et al*., 2013). This analysis investigated 86 *Vibrio* spp. and revealed one super-clade, 22 clades and 4 orphan clades (Figure 1). The species within each clade shared >80.5% average nucleotide sequence identify (ANI), and >92% average amino acid identity (AAI). The highest ANI (98.3%) has been showed in the sequence comparison between *V. anguillarum* and *V. ordalii*.

V. cholerae, *V. parahaemolyticus,* and *V. vulnificus* are considered the most significant human pathogenic bacteria (Cook, 1991). Pathogenic vibrios cause diarrheal disease due to gastrointestinal infection. In addition, extraintestinal infection has been reported to cause by *V. alginolyticus*, *V. cincinnatiensis*, *V. vulnificus* and *V. metschnikovii* (Oliver and Kaper, 1997; Shinoda, 1999). In *V. cholerae* (serotype O1), the pathogenic mechanism of cholera is caused by cholera toxin which is the major virulence factor secreted in the epithelial cells of small intestine after bacterial colonization. *V. parahaemolyticus* (serotype O3:K6) has been reported in 1996 to cause pandemic infection in India and spread all over the world. Luminous vibriosis in aquatic animals such as shrimp is caused by *V. harveyi*. In addition, *V. anquillarum* is the pathogen of sea bass, sea bream, mullet and rainbow trout (De la Peña *et al*., 1993). Pathogenic vibrios could elaborate a series of virulence factors including enterotoxin, cytotoxin, protease, siderophore, adhesive factor, haemagglutinin and hemolysin (Thompson *et al*., 2004).

Vibrio species persist in the aquatic environment and associate with the environmental abiotic and biotic components which is the public health concern (Lipp *et al*., 2002; Johnson, 2013). Haley *et al*. (2014) reported that increase of water temperature correlated with the rising of vibrios population in water and plankton. When the temperature of seawater was below 13-15°C, *V. parahaemolyticus* was rarely isolated and probably existed in a viable but non-culturable state (VBNC) and could not be cultured on common media (Kaneko and Colwell, 1975). Many researchers reported the abundance of *V. parahaemolyticus* and other vibrios during summer in temperate zone, when temperature was above 25°C (CDC, 1998; 1999;

Khan *et al*., 2002), However, the organisms were prevalent throughout the year in the tropical zone (Elhadi *et al*., 2004). Investigation of Chesapeake Bay demonstrated that *V. parahaemolyticus* and *V. vulnificus* populations were associated with oyster, sediment, and surface water and during a hurricane event, they were influenced by wave and sediment resuspension (Shaw *et al*., 2014).

Vibrios can be detected in the intestinal system of aquatic animals. They were part of the normal flora of blue crab, shrimp and shellfish (Davis and Sizemore, 1982; Vandenberghe *et al*., 1999; Olafsen *et al*., 1993). Investigation of raw oyster revealed all of them carrying *V. parahaemolyticus* and 67% of them contained *V. vulnificus*. Interestingly, 50% and 25% of cooked oyster also contained *V. parahaemolyticus* and *V. vulnificus*, respectively (Lowry *et al*., 1989). A survey of frozen raw shrimp in Mexico, China and Ecuador demonstrated over 63% of them contained *V. parahaemolyticus* and *V. vulnificus* (Berry *et al*., 1994). In addition, determination of *Vibrio* species in raw oysters from the coast of Brazil revealed that around 81%, 77%, 31%, 27%, 19%, 12% and 12% of them contained *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae* non-O1, *V. fluvialis*, *V. furnissii*, *V. mimicus* and *V. vulnificus*, respectively (Matte *et al.*, 1994).

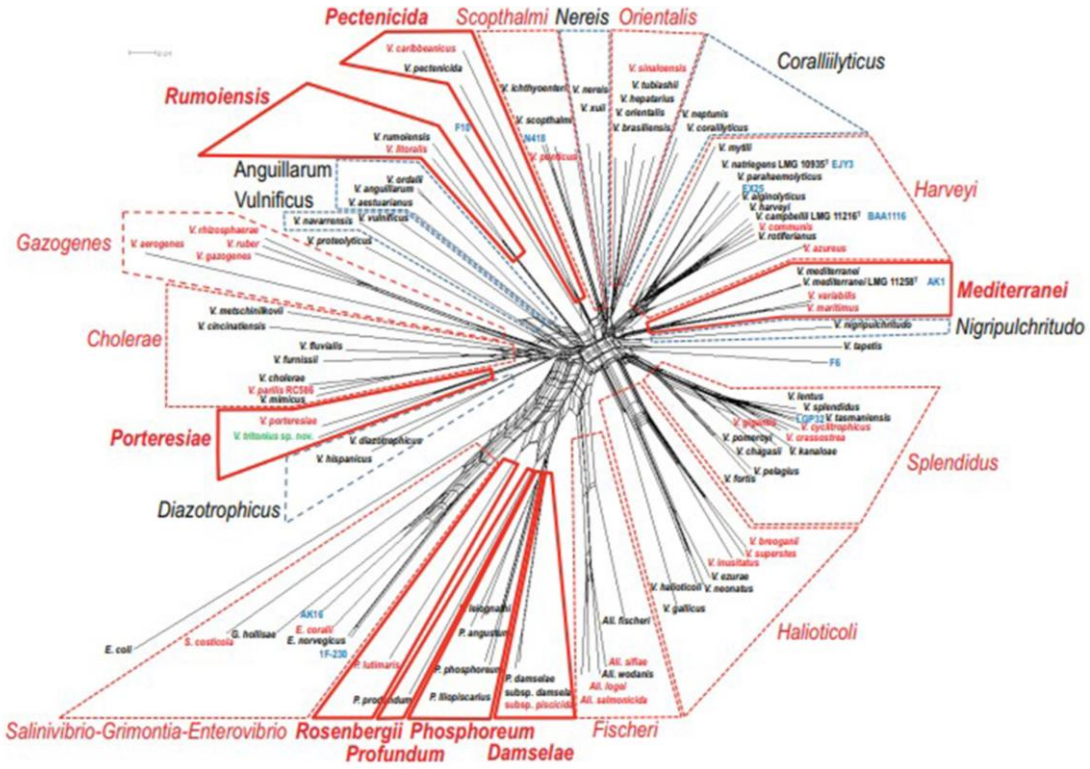

Figure 1 The latest vibrios phylogeny based on multilocus housekeeping gene sequences.

[Source: From reference (Sawabe *et al*., 2013)]

Isolation and identification

Bacteria in the genus *Vibrio* produce many extracellular enzymes including gelatinase, amylase, chitinase and DNase (Joseph *et al*., 1982). The important pathogens such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* could decarboxylate lysine and ornthine but are negative for arginine dihydrolase. Most vibrios are sensitive to the vibriostatic agent O/129 (2,4-diamino-6,7-di isopropylpteridine) (Baumann *et al*., 1984; Farmer III, 1992).

Vibrios can be isolated from clinical and environmental samples. Sample enrichment should be performed to increase the numbers of vibrios prior to plat on a selective agar. An enrichment medium is alkaline peptone water (APW: 1% peptone, 1% NaCl, pH 8.6) (Joseph *et al*., 1982). The high pH of this medium and sodium chloride (NaCl) concentration can inhibit other bacteria. Most vibrios can grow in media with NaCl and require 2-3 % NaCl (w/v) for optimal growth except

*V. cholera*e, *V. mimicus, V. hispanicus, V. fluvialis, V. furnissii* and *V. metchnikovii* which can grow with a minimum NaCl concentration approximate 0.5% (w/v) (Baumann *et al*., 1984; Farmer III, 1992). Vibrios are generally culturable on tryptic soy agar (TSA) supplemented with NaCl, marine agar and the selective media such as thiosulfate citrate bile salt sucrose (TCBS) or chromogenic agar (Kobayashi *et al.*, 1963). However, some vibrios cannot grow on TCBS such as *V. panaeicida* and some vibrios grow poorly such as *V. cincinnatiensis* and *V. metchikovii*. Those grown well on TCBS use sucrose as a carbon source and form yellow colonies while sucrose non-fermenting strains exhibit green colonies (Table 1). A CHROMagar Vibrio (CV agar) has been developed and various species of *Vibrio* generate different color colonies depending on the reaction of the bacterial beta-galactosidase and the substrate in the medium (Figure 2, Table 1) (Elhadi, 2013; Hara-Kudo *et al*., 2001). Identification of human pathogenic *Vibrio* can be performed using NaCl concentrations, nitrate reduction, oxidase production, *myo*-inositol fermentation, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase production (Robert-Pillot *et al*., 2004).

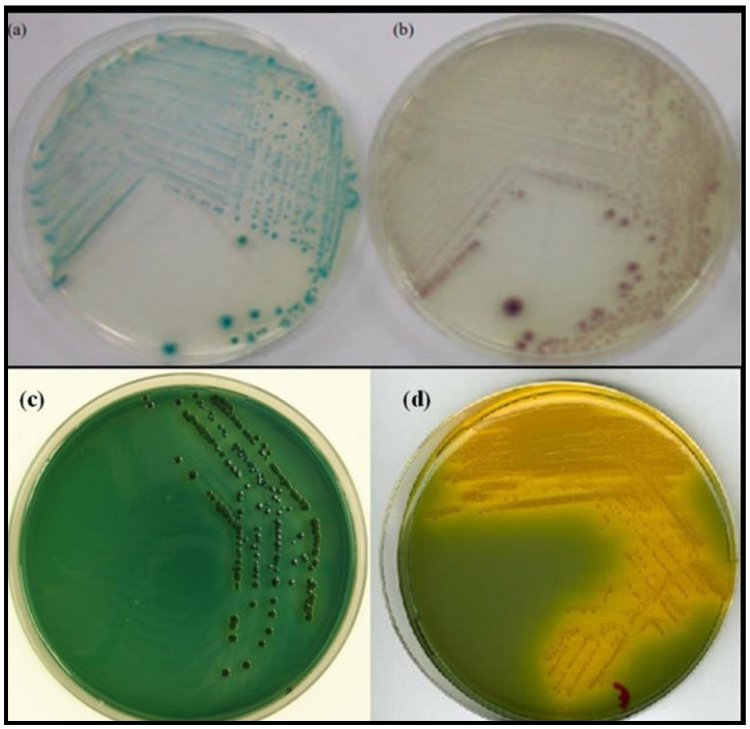

Figure 2 Illustrate *V. cholerae* (a) and *V. parahaemolyticus* (b) on CV medium, *V. cholerae* (c) and *V. parahaemolyticus* (d) on TCBS medium.
[Source: From reference (Elhadi, 2013)]

Table 1 Colony morphologies of various bacteria grown on CV and TCBS medium.

Species	No. of strains tested	CV medium		TCBS medium	
		Size of colony[a] (mm)	Color of colony	Size of colony (mm)	Color of colony
Citorobacter freundii	1	NG	NG	NG	NG
Edwardsiella tarda	1	NG	NG	NG	NG
Enterobacter cloacae	2	NG	NG	NG	NG
Escherichia coli O157: H7	2	NG	NG	NG	NG
Klebsiella ornithinolytica	1	NG	NG	NG	NG
Klebsiella oxytoca	1	NG	NG	NG	NG
Photobacterium damselae	1	NG	NG	NG	NG
Proteus mirabilis	2	Minute	Milk white	Minute	Blue-green
Providencia rettgeri	1	1	Milk white	Minute	Blue-green
Pseudomonas aeruginosa	1	NG	NG	NG	NG
Salmonella enteritidis	2	NG	NG	NG	NG
Serratia marcescens	1	NG	NG	NG	NG
Shigella sonnei	2	NG	NG	NG	NG
Vibrio alginolyticus	4	5-6	Milk white	3-4	Yellow
Vibrio cholerae O1	2	3	Pale blue	3	Yellow
Vibrio hollisae	1	4	Milk white	3	Green
Vibrio mimicus	2	3-4	Pale blue	1-2	Green
Vibrio parahaemolyticus	68	3-5	Violet	2-4	Green
Vibrio vulnificus	1	5	Pale blue	1	Green

[a] No growth.

[Source: From reference (Hara-Kudo *et al*., 2001)]

Vibrio parahaemolyticus

Importance and discovery

V. parahaemolyticus was first discovered by Tsunesaburo Fujino in 1950 as a causative agent of foodborne disease of a large outbreak in Japan which recorded 272 illnesses with 20 deaths after consumption of *shirasu* (*Engraulis japonica* Hottuyn) (Fujino *et al*., 1953). Human pathogenic *V. parahaemolyticus* strains are transmitted by consumption of raw or undercooked seafood causing acute gastroenteritis (Newton *et al*., 2012; Zarei *et al*., 2012). In rare cases, *V. parahaemolyticus* causes wound infection, ear infection or septicemia that can be life-threatening to individuals with pre-existing medical conditions (Zhang and Orth, 2013). *V. parahaemolyticus* possesses two haemolysins virulence factors, TDH (encoded by *tdh*), a poreforming protein that contributes to the invasiveness of the bacterium in humans, and TRH (encoded by *trh*) which plays a similar role as TDH in the disease pathogenesis. *V. parahaemolyticus* has been reported to be responsible for 20–30% of food poisoning in Japan and seafood borne diseases in many Asian countries (Alam *et al*., 2002). This bacterium is also recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States (Kaysner and DePaola, 2001; Newton *et al*., 2012).

Recently, *V. parahaemolyticus* has been demonstrated to cause acute hepatopancreatic necrosis disease (AHPND) in cultured shrimp which leads to a significant loss of shrimp production in the Southeast Asian countries (Flegel, 2012, Leano and Mohan, 2012, NACA-FAO, 2011 and Lightner *et al*., 2012) and Mexico (Nunan *et al*., 2014). The disease was first reported in China in 2009 and spread to Vietnam (2010), Malaysia (2011), Thailand (2012) and Mexico (2013). This disease affects the hepatopancreas of black tiger shrimp (*P. monodon*) and white leg shrimp (*P. vannamei*) within 20–35 days after post larvae stocking and frequently causes up to 100% mortality (Tran *et al*., 2013). The total production of shrimp was estimated to fall below 1 million tons in China and 600,000 tons in Vietnam due to the influence of AHPND (FAO, 2013). AHPND symptom includes a pale and atrophied hepatopancreas (HP) with an empty stomach and midgut. Histological examination

demonstrates sloughing of the HP tubule epithelial cells into the HP tubule lumens (Lightner *et al*., 2012; Tran *et al*., 2013).

General characteristics

V. parahaemolyticus is a member of the Vibrionaceae family. It is a gram- negative, halophilic, non-spore forming, curved rod-shaped bacterium that is 0.5-0.8 μm in width and 1.4-2.4 μm in length. This organism survives in temperature ranging from 5 to 44 ºC, at pH ranging from 4.8-11.0 and in the presence of 0.5-8% NaCl. However, the optimal growth of *V. parahaemolyticus* usually occurs at 30-35ºC and pH 7.6-8.6 with concentration of 1.5-3% NaCl (Table 2) (FAO/WHO, 2011). *V. parahaemolyticus* is an oxidase-positive, facultative anaerobic bacterium that can ferment glucose without gas production (Joseph *et al*., 1982). It possesses a polar flagellum which enables the bacterium to move in liquid media whereas its lateral flagella allow it to migrate across semi-solid surfaces by swarming (Farmer III, 1992). Virulent strains isolated from patients have been reported to produce TDH and/or TRH, which those two characteristics are rarely observed in the environmental isolates. TDH-producing isolates are known as Kanagawa positive and can be identified by detection β-hemolysis bacterial colonies on a Wagatsuma blood agar (Okuda *et al.*, 1997).

Table 2 Characteristics for survival or growth and stress condition of *V. parahaemolyticus*.

Parameters	Optimum conditions	Extreme conditions
Temperature (°C)	30-35	5-44
pH	7.8-8.6	4.8-11.0
Water activity (a_w)	0.981	0.940-0.996
NaCl (%)	1.5-3	0.5-10.0

[Source: From reference (FAO, 2011) with some modification]

Isolation and identification

V. parahaemolyticus can be isolated from both clinical and environmental samples such as food, sediment and water using traditional enrichment with alkaline peptone water (APW) at pH between pH 8.5 and 9.0 with the concentration of NaCl 1-3% (Kaysner and DePaola, 2004). Raghunath *et al*. (2009) compared sodium taurocholate broth (ST broth) and APW to isolate pathogenic *V. parahaemolyticus* strains from seafood samples. *V. parahaemolyticus* carrying the *tdh* gene were isolated after enrichment samples using ST broth (6.9%) and APW (3.4%) and *V. parahaemolyticus* carrying the *trh* gene were isolated from the samples enrichmented in ST broth (20.7%) and APW (13.8%). The results revealed that isolation of pathogenic *V. parahaemolyticus* from seafood could be improved by ST broth. In addition, a non-selective enrichment broth (trypticase soy broth) and a selective enrichment broth (salt polymyxin broth–SPB) were combined to isolate *V. parahaemolyticus*. This two step enrichments were more effective to isolate *V. parahaemolyticus* than the one step enrichment in SPB alone. SPB contains polymyxin B sulfate that inhibits the growth of Gram-positive organisms (Hara-Kudo *et al*., 2001). Subsequently, plating on selective agar such as TCBS or CV medium can be performed to isolate this bacterium. Specific green colonies of *V. parahaemolyticus* on TCBS or mauve colonies on CV agar can be identified by biochemical tests which include the production of oxidase, flagella, lysine decarboxylase, indole, H_2S without gas in triple sugar iron (TSI) agar and not utilize lactose in TSI. PCR targeted to the *toxR* or *tlh* genes can be applied to confirm the genus and species of this bacterium (Kim *et al*., 1999; Bej *et al*., 1999). Finally, serotyping using antibody against K and O antigens of *V. parahaemolyticus* can be implemented in epidemiological investigation.

Immunological-based method such as immunomagnetic bead technique has been developed for rapid detection of pathogenic *V. parahaemolyticus* in the environments. This method is useful for epidemiological determination during the outbreaks of *V. parahaemolyticus* to develop the risk-based approaches for controlling this bacterium in seafood. *V. parahaemolyticus* can be differentiated by serotyping with 13 O groups and 71 K types (Table 3) (Kaysner and DePaola, 2004). Since 1996,

pandemic infections caused by *V. parahaemolyticus* serotype O3:K6 have been reported worldwide (Matsumoto *et al*., 2000; Okuda *et al*., 1997) and other serotypes including O4:K68, O1:K25, O1:KUT have been demonstrated to be originated from the O3:K6 clone (Nair *et al*., 2007). Application of immunomagnetic method successfully isolated a pandemic O3:K6 strain from shellfish in southern Thailand (Vuddhakul *et al*., 2000) including isolation of O3:K6 strains in Japan (Hara-Kudo *et al*, 2001).

Table 3 Antigenic scheme of *V. parahaemolyticus*.

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	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61
12	52

[Source: From reference (Kaysner and DePaola, 2004)]

Molecular identification of human pathogenic *V. parahaemolyticus*

DNA hybridization

DNA hybridization is a molecular-based method for confirmation of *V. parahaemolyticus* in clinical specimens, food and environmental samples (Table 4). Ellison and co-workers (2001) performed direct plating to isolate *V. parahaemolyticus* and confirmed using alkaline phosphatase-labelled *tlh* (gene encoding a thermolabile haemolysin) probe which detected more *V. parahaemolyticus* in retail oysters than cultural methods.

Polymerase chain reaction (PCR)

PCR is a high sensitivity and specificity molecular technique for detection or identification of bacteria from clinical and environmental samples (Nelapati *et al*., 2012). PCR targeting to either the *toxR* or *tlh* gene has been used to identify *V. parahaemolyticus* (Kim *et al*., 1999; Bej *et al*., 1999). PCR specific to the *toxRS* sequences is unique to detect the pandemic O3:K6 *V. parahaemolyticus* strains (Matsumoto *et al*., 2000). For detection of pathogenic strains, PCR amplification of *tdh* and *trh* has been employed for detecting virulence genes of this bacterium in seafood (Tada *et al*., 1992). To detect the pandemic strains of *V. parahaemolyticus*, GS-PCR is used for identification of pandemic O3:K6 including 2 additional serotypes designated as O4:K68 and O1:KUT (Matsumoto *et al*., 2000).

Loop-mediated isothermal amplification (LAMP)

LAMP is a novel modified PCR technique but its advantage is over PCR for short reaction time, non-requirement of specific equipment, high sensitivity and specificity as well as low susceptibility to inhibitors (Notomi *et al.,* 2000). Amplification reaction of LAMP is performed using six specific primers to amplify six regions within a target DNA (Notomi *et al*., 2000). The primer set composed of outer primers F3 and B3, forward inner primer (FIP) and backward inner primer (BIP), forward loop primer (LF) and backward loop primer (LB) (Figure 3) (Mori and Notomi, 2009). FIP consists of F1c sequence complementary to F1 and F2 sequence; BIP consists of B1c sequence complementary to B1 and B2 sequence.

This technique could detect *tlh*, *tdh*, *trh* (Yamazaki *et al*., 2008; 2010), *rpoD* and *toxR* genes (Nemoto *et al*., 2011; Chen and Ge, 2010) in natural or artificially inoculated *V. parahaemolyticus* in shrimp. For identification of human pathogenic *V. parahaemolyticus*, LAMP was performed targeted to the *tdh* gene of *V. parahaemolyticus*. The specificity of the LAMP technique was evaluated using 32 strains of tdh^+ *V. parahaemolyticus*, one strain of tdh^+ *G. hollisae*, 10 strains of tdh^- *V.parahaemolyticus* and 94 strains of non-*Vibrio* species. The results showed that no false-positive was observed with any tdh^- *V. parahaemolyticus* or non-*Vibrio* species (Nemoto *et al*., 2009). Therefore, this technique is a potentially valuable tool for rapid

detection of *V. parahaemolyticus*, which is the human food-borne pathogen in seafood**.**

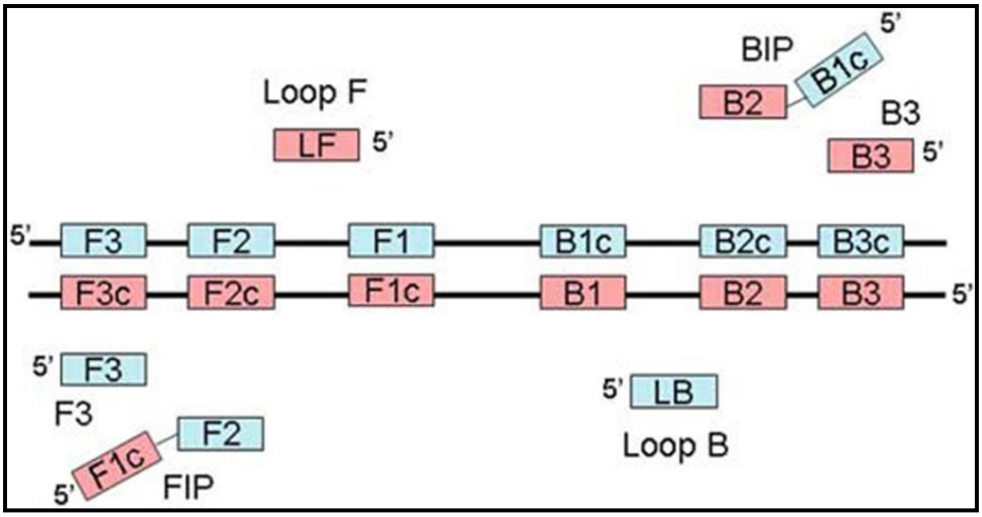

Figure 3 Schematic representations of loop-mediated isothermal amplification (LAMP) primers. The inner primers FIP (BIP) are composed of F2 (B2) and F1c (B1c). The outer primers are designed at the region of F3 and B3. The loop primers are designed between F1c (B1c) and F2c (B2c).

[Source: From reference (Mori and Notomi, 2009)]

Table 4 Molecular-based detection methods of *V. parahaemolyticus*.

Methods	Targets	Sensitivity and specificity	Advantages and disadvantages
DNA hybridization	*tlh*, *tdh*	Sensitivity: Higher than culture methods alone Specificity: Based on the target, it can be used to detect environmental, pathogenic or both *V. parahaemolyticus*	Advantages: Widely accepted method for identification of *V. parahaemolyticus*. Imparts enhanced rapidity and specificity compared to culture methods alone Disadvantages: It is dependent on culture methods, which affects the rapidity of detection
Polymerase chain reaction (PCR)	*tdh*, *trh*, *toxR*, *orf*8	Sensitivity: High, however, dependent on effective target separation and enrichment Specificity: Highly specific, can be used to detect total, environmental, pathogenic isolates or even specific serovars and can be multiplexed	Advantages: Rapid, specific, sensitive, allows for multiplexing, allows for differentiation of pathogenic and environmental isolates and can be optimised to detect specific serovars Disadvantages: Can only be performed by highly skilled personnel, theoretical high sensitivity can be hindered by nonoptimised separation and enrichment protocols
Loop-mediated isothermal amplification (LAMP)	*tdh* , *tlh*, *trh*, *rpoD*, *toxR*	Sensitivity: Higher than culture, comparable to PCR Specificity: Based on the target, it can be used to detect environmental, pathogenic or both *V. parahaemolyticus*	Advantages: Rapid, sensitive, simpler to perform than PCR, can be performed at one single temperature and does not require cycling Disadvantages: Similar to PCR, it is greatly affected by methods of target separation and enrichment.

[Source: From reference (BAM, Bacteriological Analytical Manual) with some modification]

Genome analysis

Analysis of the *V. parahaemolyticus* genome sequences reveals the genome consisting of two circular chromosomes with 45.4% GC content for each and included 4,832 genes (Table 5) (Makino *et al*., 2003; Broberg *et al*., 2011). The hypothetical proteins as a large proportion and 11 copies of rRNA operons are detected on the *V. parahaemolyticus* genome. Most of the essential genes required for growth and viability of *V. parahaemolyticus* such as the essential gene encoding for normal cell function and ribosomal proteins are located on chromosome 1, while the chromosome 2 contains the several genes involved in essential metabolic pathways, which are related to transcriptional regulation and transport of various substrates for adaptation to the environmental changes (Makino *et al*., 2003; Broberg *et al*., 2011).

Table 5 Comparison of genome features of chromosome 1 and 2.

	Chromosome 1	Chromosome 2
Length of sequence (bp)	3,288,558	1.877,212
Number of coding sequence	3,080	1,752
Hypothetical proteins	1,090 (35%)	756 (43%)
rRNA operons	10	1
tRNA	112	14
Flagella coding genes for	Polar	Lateral
Super-integron gene	+	-
tdh gene	-	+
trh gene	-	+
T3SS	T3SS1	T3SS2
T6SS	T6SS1	T6SS2

[Source: From reference (Makino *et al*., 2003; Broberg *et al*., 2011) with some modification]

Human pathogenic *V. parahaemolyticus*

Ecology

V. parahaemolyticus occurs naturally in the estuarine environment associated with seawater, sediments, plankton (Pavia *et al.*, 1989) and 30 different marine animals including eel, crab, clams, oysters, lobsters, scallops, sardines, shrimp, and squid (Fedhila *et al.*, 2002). The most important modulators of environmental concentration of *V. parahaemolyticus* worldwide are salinity and water temperature (Barbieri *et al.*, 1999). The growth of this bacterium is supported by high concentration of salt between 1-8% which leads the organism to survive in different saline environments such as seawater, brackish water. Usually, the numbers of *V. parahaemolyticus* is highly detected in summer when the water temperature increase above 20ºC (West, 1989).

Epidemiology

V. parahaemolyticus was first reported in 1950 followed by the outbreak in 1956 and 1960 in Japan. It is the leading cause of food poisoning. Around 24,373 cases in Japan between 1996 and 1998 (IDSC, 1999), 1,495 cases in Taiwan between 1981 and 2003 (Anon, 2005) were reported to be infected by *V. parahaemolyticus*. In addition, this bacterium caused 31.1% of 5,770 foodborne outbreaks in China between 1991 and 2001 (Liu *et al*., 2004). However, not many *V. parahaemolyticus* infections are reported in European countries. Eight cases of *V. parahaemolyticus* gastroenteritis related to fish and shellfish consumption were reported in Spain in 1989 (Molero *et al*., 1989). The outbreak of 64 illnesses associated with raw oysters consumption occurred in 1999 in Spain (Lozano-Leon *et al*., 2003). A serious outbreak affecting 44 patients associated with consumption of shrimps imported from Asia occurred in France in 1997 (Robert-Pillot *et al*., 2004). A recent outbreak involving 80 illnesses of *V. parahaemolyticus* infection among guests attending weddings in one restaurant was reported in Spain in 2004 (Martinez-Urtaza *et al*., 2005). In the United States, *V. parahaemolyticus* was first identified as an etiological agent in 1971 after three outbreaks of 425 cases of gastroenteritis

associated with consumption of crabs in Maryland (Molenda *et al*., 1972). Between 1973 and 1998, approximately 40 outbreaks of *V. parahaemolyticus* infections were reported to the Centers for Disease Control and Prevention (CDC) (Daniels *et al*., 2000). Among them, four major outbreaks associated with raw oyster consumption occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions between 1997 and 1998. In 1998, two outbreaks occurred in Washington and Texas were associated with consumption of raw oyster (DePaola *et al*., 2000). In addition, a small outbreak of eight cases of *V. parahaemolyticus* infections was reported in Connecticut, New Jersey, and New York between July and September in 1998 as a result of eating oysters and clams harvested at Long Island Sound of New York (CDC, 1999). Recently, an outbreak of *V. parahaemolyticus* involving 177 cases occurred in the summer of 2006 was linked to contaminated oysters harvested in Washington and British Columbia (CDC, 2006). Therefore, the outbreaks of *V. parahaemolyticus* were linked to consumption of raw or undercooked oysters harvested from all the coastal waters of United States. It has been suggested that these outbreaks might be associated with warmer water temperatures, which increased *V. parahaemolyticus* levels and increased the probability of infection.

Pathogenicity

The human gastroenteritis caused by *V. parahaemolyticus* is self-limited and characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever. Infections are most commonly reported during the warmer period. The incubation period of infection is 3-24 h. Infective dose is believed to be approximately 10^6 to 10^9 cfu. Many virulence factors are thought to play a role in the pathogenicity of human pathogenic *V. parahaemolyticus* including those associated with adherence factors, various enzymes, and the products of the *tdh*, *trh* and *ure* genes (Sakazaki *et al*., 1968; Nishibuchi and Kaper, 1995; Hackney *et al*., 1980; Yamamoto and Yokota, 1989; Baffone *et al*., 2001; Honda *et al*., 1987; 1988; Suthienkul *et al*., 1995; Iida *et al*., 1997; 1998). The pathogenicity of *V. parahaemolyticus* correlates with the Kanagawa phenomenon (KP^+) due to production of the TDH (Takeda, 1982; Nishibuchi *et. al*., 1989). However, Honda and others (1987, 1988) reported that some KP-negative strains of *V. parahaemolyticus* associated with illness in humans

produced a TDH-related hemolysin designated as TRH, which was similar but not identical to the TDH protein. Both haemolysins possessed similar haemolytic activity *in vitro* and caused lysis to human erythrocytes in excessively saline medium (Sakazaki *et al*., 1968; Miyamoto *et al*., 1969; Hondo *et al*., 1987; Honda *et al*., 1988). The TRH protein was first detected in *V. parahaemolyticus* strains originated from Maldives in 1985 (Honda *et al*., 1987). Some clinical isolates contain both the *tdh* and *trh* genes, whereas most environmental isolates do not harbor either *tdh* or *trh* genes. In addition, Suthienkul and colleagues (1995) found that the *ure* gene was associated with the *trh*$^{+}$ *V. parahaemolyticus* and that the *ure* and *trh* genes were genetically linked on the chromosome of pathogenic *V. parahaemolyticus* strains (Park *et al*., 2000).

Virulence factors

Mechanisms of human infection caused by *V. parahaemolyticus* have been demonstrated to involve in virulence genes located in pathogenicity island (Vp-PAI), type III secretion systems (T3SS1 and T3SS2), type IV secretion systems (T6SS1 and T6SS2) and various enzymes (Figure 4) (Wang *et al*., 2015). However, the most important virulence factors of *V. parahaemolyticus* are TDH and TRH.

TDH encoded by *tdh* gene is frequently detected in almost all (95%) of clinical isolates and *tdh* has been used as an important marker for identification of virulent strains (Okuda *et al.*, 1997; Cook *et al.*, 2002). The *tdh* gene is located on pathogenicity island containing gene clusters including *toxR*, transposase genes, type III secretion system (T3SS)-related gene and other genes of pandemic *V. parahaemolyticus* on chromosome II (Figure 5) (Chen *et al*., 2011). The early epidemiological investigations revealed a very strong association between the Kanagawa phenomenon (KP) and TDH. KP is a beta-type haemolysis on a special blood agar medium (Wagatsuma agar) that is induced by the TDH (Sakazaki *et al.*, 1968). Five *tdh* genes designated as *tdh*1 to *tdh*5 have been reported (Nishibuchi and Kaper, 1995). TDH was produced from the *tdh*2 gene rather than the *tdh*1 gene. The nucleotide homologies of *tdh*2 with *tdh*1, *tdh*3, *tdh*4 and *tdh*5 were 97%, 98.6%, 98.6% and 98.9% respectively (Nishibuchi and Kaper, 1985; 1995). Correlation between TDH and diarrhea was demonstrated to be induced through the intestinal

chloride secretion (Nishibuchi *et al*., 1992). Raimondi and co-workers (1995) demonstrated that the TDH was one of the few enterotoxins produced by human pathogens and its action was mediated by intracellular calcium in rabbit model. TDH also raised the cytosolic free calcium concentration in non-transformed rat intestinal cells (Fabbri *et al.*, 1999). In addition, TDH was reported to possess cardiotoxic (Honda *et al.*, 1976) and cytotoxic effects (Sakazaki *et al*., 1974).

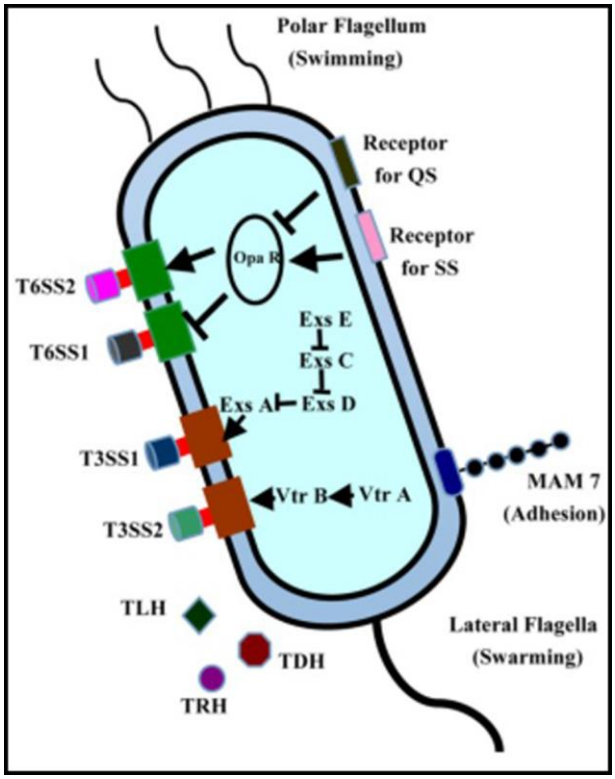

Figure 4 Structures and virulence factors of *V. parahaemolyticus*.
[Source: From reference (Wang *et al*., 2015)]

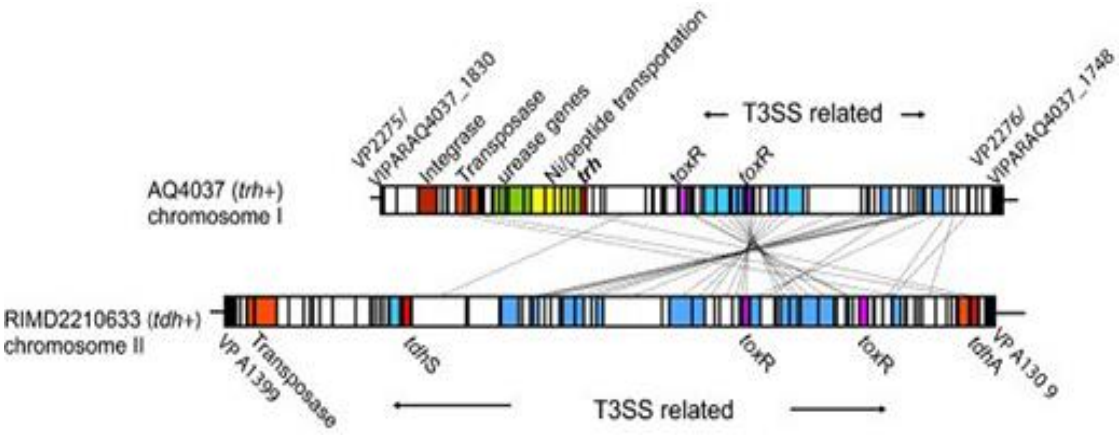

Figure 5 Gene clusters on chromosome II including the *tdh*, *trh*, *toxR* and other genes in *V. parahaemolyticus* pathogenicity islands.
[Source: From reference (Chen *et al*., 2011)]

The KP-negative *V. parahaemolyticus* strains were first detected from an outbreak of gastroenteritis in 1985, which produced hemolysin designated as TRH (Honda *et al.,* 1988). TRH is encoded by the *trh* gene which shares the 68.4% and 68% identity to the *tdh*1 and *tdh*2 gene, respectively (Nishibuchi *et al.,* 1989). The mechanism of action of TRH appears to be similar to that of TDH. TRH causes fluid accumulation in rabbit ileal loops and has cytotoxic activity in numerous tissue culture cells (Honda and Iida, 1993). However, the hemolytic activity of TRH was lower than TDH. Okuda and colleagues (1997) identified a variant of the *trh* gene called *trh*2, which was 84% homologous to the original *trh* gene (*trh*1), indicated that both the *trh*1 and *trh*2 carrying strains should be considered potentially virulent pathogens (Okuda *et al*., 1997). The immunogenicity of the *trh*2 gene product was partially identical to the *trh*1 and the *tdh* gene products. The *trh*2 gene was rarely detected in clinical and environmental isolates. The difference in the nucleotide sequences between the *trh*1 and *trh*2 genes caused the difference in the amino acid sequences and reflected the different hemolytic characteristics. The *trh*1 gene product was hemolytic to human, rabbit, sheep and calf erythrocytes, whereas the *trh*2 gene product was hemolytic to human and rabbit erythrocytes but the hemolytic activities were relatively weak (Kishishita *et al*., 1992). Normally, *V. parahaemolyticus* was urease negative, however, several studies reported the trh^{+} *V. parahaemolyticus* strains were associated with urease activity (Suthienkul *et al*., 1995; Eko, 1992;

Honda *et al*., 1992; Magalhaes *et al*., 1991; Suzuki *et al*., 1994; Huq *et al*., 1979). The *trh* gene is genetically linked to *ure* gene cluster associated with a nickel transportation system (Figure 6) (Park *et al*., 2000). Early studies showed that urease induced the accumulation of intestinal fluid in the rabbit ileal loops and caused gastrointestinal inflammatory lesions (Cai and Ni, 1996; Osawa *et al*., 1996), and was involved in the formation of ammonia during the process of infection (Levin, 2006). Hence, the urease-positive phenotype of *V. parahaemolyticus* could be the marker for potential pathogenic *trh*$^{+}$ *V. parahaemolyticus* strains (Park *et al*., 2000).

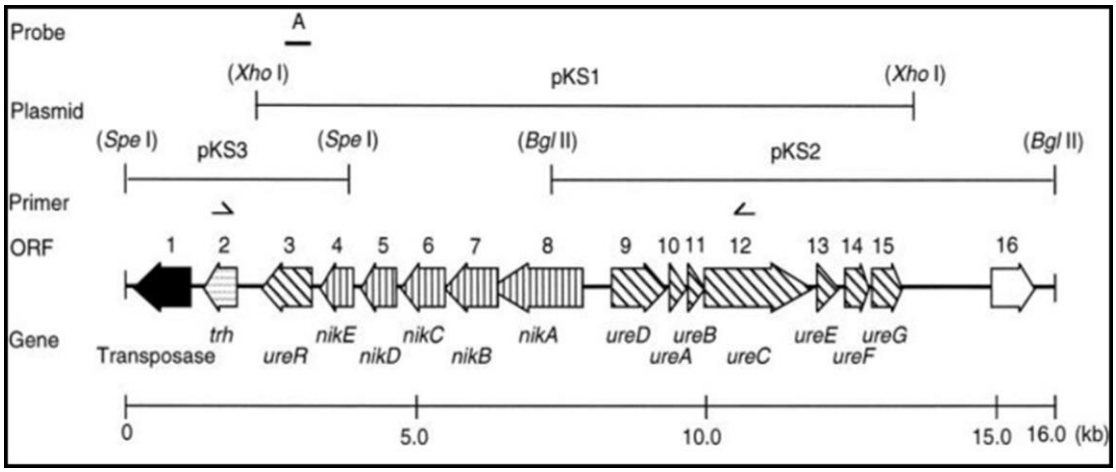

Figure 6 Schematic representation of the region containing the *trh* and *ure* gene cluster.
[Source: From reference (Park *et al*., 2000)]

Type III secretion systems (T3SSs) have been reported in Gram negative bacteria and associated with pathogenicity. They are needle-like bacterial structure used to inject bacterial effector proteins such as toxin directly into the membrane and cytoplasm of host cells (Cornelis, 2006). The proteins of T3SS1 and T3SS2 in *V. parahaemolyticus* possess cytotoxicity and enterotoxicity, respectively (Park *et al*., 2004). The T3SS1 is present in all environmental and clinical *V. parahaemolyticus* strains while T3SS2 is only detected in pathogenic *V. parahaemolyticus* strain and associated with *tdh* gene (Makino *et al*., 2003). T3SS-related genes in *V. parahaemolyticus* include the genes encoding the apparatus proteins (*vscCJQRSTU* and *vcrD*), ATPase (*vscN*), protein translocons (*vopBD*), and effectors (*vopCLP*) (Iida *et al*., 2006; Kodama *et al*., 2008; Livermans *et al*., 2007;

Park *et al*., 2004). A series of T3SS1 gene including VopQ (VP1680), VPA0450, and VopS (VP1686) involves in autophagy, membrane blabbing, cell rounding and cell lysis during infection (Caburlotto *et al*., 2009). T3SS2 is detected in clinical isolates and associated with pandemic *V. parahaemolyticus* strains (Paranjpye *et al*., 2012). The effectors of T3SS2 are VopA/VopP (VPA1346), VopL (VPA1370), VopT (VPA1327), VpoV (VPA1357), VopC (VPA1321). They are mostly associated with the enterotoxicity, though they have been shown to cause cytotoxicity in intestinal cell lines such as Caco2 cells and HCT cells.

Type VI secretion system (T6SS) is a macromolecular transenvelope machine encoded within the genomes of Gram negative bacteria. It is a complex molecular machine that utilizes a bacteriophage-like cell-puncturing device to inject effector proteins into host cells (Coulthurst, 2013). Conserved structural elements for the T6SS injectisome is composed of a transmembrane of VipA and VipB heterodimeric proteins, a surface-exposed Hcp tube capped with a trimer of VgrG proteins (Basler *et al*., 2012). Two types of T6SS are detected in *V. parahaemolyticus* and both are highly regulated systems for a several functions including virulence, interbacterial competition and adhesion to host cell (Bingle *et al*., 2008; Yu *et al*., 2012). The T6SS1 is identified on chromosome I and predominantly detected in clinical isolates, while T6SS2 is observed in all isolates of *V. parahaemolyticus* (Krachler and Orth, 2011). Salomon and colleagues (2013) reported that two *V. parahaemolyticus* T6SSs were differentially regulated by quorum sensing. T6SS1 was repressed by quorum sensing and induced by surface sensing, while these regulatory networks reciprocally regulated T6SS2.

V. parahaemolyticus causing AHPND (AHPND *V. parahaemolyticus*)

Epidemiology

In China, acute hepatopancreatic necrosis disease (AHPND) was firstly reported to cause mass mortality in shrimp in 2009 and subsequently it was detected in Vietnam (2010), Malaysia (2011), Thailand (2012), and Mexico (2013). This catastrophic disease affected China to lose 80% of shrimp products. In Vietnam, the economic lost was estimated around 570,000 to 7,200,000 USD in 2011 and 2012. In Thailand, approximately 7% of total shrimp production was lost especially in coastal area during 2012 (Lightner *et al.*, 2013). The spread of disease to the Western Hemisphere, Mexico, was a major concern of shrimp producers in that region (De Schryver *et al.*, 2014). To confirm the etiologic organism of AHPND, both immersion and oral challenge studies were applied to the *P. vannamei* to induce AHPND characteristics (Tran *et al.*, 2013). Although the causative agent has already been identified as *V. parahaemolyticus*, the incidence of AHPND remains exist because of some factors such as high stocking density of shrimp, sensitivity of shrimp species, lack of water treatment and pond preparation, stress during transportation or storage of shrimp post larvae, low oxygen condition and feed mismanagement (De Schryver *et al*., 2014).

Etiology

After spread of AHPND in Southeast Asia, pure isolates from the AHPND-infected shrimp in Vietnam were evaluated using Koch's postulates (Tran *et al*., 2013). The bacterium that induced 100% mortality in shrimp with typical AHPND pathology was confirmed as *V. parahaemolyticus* by reverse gavage test using bacterial toxin in the culture broth. It has been proposed that AHPND *V. parahaemolyticus* colonizes shrimp stomach and secretes toxin that causes shrimp hepathopancreas necrosis and degeneration.

Pathogenicity

AHPND has been reported in *Penaeus monodon*, the black tiger shrimp and *Penaeus vannamei*, the white leg shrimp (OIE, 2013) and affects shrimp post larvae within 20-35 days after stocking. The symptoms in shrimp include pale and atrophy of hepatopancreas together with an empty stomach and midgut (Figure 7) (Tran *et al.*, 2013). In addition, abnormal behavior such as lethargy, sluggishly swimming along the dikes, spiral swimming and decrease preening and feeding are also observed in infected shrimp. Finally, infected shrimp sink to the pond bottom and die. Hepatopancreas is the main target in this disease, histological examination reveals proximal to distal sloughing of hepatopancreas cells, lacking of B, F and R cells, impaired E-cell mitosis, enlarged hepatopancreas nuclei and infiltration of haemocytes (Figure 8) (Flegel, 2013). These lead to massive secondary bacterial infection and complete devastation of hepatopancreas at the fatal phase of the disease.

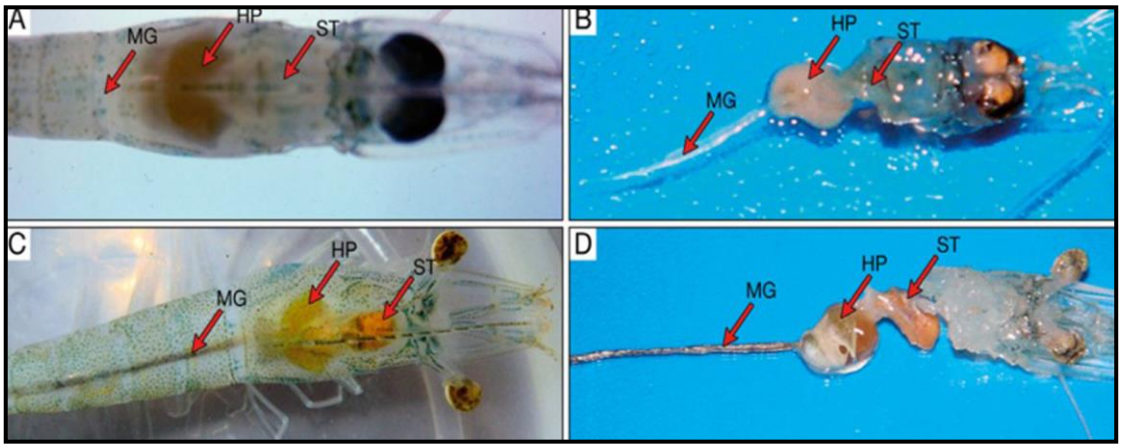

Figure 7 Gross signs of *P. vannamei* affected by AHPND (A and B) compared to normal shrimp (C and D). MG, Midgut; HP, hepatopancreas; ST, stomach.
[Source: From reference (Tran *et al.*, 2013)]

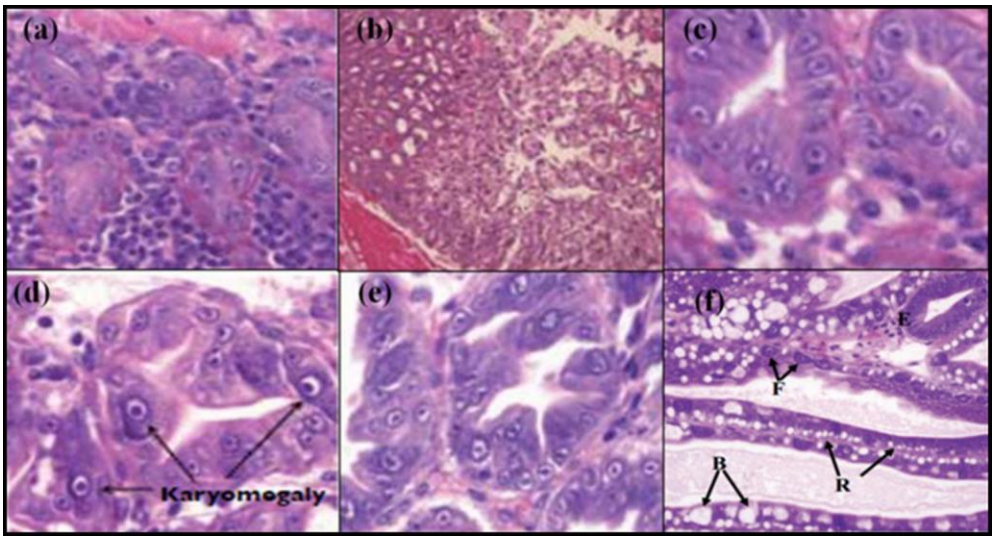

Figure 8 Key diagnostic features of AHPND-affected hepatopancreas (HP) with sloughing of HP cells (a), lack of B, F and R cells (b), lack of E-cell mitosis (c), enlarged HP nuclei (d) and the infiltration of haemocytes (e) compared with normal HP (f) with intact tubules and distinct F, B and R cells.
[Source: From reference (Flegel, 2013)]

Virulence factors

Genome sequences analysis of AHPND *V. parahaemolyticus* isolates demonstrated the 69 kb plasmid encoded genes homologous to *Photorhabdus* insect-related (*pir*) toxin genes. This plasmid containing genes is detected in all AHPND *V. parahaemolyticus* strains but is absent in non-pathogenic strains (Yang *et al*., 2014; Han *et al*., 2015). The structure of *pir* toxin genes region of AHPND *V. parahaemolyticus* strain is showed in Figure 9 (Hirono *et al*., 2016). The plasmid has a GC content of 45.9% with a copy number of 37 per bacterial cell and consists of 92 open reading frames that encodes for several essential proteins including mobilization proteins, replication enzymes, transposases and virulence-associated proteins similar to Pir toxins (Figure 10) (Han *et al*., 2015). The GC content of the toxin genes is 38.2% which is lower than the GC content of the rest of the plasmid indicating that the presence of these genes might be occur by horizontal gene transfer. The Pir toxins act as binary proteins which are first identified in *Photorhabdus luminescens*, a bacterium that colonizes the intestine of entomopathogenic nematodes

in the family *Heterorhabditidiae*. The nematode larvae could attack insect and release toxins that play a role in cytotoxicity against insect midgut cells (Li *et al.*, 2014). Shrimp and insect are both arthropods, AHPND *V. parahaemolyticus* affects shrimp in similar pathology to the Pir toxins in insect. Analysis by SDS-PAGE of the toxin active fractions derived from AHPND *V. parahaemolyticus* indicates 13 and 50 kDa of the Pir A and B related toxins encoded by *pirA*-like (336 bp) and *pirB-like* (1,317 bp) genes, respectively (Sirikharin *et al*., 2015).

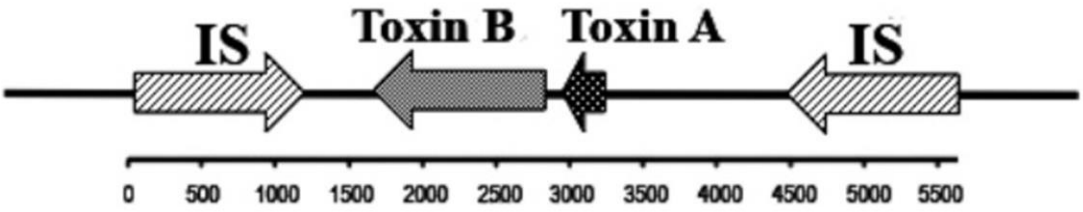

Figure 9 The structure of *pir* toxin genes regions of AHPND *V. parahaemolyticus*.
[Source: From reference (Hirono *et al*., 2016)]

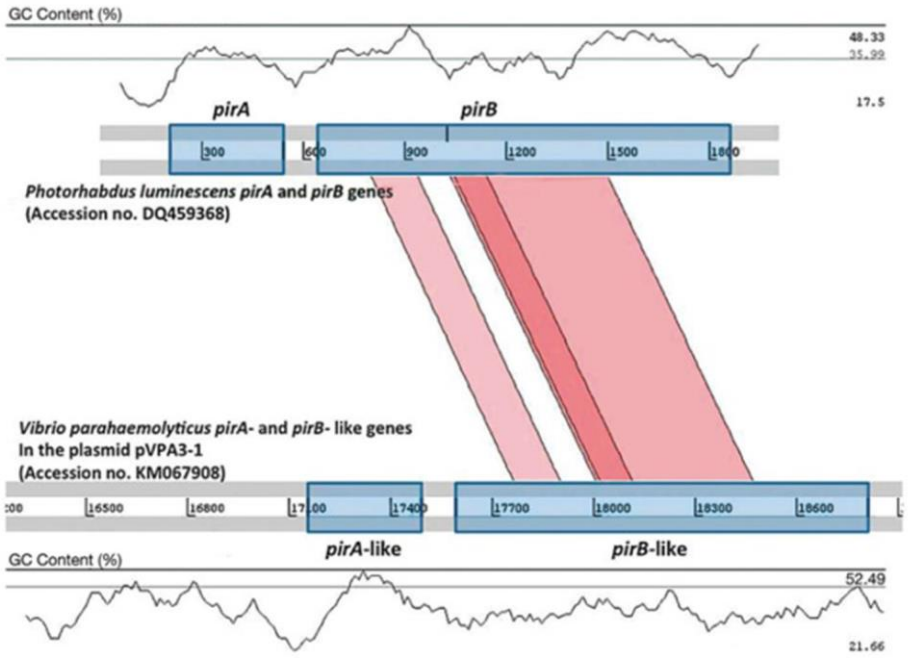

Figure 10 Comparative analysis of the *pirA*- and *pirB*-like genes in AHPND *V. parahaemolyticus* and the *pirA* and *pirB* genes of *P. luminescens*.
[Source: From reference (Han *et al*., 2015)]

Isolation and identification

AHPND *V. parahaemolyticus* is a halophilic gram negative bacterium detected mostly in infected shrimp, sediment and water in shrimp ponds. AHPND *V. parahaemolyticus* lacks of the *tdh* and *trh* genes and is not human pathogen (FAO, 2013). Isolation of this bacterium includes enrichment prior to plating on a selective agar such as TCBS or CV medium. For confirmation of AHPND *V. parahaemolyticus*, PCR technique is suggested. Flegel and Lo (2014) demonstrated two PCR detection methods using AP1 and AP2 primers targeted to DNA plasmid sequences of AHPND *V. parahaemolyticus*. AP2 was superior to AP1 with 97% positive predictive value for the detection. Subsequently, AP3 primer based on 336 bp *toxA* gene sequences of protein detected in cell-free culture broth of AHPND *V. parhaemolyticus* has been developed, which gave 100% sensitivity and 100% specificity for detection (Sirikharin *et al.*, 2014). However, using one-step PCR, the sensitivity of detection low levels of AHPND *V. parhaemolyticus* is poor. To solve this problem, a new two-step or nested-PCR using AP4 primer has been developed targeting to the 1,269 bp *toxA* and *toxB* genes located on AHPND *V. parahaemolyticus* plasmid sequences (Dangtip *et al*., 2015).

Molecular typing methods

DNA typing is a useful technique for epidemiological investigation the transmission of bacteria. In addition, it is also applied for surveillance and evaluation of management systems (Maslow and Mulligan, 1996). Several criteria including stability, reproducibility, type ability and discriminatory properties are considered for evaluation the techniques (Struelens, 1998).

Pulse field gel electrophoresis (PFGE)

PFGE technique has been considered as a gold standard tool for genome fingerprinting of microbial pathogens (Maslow and Mulligan, 1996). The advantage of this technique is the elongated and oriented configuration of large DNA molecules in agarose gel at finite field strengths and the genome size can be measured, in which this cannot be determined by other techniques (Birren and Lai, 1993). The

genomic DNA for PFGE is obtained by digestion of total bacterial DNA encased in agarose blocks and electrophoreses using PFGE (Figure 11) (Tenover *et al.*, 1997). PFGE differs from conventional agarose electrophoresis in that the orientation of the electric field across the gel is periodically changed in contrast to being unidirectional and constant in standard electrophoresis (Carle *et al.*, 1986). The contour-clamped homogeneous electric field electrophoresis (CHEF) accomplished by a 24-electrode arrangement produces a highly uniform electrophoresis gradient causing DNA molecules to reorient, most commonly over an angle of 120º. Field inversion gel electrophoresis (FIGE) does not require a special electrophoresis chamber because the electric current alternates at an angle of 180º rather than 120º (Figure 12) (Goering, 2004).

For differentiation between pandemic and non-pandemic strains *V. parahaemolyticus*, PFGE of *Not*I digested genomic DNA was successfully employed (Wong and Lin, 2001). It is more reliable than group-specific PCR (GS-PCR) and *orf8*-PCR because it produces many diverse patterns and organizes the pandemic strains in closely related clusters (Yeung *et al*., 2002).

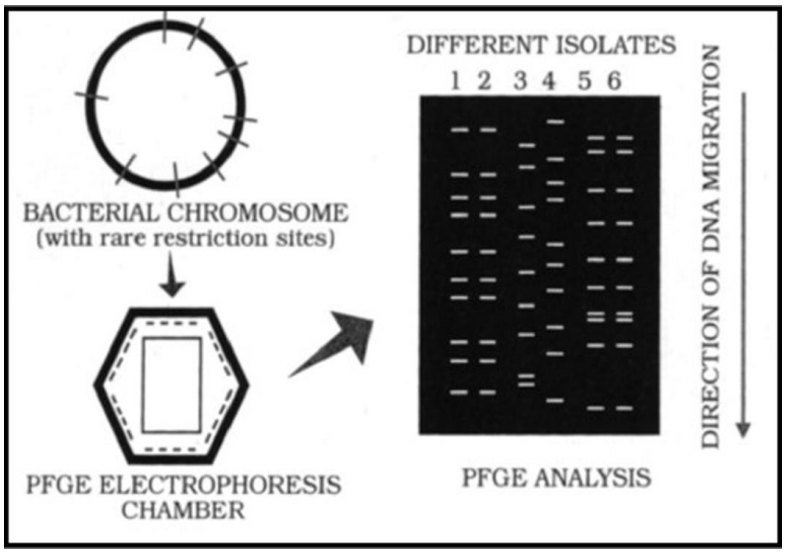

Figure 11 Schematic drawing of PFGE technique.
[Source: From reference (Tenover *et al*., 1997) with some modification]

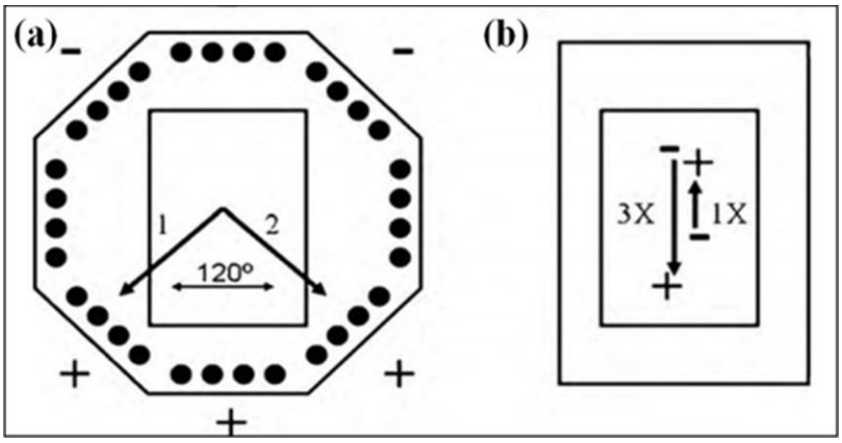

Figure 12 Schematic drawing illustrating the difference between instrumentation used for PFGE by contour-clamped homogeneous electric field electrophoresis (CHEF) (a) and field inversion gel electrophoresis (FIGE) (b).
[Source: From reference (Goering, 2004)]

Clustered regularly interspaced short palindromic repeat (CRISPR) analysis

CRISPR is the segments of prokaryotic DNA containing short and repetitive base sequences derived from virus or plasmid after previous exposures (Westra *et al.*, 2012). CRISPR-associated system (*cas*) genes are normally located next to CRISPR sequences (Ledford, 2015; Hendel *et al.*, 2015). The CRISPR-Cas system plays a key role in the prokaryotic immune system against exogenous foreign genetic elements such as plasmids and bacteriophages (Garneau *et al.*, 2010. CRISPR-Cas system can be used for modification of genes within organisms called genome editing technology (Figure 13) (Horvath and Barrangou, 2010). In addition, it can be used for bacterial typing based on variable numbers of spacer containing sequences from previously encountered foreign DNA (Shariat and Dudley, 2014). Analysis of the spacer acquisition in each strain can be provided the evolutionary and epidemiological information to determine phylogenetic relationships of pathogenic bacteria such as *Yersinia pestis, Erwinia amylovora, Escherichia coli* and *Salmonella enterica* (Cui *et al.*, 2008; McGhee and Sundin, 2012; Yin *et al.*, 2013; Fricke *et al.*, 2011)

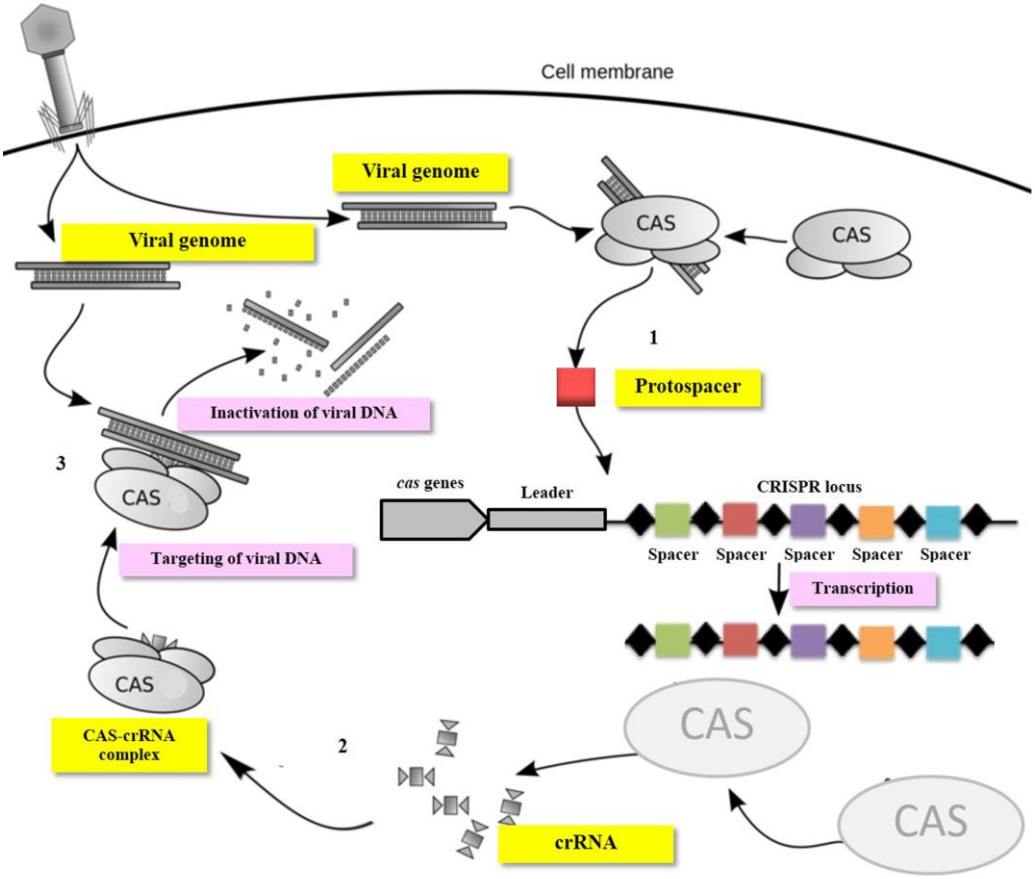

Figure 13 Three-step of anti-exogenous DNA pathway by CRISPR-Cas systems. [Source: From reference (Horvath and Barrangou, 2010) with some modification]

***Bdellovibrio* and like organisms (BALOs)**

In natural environments, a unique interaction between prey (the organism that is being attacked) and a predator (an organism that is hunting) usually occur. Predation is importance for ecological balance and nutrient acquisition (Jurkevitch *et al*., 2000)

Bdellovibrio and like organisms (BALOs) are Gram-negative small rod or vibrio-shaped, highly motile bacteria that obligatory prey on other Gram-negative bacteria. They possess one sheathed polar flagellum which enables them to swim rapidly and hunt many preys (Stolp and Starr, 1965) . BALOs are widespread in saltwater nature and have been isolated from oceans, estuaries, coastal waters, salt lakes, aquarium tanks, gills of blue crabs, biofilm of oyster and animal faeces (Jurkevitch *et al.*, 2000; Williams and Piñeiro, 2007; Piñeiro *et al.*, 2007). They are classified into two families, *Bdellovibrionaceae* consisting of the genus *Bdellovibrio* and *Bacteriovoracaceae* consisting of the genera *Bacteriovorax* and *Peredibacter* (Davidov and Jurkevitch, 2004).

BALOs attach bacterial hosts, penetrate through their cell walls to form bdelloplast, multiply and finally lyse and kill their hosts (Snyder *et al.*, 2002). The ability to lyse prey cells makes BALOs to be potential agents for controlling aquatic pathogens (Williams and Piñeiro, 2007). BALOs reduce microbial density and alter microbial communities through predation (Cao *et al.*, 2012). Some *Bdellovibrio* strains have been reported to control *Aeromonas hydrophila* in *P. vannamei* cultivation (Cao *et al*., 2012).

OJECTIVES

1. To characterize *trh*$^{+}$ *V. parahaemolyticus*
2. To characterize AHPND *V. parahaemolyticus*
3. To develop rapid method for detection AHPND *V. parahaemolyticus*
4. To isolate BALOs and evaluate the potential for using as a biocontrol of AHPND *V. parahaemolyticus*

CHAPTER 2

RESEARCH METHOLOGY

MATERIALS AND METHODS

1. Equipments

Equipments	Company
ABI 3130 genetic analyzer	Applied Biosystems, Courtaboeuf, France
Autoclave	Tomy, Japan
Autopipette	Gilson, France
Balances	Denver Instrument Company, NY, USA
Centrifugation (H-103N)	Kokusan, Japan
CHEF_DRIII system	Bio-Rad Laboratories, MA, USA
Densimat	bioMerieux, Italy
Digital Dry Bath (Accublock)	Labnet International Inc., NJ, USA
Electrophoresis apparatus	Bio-Rad Laboratories, MA, USA
Freezer (4°C, -20°C and -80°C)	Sanyo ,Japan
Gel Documentation	Syngene, MD, USA
Hot air sterilizer	BINDER, Germany
Hot plate-Stirrers	Fisher Scientific, PA, USA
Incubator (MIR-262)	Sanyo, Japan
Laminar airflow cabinet (ABS 1200A)	ASTEC microflow, UK
Loopampreal-time turbidimeter (LA-500)	Eiken Chemical Co., Ltd. Tokyo, Japan
Microcentrifuge (Hettich Mikro 22R)	Andreas Hettich GmbH & Co., KG, Germany
MiniVE electrophoresis system	GE Healthcare Bio-Sciences, NJ, USA
Olympus CH40 light microscope	Olympus, Japan
Orbital shaker (TPM-2)	SARSTEDT, Germany
pH meter	Metrohm, Switzerland
Power supply (PowerPac Basic)	Bio-Rad Laboratories, MA, USA

Equipments	Company
Refractometer	Nippon Optical Works, Japan
Refrigerated Microcentrifuge	Hettich, Germany
Shaking incubator	Labline Instrument Co., Ltd. IL, USA
Spectrophotometer (LUMIstar Omega)	BMG LABTECH, Germany
Spectrophotometer (MaestroNano)	GMI, Inc., MN, USA
Spectrophotometer (U2000 UV/VIS)	Hitachi Instruments Inc., CT, USA
Thermal Cycler (Gene Atlas)	Astec, Fukuoka, Japan
Thermo cycler (T1000)	Bio-Rad Laboratories, MA, USA
Ultrasonic Sonicator (VCX-130)	Sonics & Materials, Inc., CT, USA
Votex-Genie 2	Scientific Industries, Inc., NY, USA
Waterbath (1235)	Sheldon Manufacturing, Inc., OR, USA
0.2 and 0.45-µm Millipore	Millipore Filter Corp., MA, USA
96-well microtiter plates (Nunclon)	Sigma-Aldrich, Inc., Mo, USA

2. Microbiological media

All mycological media used in this study were purchased from Difco (USA), Merck (Germany) and CHORMagar Microbiology (France).

3. Enzyme, antibodies and other reagents

All antibodies, enzymes and other reagents used in this study are analytical grade.

Reagents	Company
Anti-K antibodies	Denka Seiken-Tokyo, Japan
Anti-O antibodies	Denka Seiken-Tokyo, Japan
Betaine	Sigma-Aldrich, Inc., MO, USA
BigDye terminator v3.1 cycle sequencing kit	Applied Biosystems, Courtaboeuf, France
Bst DNA polymerase	New England Biolabs Inc., MA, USA
Buffer *Taq* Polymerase	Promega, USA
dNTPs	Promega, USA

Reagents	Company
Ex *Taq*	Takara Biochemicals, Tokyo, Japan
Ex *Taq* buffer	Takara Biochemicals, Tokyo, Japan
FastGene dye terminator removal kit	Nippon Genetics, Tokyo, Japan
HiDi formamide	Applied Biosystems, Courtaboeuf, France
*Not*I restriction enzyme	TOYOBO Co., Ltd. Osaka, Japan
Performanceoptimized polymer 7	Applied Biosystems, Courtaboeuf, France
Proteinase K	Bio-Rad Laboratories, Hercules, CA, USA
Taq DNA polymerase	Promega, USA

4. Oligonucleotide primers

All oligonucleotide primers used in this study were synthesized by Eurofins MWG Operon, Ebersber, Germany except HPLC purification primers for LAMP were synthesized by Hokkaido, System Science Co., Ltd. Hokkaido, Japan.

5. Other chemical substances

Chemical substances	Company
Absolute ethanol	Merck, Germany
Agarose	Gibco, NY, USA
Ammonium chloride	Nacalai Tesque Inc., Japan
Bovine serum albumin	Nacalai Tesque Inc., Japan
Calcium chloride	Nacalai Tesque Inc., Japan
Copper (II) sulfate pentahydrate	Nacalai Tesque Inc., Japan
Folin-Ciocalteau reagent	Nacalai Tesque Inc., Japan
Glutaraldehyde	Nacalai Tesque Inc., Japan
Lambda DNA/*Hin*dIII marker	Bio-Rad Laboratories, MA, USA
Low-melting agarose	Invitrogen, Carlsbad, CA, USA
Phenol	Nacalai Tesque Inc., Japan
Pulsed-Field Certified agarose	Bio-Rad Laboratories, Hercules, CA, USA
Sodium acetate	Nacalai Tesque Inc., Japan
Sodium azide	Nacalai Tesque Inc., Japan

Chemical substances	Company
Sodium carbonate	Nacalai Tesque Inc., Japan
Sodium hydroxide	Promega, USA
Sodium hypochlorite	Nacalai Tesque Inc., Japan
Sodium nitroprusside	Nacalai Tesque Inc., Japan
Sodium tartrate	Nacalai Tesque Inc., Japan
Tris base	Sigma Aldrich, Inc., MO, USA
Urea	Nacalai Tesque Inc., Japan
1 kb DNA Ladder	New England Biolabs Inc., MA, USA
100 bp DNA Ladder	New England Biolabs Inc., MA, USA

METHODS

Overall

This study was divided into three parts. Part I, characterization of human pathogenic *V. parahaemolyticus* isolates from clinical samples. Part II, characterization of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease in shrimp. In addition, LAMP technique to detect *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease was performed. Part III, isolation and identification of *Bdellovibrio* and like organisms (BALOs) against shrimp pathogenic *V. parahaemolyticus* (Figure 14).

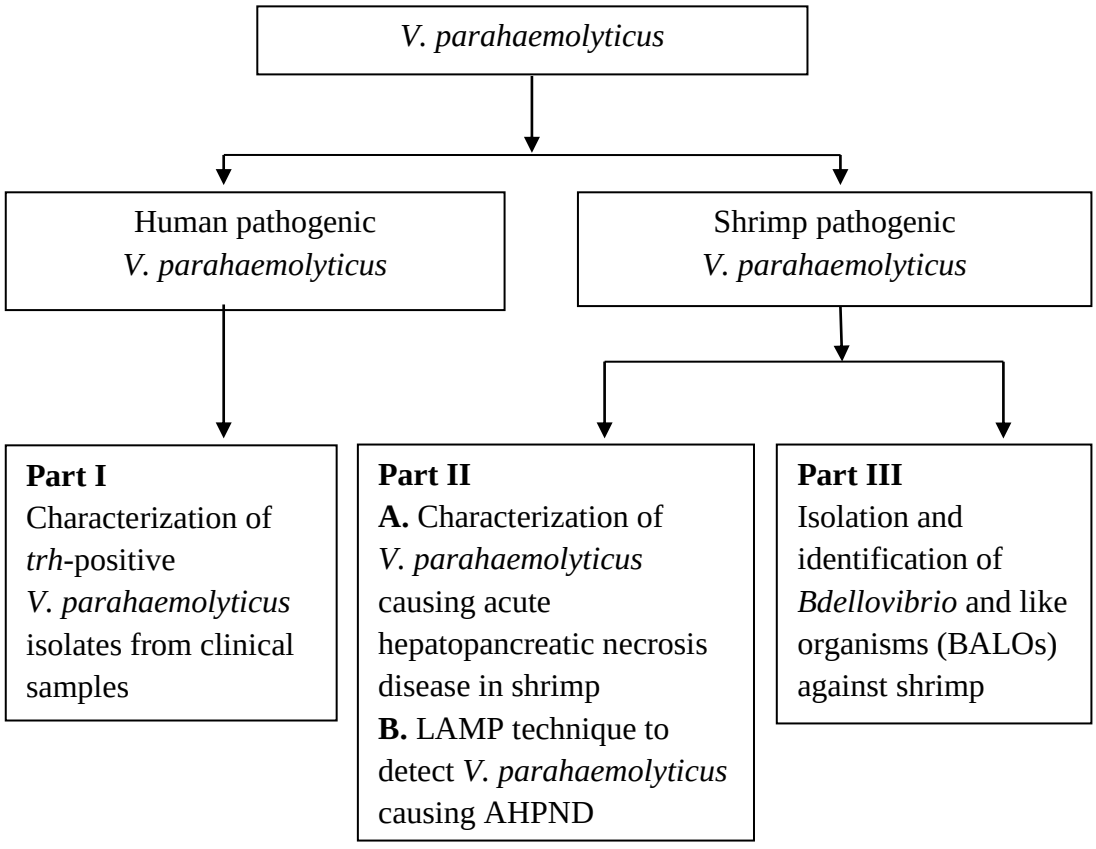

Figure 14 Schematic representation of all study.

Part I Characterization of human pathogenic *V. parahaemolyticus* from clinical samples

To examine the pathogenicity of *trh*$^+$ *V. parahaemolyticus* isolated from clinical samples, the urease production, haemolytic activity and biofilm formation were characterized. In addition, clustered regularly interspaced short palindromic repeats (CRISPRs) was performed to differentiate clinical *trh*$^+$ *V. parahaemolyticus* strains (Figure 15).

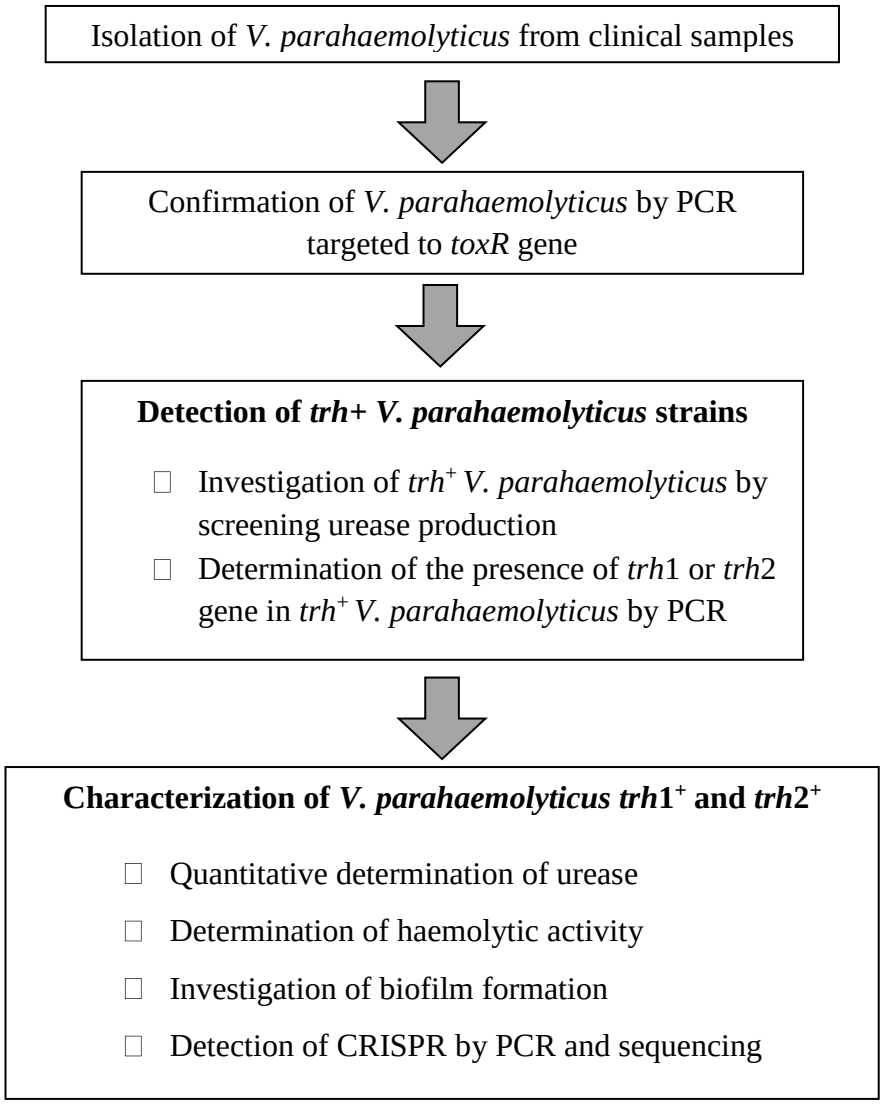

Figure 15 Schematic representation of Part I investigation.

1. Isolation and identification of *V. parahaemolyticus* in clinical samples

Clinical *V. parahaemolyticus* isolates from various countries including Thailand, India, Singapore, Hong Kong, Bangladesh, Vietnam, Philippines, Malaysia, Maldives and United States of America during 1886 to 2012 were obtained from the stock culture of Prof. Dr. Nishibuchi's lab, Kyoto University, Japan and Prof. Dr. Varaporn's lab, Prince of Songkla University. Each isolate was confirmed as *V. parahaemolyticus* by PCR targeted to the *toxR* gene (Kim *et al*., 1999).

2. Confirmation of *V. parahaemolyticus* by PCR targeted to *toxR* gene

For confirmation of *V. parahaemolyticus*, a pair of primers T4 (5′-GTCTTCTGACGCAATCGTTG-3′) and T7 (5′-ATACGAGTGGTTGCTGTCATG-3′) was used to detect 368-pb amplicon of *toxR* gene (Kim *et al*., 1999). PCR reaction consisted of 2.0 µl of DNA template, 1.6 µl of 2.5 mM dNTPs, 4 µl of 2 µM pirmer-mix, 0.1 µl of *Taq* DNA polymerase (5 U/µl) (Promega, USA), 4.0 µl of 5x reaction buffer and 8.3 µl of sterile MilliQ water. Amplification was performed with a single cycle at 96°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, an extension at 72°C for 1.5 min and a final extension at 72°C for 7 min. A 7 µl of PCR product was mixed with 6x gel loading buffer (Appendix B7) and resolved by electrophoresis in a 1.5% agarose gel in 1x Tris-borate-EDTA (TBE) buffer (Appendix B16). Then, the gel was stained with ethidium bromide (Appendix B6) and the amplicons were detected using a UV transilluminator.

3. DNA preparation

For any PCR method, bacterial genomic DNA was extracted by boiling. In brief, each bacterial isolate was culture in Luria-Bertani (LB) broth (Appendix A6) containing 1% NaCl with shaking (160 rpm) at 37°C overnight and 1 ml of the culture was centrifuged (7,000 × *g*) to remove supernatant. The pellet was washed with 0.85% NaCl and resuspened in sterile MilliQ water. Then, the cell suspension was boiled at 100°C for 10 min and centrifuged (14,500 × *g*) to obtain the boiled supernatant. After 10-fold dilution in sterile MilliQ water, the diluted supernatant was used as DNA template for PCR amplification.

4. Detection of *trh*$^+$ *V. parahaemolyticus* strains

4.1 Screening urease-positive *V. parahaemolyticus*

Urease test was used for screening the presence of *trh* gene in *V. parahaemolyticus.* Briefly, a single colony of *V. parahaemolyticus* was inoculated into urea slant agar (Appendix A13) and incubated at 37°C for 18-24 h. The production of urease is indicated by an intense red or pink color on the slant.

4.2 Determination of *trh*1 or *trh*2 gene in *V. parahaemolyticus*

Urease-positive *V. parahaemolyticus* were determined the presence of *trh*1 or *trh*2 gene by PCR assay. In this study, two PCR primer sets were designed base on *trh1* and *trh2* sequences available in the National Center for Biotechnology Information (NCBI) GenBank database using Primer3 Software. The *trh*1 primers: Trh1-F1 (5′-CTGAATCACCAGTTAACGC-3′) and Trh1-R1: (5′-GGCGTTTRATCCAAATAC-3′) generated a PCR product of 313-bp and the *trh*2 primers: Trh2-F2 (5′-CAATCAAAACTGAATCCCC-3′) and Trh2-R3 (5′-CATCAACAAAAMATTTTACCGA -3′) provided an amplicon of 276-bp. The *trh*1 and *trh*2 amplification was carried out separately with a reaction mixture consisting of 2.0 µl of DNA template, 1.6 µl of 2.5 mM dNTPs, 2 µl of 2 µM of each primer, 0.1 µl of *Taq* DNA polymerase (5 U/µl) (Promega, USA), 4.0 µl of 5x reaction buffer and 8.3 µl of sterile MilliQ water. The reaction was performed as follows: 5 min for a hot start at 96°C, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min and final extension at 72 °C for 7 min. Electrophoresis was performed on a 1.5% agarose gel and the amplicons were detected using a UV transilluminator.

To determine the specificity of the *trh*1 or *trh*2 primers, PCR products of the amplification were purified and sequenced. Briefly, 45 µl of PCR product was purified using ethanol/sodium acetate (Appendix B13) precipitation method (Appendix C2) and then 2 µl of purified DNA was used to perform the sequencing reaction (Appendix C3) by mixing with BigDye Terminator v3.1 ready reaction mix (Applied Biosystems, Courtaboeuf, France) and either forward primer of Trh1-F1 or Trh2-F2. Cycle sequencing was performed in a Thermal Cycler Gene Atlas (Astec,

Fukuoka, Japan), including initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10s, annealing at 50°C for 5 s, extension at 60°C for 4 min and final extension at 60°C for 7 min. A 20 µl of sequencing product was purified using FastGene dye terminator removal kit (Nippon Genetics, Tokyo, Japan) to remove the dye terminators. A 5 µl of purified sequencing product was mixed with 5 µl of HiDi formamide (Applied Biosystems, Courtaboeuf, France) and loaded onto the ABI3130 capillary sequencer using a 36 cm capillary filled with performanceoptimized polymer 7 (Applied Biosystems, Courtaboeuf, France). Then, the nucleotide sequences were obtained and analyzed with the database at NCBI using the Basic Local Alignment Search Tool (BLAST) program.

5. Characterization of *trh*1$^+$ and *trh*2$^+$ *V. parahaemolyticus*

5.1 Quantitative determination of urease

Urease was quantified using a colorimetric assay based on the reaction of ammonia and phenol in the presence of hypochlorite which yields a blue product of indophenol (Weatherburn, 1967). Briefly, bacteria were inoculated into LB broth supplemented with 3% NaCl and 0.1% urea and incubated at 37°C. Overnight cultures were concentrated by centrifugation at 10,000 x *g* for 10 min, washed twice with 50 mM HEPES buffer (pH 7.5) (Appendix B9), resuspended in the same buffer, and lysed by sonication. Subsequently, 50 µl of supernatant obtained after centrifugation at 12,000 x *g* for 20 min at 4°C was mixed with 200 µl of 25 mM of urea in HEPES buffer (pH 7.5) (Appendix B18) and incubated at 37°C for 30 min. Ammonia released from the lysate was determined by adding 400 µl of solution A [1% (w/v) phenol, 170 µM sodium nitroprusside] (Appendix B14) followed by 400 µl of solution B [125 mM NaOH, 0.05% (v/v) NaOCl] (Appendix B15). Then, the tubes were incubated at 37°C for 30 min and the absorbance was measured on a Hitachi U 2000 Double-Beam UV/VIS spectrophotometer (Hitachi Instruments Inc., CT, USA) at a wavelength of 625 nm. Ammonium chloride (NH_4Cl) in the same buffer was used as standard. For total protein determination, the total protein concentration from the same lysate was determined by Lowry's method (Lowry *et al*., 1951) with bovine serum albumin (BSA) as the standard. Briefly, the lysate (200 µl) was mixed with 1 ml of reagent C [2% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO_4$•$5H_2O$ in 0.1% (w/v) aqueous solution

of sodium tartrate] (Appendix B11) and incubated at room temperature (30°C) for 30 min, and then 100 µl of reagent D [1 N Folin-Ciocalteau reagent] (Appendix B12) was added into the reaction. After incubation at room temperature for 30 min, the absorbance was measured at a wavelength of 750 nm. All experiments were done in duplicate.

Ammonia concentration was determined by comparison to a standard curve based on the absorbance of NH_4Cl standard. The total protein concentration was determined with BSA standard curve. Urease activity was calculated as micromoles of NH_3 per minute per milligram of protein.

5.2 Determination of haemolytic activity

Haemolytic activity of *V. parahaemolyticus* isolates carrying only the *trh* gene was examined using the blood agarose assay (Nishibuchi and Kaper, 1985) with some modification. Briefly, *V. parahaemolyticus* was grown in 5 ml of LB broth supplemented with 2% NaCl at 37°C for 18 h, the pellet was harvested and resuspended in 1 ml of phosphate-buffered saline (PBS, pH 7.0) (Appendix B10). Then, the cell was disrupted by sonication and the supernatant obtained by centrifugation at 8,000 x *g* for 15 min was determined. Fifty microlitre of the supernatant was added in a well of various concentration of human blood agarose plate (Appendix C1) (0.25%. 0.5% and 1% red blood cells). After incubation for 24 h at 37°C**,** a clear zone around the well was indicated as haemolytic activity. High haemolytic activity (+3) was defined as the isolates can lyse all three concentration of erythrocytes whereas medium (+2) and low (+1) haemolytic activities were defined as the isolates can haemolyse two (0.5% and 0.25%) and one (0.25%) blood concentrations, respectively.

5.3 Investigation of biofilm formation

Biofilm formation was conducted with a slight modification (Nesper *et al*., 2001). Briefly, *V. parahaemolyticus* was grown overnight in LB broth supplemented with 2% NaCl. Then, the bacterial culture was adjusted to be 10^8 cfu/ml and 1 ml of the culture was inoculated in the 96-well microtiter plates. After incubation at 37°C for 24 h, the wells were washed thrice with distilled water to

remove unattached bacterial cell and the wells were fixed with 2.5% glutaraldehyde (Appendix B8). Then, the cells were stained with crystal violet (0.4% w/v) (Appendix B4) for 15 min, after washing with distilled water and dry for 1 h, the cells were destained with ethanol-acetone (80:20) (Appendix B5). Biofilm formation was quantified by measuring the optical density (OD) at 570 nm using a LUMIstar Omega spectrophotometer (BMG LABTECH, Germany). LB broth supplemented with 2% NaCl was used as control. The experiment was performed in triplicate.

5.4 Detection of CRISPR by PCR and sequencing

In this work, primers to detect CRISPR of *V. parahaemolyticus* were designed from the CRISPR sequences of *V. parahaemolyticus* serotype O3:K6 (strain RIMD 2210633) obtained from the CRISPR database website (http://crispr.u-psud.fr/) (Grissa *et al.*, 2007). The forward primer: VpCRISPR_3-F (5′-ATGCATTCCAAAGCTACCACTC-3′) and the reverse primer: VpCRISPR_705-R (5′-GCCTACCAGATAGCAAGTGTCC-3′) were designed to amplify a 592-bp region. The PCR reaction mixture consisted of 5 µl of 10x *Ex Taq* Buffer, 0.25 µl of *Ex Taq* DNA polymerase (5 U/µl) (Takara Biochemicals, Tokyo, Japan), 4 µl of 2.5 mM dNTPs, 1 µl of each primer (10 µM), and 5 µl of DNA template in a total volume of 50 µl. The PCR reaction was performed with the following conditions: initial denaturation cycle for 1 min at 94°C, followed by 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR product was purified using ethanol/sodium acetate precipitation method for sequencing as described above.

CRISPR pattern including the direct repeats (DRs) and spacers in *trh*$^{+}$ *V. parahaemolyticus* strains was investigated using the CRISPRfinder tool (http://crispr.i2bc.paris-saclay.fr/Server/). The DR sequences in each isolate were analyzed to classify based on the similarity of consensus direct repeat sequences (CDRs). In addition, all spacer sequences were investigated using the CRISPRTarget tool (http://bioanalysis.otago.ac.nz/CRISPRTarget/) and were used for determination of the phylogenetic relationship.

In this work, CRISPR-virulence typing was constructed and compared to profiles obtained by CRISPR typing alone. CRISPR-virulence typing was constructed based on the CRISPR spacer sequences and the presence of *tdh*, *trh*1 and *trh*2 genes. A profile of each isolate was created using a binary matrix of presence or absence of spacer sequences and virulence genes. The dendrogram was constructed using BioNumerics 7.0 software (Applied Maths, Saint-Martens-Latem, Belgium) with the UPGMA algorithm using the Dice similarity coefficient. The discriminatory power index (DI) of CRISPR typing alone and CRISPR-virulence typing were assessed by Simpson's diversity index (Hunter and Gaston, 1988) and given by the following equation:

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

N is the total number of isolates in the sample population

s is the total number of types described

nj is the number of isolates belonging to the *j*th type

6. Statistical analysis

Urease activity and correlation between urease and haemolytic activities of *trh*$^+$ *V. parahaemolyticus* isolates were determined by the independent samples *t*-test analysis using SPSS 11.5 software. The differences observed were considered statistically significant at $p < 0.05$.

Part II Characterization of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) and rapid technique to detect AHPND *V. parahaemolyticus* in shrimp

To obtain information on epidemiology and pathogenicity of AHPND *V. parahaemolyticus*, this bacterium was isolated from diseased shrimp and characterized. The virulence factors, serotyping, genotyping and antibiotic susceptibility were investigated. In addition, rapid detection was evaluated (Figure 16).

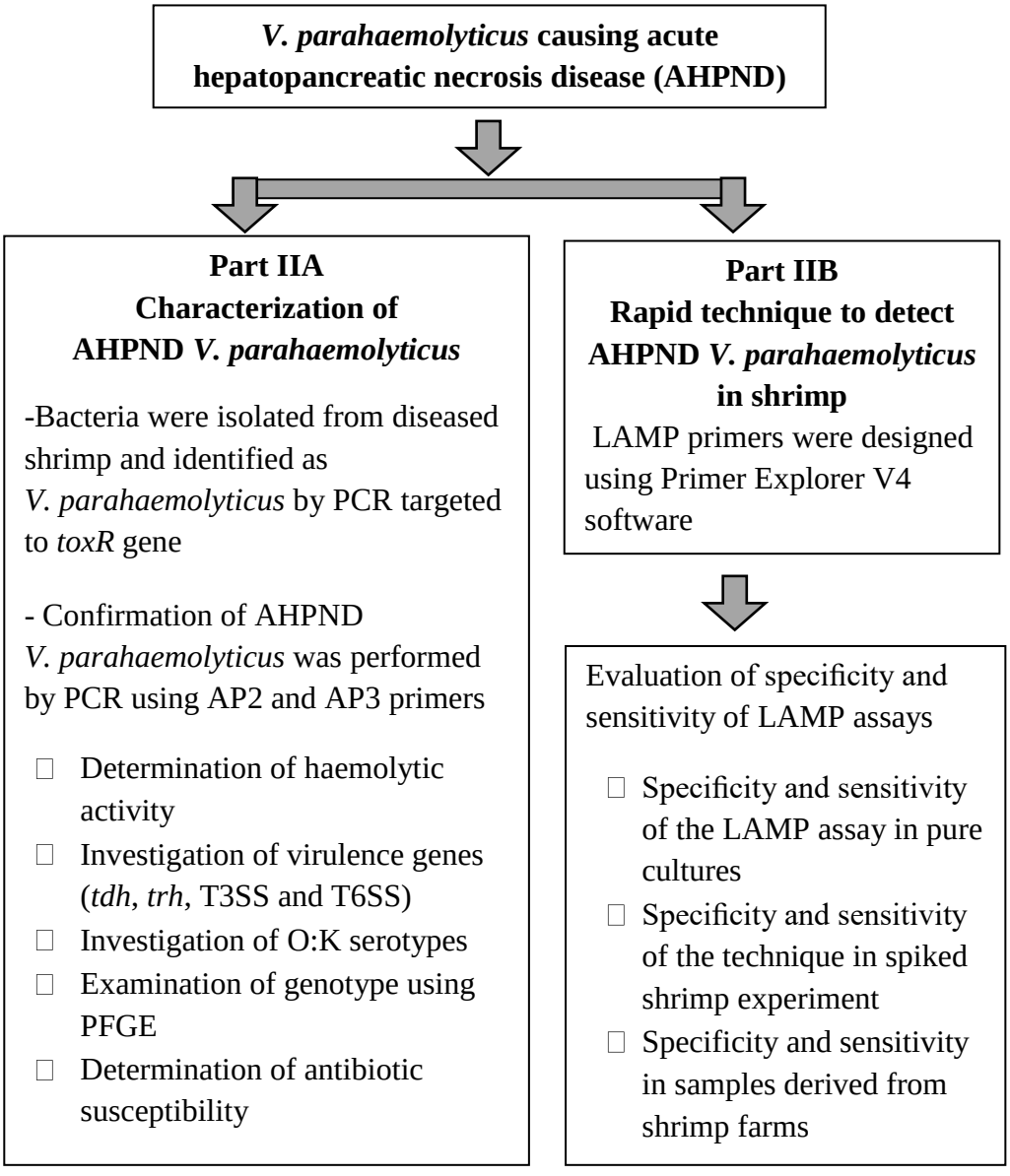

Figure 16 Schematic representation of Part II investigation.

Part IIA Characterization of shrimp pathogenic *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND)

1. Sample collection

Shrimp samples were obtained from 11 ponds of five different farms in two Southern provinces, Pattani and Songkhla. All collected shrimp ponds exhibited the AHPND phenomenon by massive death of shrimp one month after the release of post-larvae to the earth pond. Five to seven diseased shrimps that showed specific signs of AHPND were collected from each pond and their intestines and hepatopancreases were dissected separately. Each sample was plated onto CHROMagar Vibrio (Appendix A2) and the plate was incubated at 30°C overnight. Five mauve colonies per plate were selected and confirmed as *V. parahaemolyticus* using PCR targeted to the *toxR* gene (Kim *et al*., 1999). In addition, the 63 and 66 isolates of clinical and environmental *V. parahaemolyticus*, respectively, which obtained from stool cultures at Hat Yai hospital, Songkhla and from shrimp and mollusks collected in Hat Yai city, Songkhla during 2008 to 2014 (Thongjun *et al*., 2013; Thongchan *et al*,. 2013) were also used in this study.

2. Molecular confirmation of AHPND *V. parahaemolyticus*

For confirmation of AHPND *V. parahaemolyticus*, the tested isolates were subjected to PCR using the specific AP2 primers (AP2F: 5′-TCACCCGAATGCTCGCTTGTGG-3′ and AP2R: 5′-CGTCGCTACTGTCTAGCTGAAG-3′) that targeted to the unique DNA sequences derived from the plasmid of AHPND bacteria (Joshi *et al*., 2014) and AP3 primers (AP3F: 5′-ATGAGTAACAATATAAAACATGAA- 3′ and AP3R: 5′-ACGTGGTAATAGATTGTACAGAA- 3′) specific to *pirA* toxin gene of AHPND *V. parahaemolyticus* (Sirikharin *et al*., 2014). PCR reaction consisted of 2.0 µl of DNA template, 1.6 µl of 2.5 mM dNTPs, 2 µl of each primer (2 µM), 0.1 µl of *Taq* DNA polymerase (5 U/µl) (Promega, USA), 4.0 µl of 5x reaction buffer and 8.3 µl of sterile MilliQ water. The amplification of the AP2 primers was performed with a single cycle at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, an extension at 72°C for 1 min and a final extension

at 72°C for 10 min. For the AP3 primers, the amplification condition was started at 95°C for 5 min and then 30 cycles of denaturation at 94°C for 30s, annealing at 53°C for 30s and extension at 72°C for 5 min was subsequently performed. The thermocycle finished with one cycle at 72°C for 5 min. A 10 µl of PCR product was resolved by electrophoresis in a 1% agarose gel.

3. Determination of haemolytic activity

To investigate any haemolytic activity of AHPND *V. parahaemolyticus*, a single colony of all tested isolates was inoculated into 5% sheep blood agar (SBA) (Appendix A10) and prawn blood agar (PBA) (Appendix A9 and B3) (Chang *et al*., 2000). Then, the plate was incubated at 37°C 18 h for SBA agar and 30°C 7 days for PBA. Clear zones around the bacterial colonies were evaluated for complete haemolysis. In addition, five isolates of environmental *V. parahaemolyticus* isolates were also selected to evaluate the haemolytic activity in this study.

4. Detection of virulence genes

To examine the presence of virulence genes including *tdh*, *trh*, (Tada *et al*., 1992), *vcrD*1 (T3SS1) (Yu *et al*., 2013), *vcrD*2 (T3SS2) (Okada *et al*., 2009), *vipA*1 (T6SS1) and *vipA*2 (T6SS2) (Salomon *et al*., 2013) which are involved in the *V. parahaemolyticus* pathogenesis, PCR was performed using primer pairs as described in Table 6. The PCR mixture consisted of 2.0 µl of DNA template, 1.6 µl of 2.5 mM dNTPs, 2 µl of 2 µM of each primer, 0.1 µl of *Taq* DNA polymerase (5 U/µl) (Promega, USA), 4.0 µl of 5x reaction buffer and 8.3 µl of sterile MilliQ water. The reactions were performed with a T100 Thermo cycler (Bio-Rad Laboratories, MA, USA) as follows: 5 min for initial denaturation, followed by 35 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 55°C 1 min for *tdh* and *trh* genes, 55°C 30 s for *vcrD*2, 58°C 1 min for *vcrD*1 and *vipA*2, 60°C 1 min for *vipA*1, and extension at 72°C for 1.5 min and final extension at 72°C for 7 min. Electrophoresis was performed on a 1.5% agarose gel and the amplicons were detected using a UV transilluminator.

Table 6 Primers used for detection virulence genes of AHPND *V. parahaemolyticus*.

Target genes	Primers	Primer sequence (5′ to 3′)	Product size (bp)	References
tdh	D3	CCACTACCACTCTCATATGC	251	Tada *et al*., 1992
	D5	GGTACTAAATGGCTGACATC		
trh	R2	GGCTCAAAATGGTTAAGCG	250	Tada *et al*., 1992
	R6	CATTTCCGCTCTCATATGC		
vcrD1	vcrD1F	CTGCTGGTCTTGTTCGCTCT	493	Yu *et al*., 2013
	vcrD1R	TCTGGTCGCTTCCTTCTGTG		
vcrD2	vcrD2F	GGTAACACTGCCTGGTGTGGTCATCG	159	Okada *et al*., 2009
	vcrD2R	GTCTCTCAAAGTCTTCAAACTCACCTGC		
vipA1	vipA1F	CACGTGACGGCTCGGTGG	500	Salomon *et al*., 2012
	vipA1R	CTCTTCTTTCGCGTCTTGGTCG		
vipA2	vipA2F	CGAGTATCCACTCGAAACTTTC	524	Salomon *et al*., 2012
	vipA2R	TTCTGCTCCCTCAGTACTTTCTG		

5. Serotyping

In order to determine the somatic (O) and capsular (K) antigens of *V. parahaemolyticus*, the slide agglutination using anti-O and anti-K antibodies (Denka Seiken, Tokyo,Japan) was performed following the protocol of BAM with slight modification. Briefly, the tested isolates were grown on trypticase soy agar (TSA) (Appendix A12) containing 3% NaCl at 37°C for 18 h. For determination of O serotypes, bacterial cells were washed with a solution containing 3% NaCl and 5% glycerol and the suspension was autoclaved at 121°C for 1 h. Then, the cell pellet was obtained by centrifugation at 8,700 × *g* for 15 min and was resuspended in 3% NaCl solution. A heavy suspension was subjected for the agglutination with specific anti-O antibodies. For K antigen, bacterial cells were washed with 3% NaCl solution and a smooth heavy cell suspension was tested first with pooled K antisera (I-IX). The positive isolate of the pooled K antisera was then subjected to test against the monovalent K antiserum.

6. Genotype investigation using pulse field gel electrophoresis (PFGE)

DNA profiles of all AHPND *V. parahaemolyticus* isolates including some environmental and clinical *V. parahaemolyticus* isolates were investigated using the PFGE technique with some modification (Appendix C4) (Bhoopong *et al*., 2007). In brief, *V. parahaemolyticus* isolates were grown on TSA supplemented with 1% NaCl at 30°C (AHPND and environmental isolates) or 37°C (clinical isolates) overnight. The bacterial cells were suspended in 500 µl of suspension buffer (Appendix B2) and adjusted the OD600 to 0.1 to 0.2. After incubation at 55°C for 10 min, a 20 µl of the proteinase K (20 mg/ml) was added to digest the native proteins. The cell suspension was mixed by inversion and incubated at room temperature (30°C) for 5 min. An agarose plug was prepared by mixing equal volumes of a bacterial suspension with 1.2% low-melted-point-agarose (LAM) in 0.5x TBE buffer and was transferred to a disposable plug mold. After solidification, an agarose plug was soaked in 5 ml of cell lysis buffer (Appendix B1) and incubated at 55°C for 2 h. Then, an agarose plug was washed 3 times with Tris-EDTA (TE) buffer (pH 8.0) (Appendix B17). The DNA was cleaved by the *Not*I restriction

enzyme (TOYOBO Co., Ltd. Osaka, Japan), and the digested DNA fragments were separated on 1% Pulse-Field Certified agarose gel using 0.5x TBE buffer on a CHEF-DRIII system (Bio-Rad Laboratories, MA, USA). Electrophoresis was performed for 19 h at a constant voltage of 200 V (6 V/cm) with a field angle of 120 at 14°C and a pulse time of 2.2 to 54.2 s. After electrophoresis, the gel was stained with ethidium bromide and DNA was visualized with a UV transilluminator. Gel images were analyzed and a dendrogram was constructed based on the UPGMA with BioNumerics software version 7.0 (Applied Maths, Saint-Martens-Latem, Belgium).

7. Antibiotic susceptibility testing

A total of 33 isolates of AHPND *V. parahaemolyticus* and 16 isolates of environmental *V. parahaemolyticus* were subjected to antimicrobial susceptibility testing by standard disc diffusion method (CLSI, 2010) with 7 antibiotics including ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulphamethoxazole/trimethoprim (25 μg), gentamycin (10 μg), erythromycin (15 μg) and norfloxacin (10 μg) that historically and currently are often used in aquaculture. In brief, the tested isolates were cultured on Luria-Bertani (LB) agar (Appendix A5) supplemented with 1.5% NaCl. Three to four colonies were selected and inoculated in Mueller Hinton broth (Appendix A8) containing 1.5% NaCl. After incubation with shaking (160 rpm) for 3 h, the bacterial suspension was adjusted to be 10^8 cfu/ml to make a bacterial lawn on Mueller Hinton agar (Appendix A7). The antibiotic-loaded paper discs (Oxoid) were placed onto the bacterial lawn and the plate was incubated at 28°C for 24-28 h. The diameter of the inhibition zone was recorded and interpreted according to the interpretive chart provided by the Performance Standards for Antimicrobial Disk Susceptibility Tests (CLSI, 2010). In this study, *Escherichia coli* ATCC25922 was used as a standard strain and all tests were done in duplicate.

Part IIB. Rapid technique to detect AHPND *V. parahaemolyticus*

To develop a rapid method for AHPND *V. parahaemolyticus* detection, two sets of LAMP primers were designed from the unique sequences of the plasmid and *pirA* toxin gene of AHPND *V. parahaemolyticus*. Then, specificity and sensitivity of this assay were evaluated using pure cultures, spiked shrimp experiment and samples collected from shrimp farms (Figure 16).

1. LAMP assay to detect AHPND *V. parahaemolyticus*

1.1 LAMP assay

Six primers of each of two sets were designed using PrimerExplorer V4 software (Fujitsu System Solution Ltd., Tokyo, Japan). LAMP-A2 primers were designed from the 700-bp of AP2 amplification sequences (sequences derived from the plasmid of AHPND *V. parahaemolyticus*) (Joshi *et al*., 2014) and LAMP-A3 primers were designed from the 336-bp AP3 amplification sequences (*pirA* toxin gene of AHPND *V. parahaemolyticus*) (Sirikharin *et al*., 2014). The LAMP assay was performed in 25 µl containing 2 µl of extracted DNA, 1.3 µl of primer-mix [1.6 µM of primers FIP and BIP, 0.2 µM of primers F3 and B3, 0.8 µM of primers LF and LB], 1 µl of *Bst* DNA polymerase (New England Biolabs Inc., MA, USA), 2 µl of reaction buffer [0.25 M Tris–HCl (pH8.8), 0.125 M KCl, 0.125 M $(NH_4)_2SO_4$, 2.5 % Tween 20], 2 µl of 100 mM $MgSO_4$, 4 µl of 5 M Betaine and 3.5 µl of 10 mM dNTPs. The reaction mixture was performed in a Loopamp realtime turbidimeter (Eiken Chemical Co., Ltd. Tokyo, Japan) at 65ºC for 60 min and the reaction was inactivated at 80ºC for 2 min. The reaction was suspected positive result when the turbidity reached 0.1 within 60 min. The presence of *V. parahaemolyticus* in all positive tubes was confirmed using a LAMP assay targeting the *tlh* gene as previously described (Yamazaki *et al*., 2008). Primer sets used in LAMP assay including LAMP-A2, LAMP-A3 and *tlh*-LAMP were illustrated in Table 7.

Table 7 LAMP primer sets used in this study.

Target genes	Primers	Primer sequences (5′ to 3′)	References
AP2	LAMP-A2-FIB	GGTTTTGGCTCTGGGTACACTCGTCCAACATTTTAACGACT	This study
	LAMP-A2-BIP	GCTTCCTACGTTGAAAGACCGACAGTCATTCAGCGATCC	
	LAMP-A2-F3	GCTTAATCGATTATTCAAATGCG	
	LAMP-A2-B3	CTGAGATTGATCAATCTTTATTGTC	
	LAMP-A2-LF	TCTTGCCGTTTCGCCCA	
	LAMP-A2-LB	GCGTTATCAGCTTCATATCCCC	
AP3 *(pirA*)	LAMP-A3-FIB	GGTATTGAATGGTAAGCTCCCCGGAAGTCGGTCGTAGTGT	This study
	LAMP-A3-BIP	AATGGGGTGCGCCATTTATGAAGTTTCATCACGTTGTACC	
	LAMP-A3-F3	GCAAACATACACCTATCATCC	
	LAMP-A3-B3	GCATTATCAGGGCGTTGT	
	LAMP-A3-LF	ACGTCCCGTATTCTCAATGTCT	
	LAMP-A3-LB	GCTGGCGGCTGGAAAGT	
tlh	*tlh*-FIB	ATGTTTTTAAATGAA ACGGAGCTCCGGCAA AAAACGAAG ATGGT	Yamazaki *et al*., 2008
	tlh -BIP	ACGTCGCAA AACGTTATCCGGCGAAGA ACGTAATGTCTG	
	tlh -F3	AGCTACTCGAAAGATGATCC	
	tlh -B3	GGTTGTATGAGAAGCGATTG	
	tlh -LF	ACCAGTAGCCGTCAATG	
	tlh -LB	TTAGATTTGGCGAACGAGA	

2. Evaluation of specificity and sensitivity of LAMP assay

2.1 Specificity and sensitivity of LAMP assay in pure cultures

Specificity of LAMP-A2 and LAMP-A3 primers were evaluated using pure cultures of 33 AHPND *V. parahaemolyticus* isolates, 15 *Vibrio* spp. and 5 non-*Vibrio* spp. The bacterial isolates were cultured in LB broth supplemented with 1% NaCl at 37ºC overnight. One milliliter of each pure culture was centrifuged at 10,000 x *g* for 5 min and then the cell pellet was washed with 0.85% normal saline solution. The pellet was resuspended in 500 µl of sterile distilled water and boiled at 100°C for 10 min. After centrifugation at 20,000 x *g* for 5 min at 4ºC, the supernatant was used as the DNA template to evaluate the sensitivity and specificity of LAMP assay. For sensitivity testing, AHPND *V. parahaemolyticus* in LB broth supplemented with 1 % NaCl was adjusted to be 10^8 cfu/ml and 10-fold dilution was performed to obtain a range of template concentrations (10-10^5 cfu/ml). Numbers of bacteria were confirmed by plating on TSA supplemented with 1% NaCl. All experiments were performed in triplicate and the detection limit was defined as the highest dilution of sample in all three experiments that is considered positive.

2.2 Sensitivity in spiked shrimp experiment

Normal shrimp was purchased from the local market and confirmed the absence of AHPND *V. parahaemolyticus* using PCR. Then, the shrimp was homogenized in alkaline peptone water (APW) (Appendix A1) pH 8.6 supplemented with 1% NaCl and inoculated with 100 µl of AHPND *V. parahaemolyticus* at final concentrations of 10-10^5 cfu/ml. After mixing, the supernatant was harvested and the DNA template was prepared for detection by the LAMP assay as described above.

2.3 Specificity and sensitivity in samples derived from shrimp farms

Shrimp farm samples including post-larvae, shrimp, sediment and water were collected from shrimp farms in southern Thailand. Each sample was enriched in APW pH 8.6 supplemented with 1% NaCl at a ratio of 1:10 and incubated at 30°C for 6 h. One milliliter of the culture was centrifuged at 1,000 × *g* for 1 min to

remove debris and then the supernatant was subjected to DNA extraction and LAMP assay was performed using both LAMP-A2 and LAMP-A3 as described above. In addition, all extracted DNA was confirmed by conventional PCR for comparison. Specificity was calculated as (number of samples negative by both techniques)/ (number of samples negative by both techniques + number of samples positive by LAMP but negative by PCR), and sensitivity was calculated as (number of samples positive by both techniques)/ (number of samples positive by both techniques + number of samples positive by PCR but negative by LAMP) (Lau *et al*., 2010).

2.4 Confirmation by sequencing

To confirm the specificity of the LAMP assay, LAMP product was amplified by PCR using the outer primers B3 and F3 of each primer pair. The PCR reaction was performed in 20 µl including 2 µl of extracted DNA, 4 µl of 5 x reaction buffer, 1.6 µl of 2.5 mM dNTPs, 2 µl of 2 µM of each primer F3 and B3 and 0.1 µl of 5U/µl *Taq* DNA polymerase (Promega, USA). For the cycling conditions, the initial denaturation was performed at 94ºC for 5 min, followed by 30 cycles of 94ºC for 30, 30 s at 55ºC for LAMP-A2 and 60ºC for LAMP-A3, 72ºC for 1 min and a final extension at 72ºC for 10 min. The PCR products were purified and sequenced.

Part III. Isolation and identification of *Bdellovibrio* and like organisms (BALOs) against AHPND *V. parahaemolyticus*

To determine bacteria that possess inhibitory activity toward pathogenic *V. parahaemolyticus* in shrimp, BALOs were isolated from sediment and water and inhibitory activity against AHPND *V. parahaemolyticus* was evaluated (Figure 17).

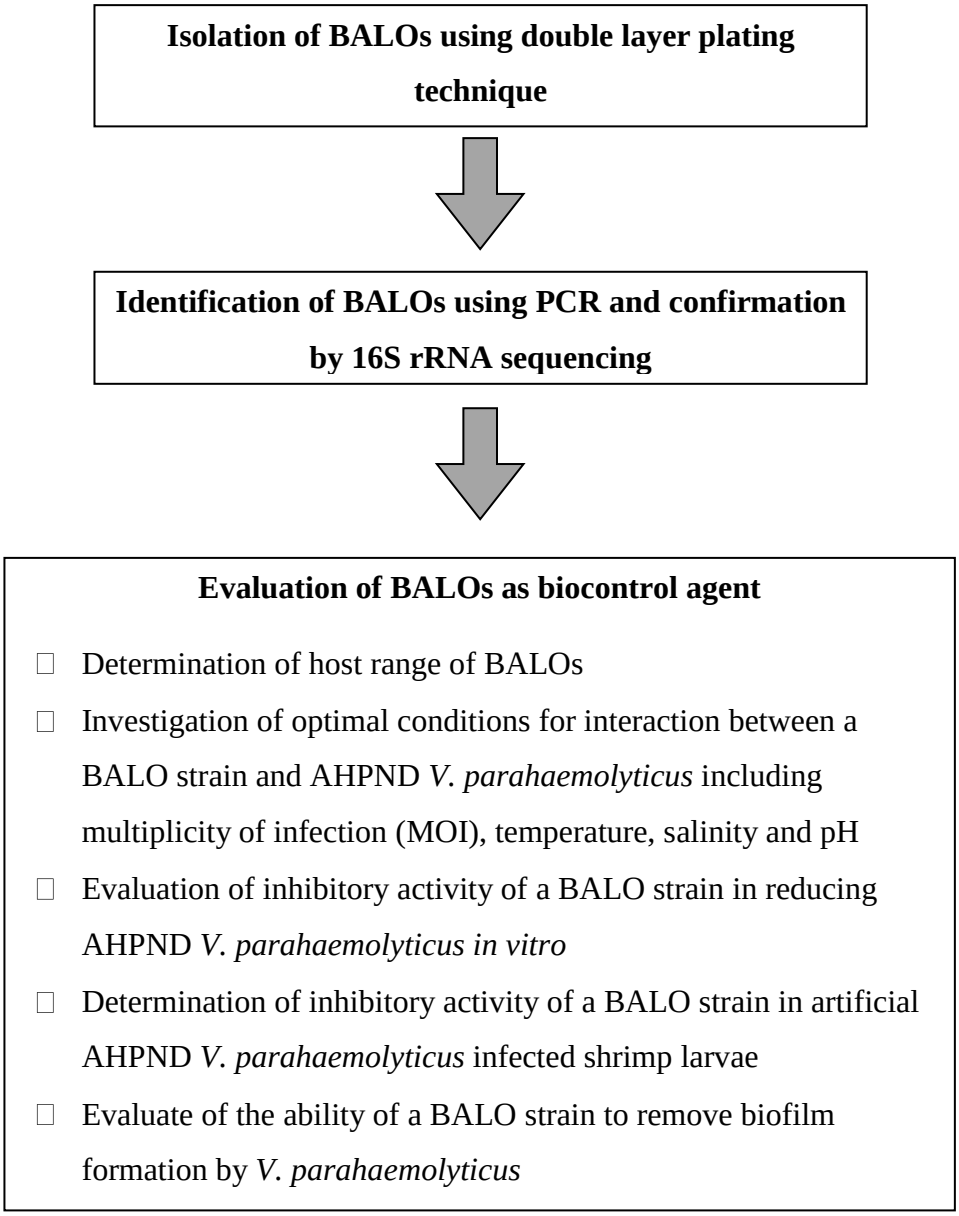

Figure 17 Schematic representation of the workflow of Part III study.

1. Isolation and purification of BALOs

Four isolates of AHPND *V. parahaemolyticus* (PSU5429, PSU5499, PSU5562 and PSU5579) obtained from AHPND shrimp (Kongrueng *et al*., 2015) were used as a mixed prey cocktail for isolation of BALOs. A total of 25 samples including 13 water and 12 sediment samples were collected from Yor Island, and shrimp farms in Songkhla, southern Thailand. Isolation of BALOs was performed using the double-layered plaque assay (Medina *et al*., 2008). Briefly, a sample of either 10 g or 10 ml of sediment or water was mixed with 100 ml of diluted nutrient broth (DNB) (Appendix A4) (Starr and Seidler, 1971) and 1 ml of prey cocktail at a concentration of 10^8 cfu/ml. After incubation with shaking (200 rpm) at 30°C for 7 days, the mixture was then centrifuged and the supernatant was filtered through a 0.45 µm membrane filter (Millipore Filter Corp., MA, USA) to remove debris and prey cells. The filtrate was concentrated by centrifugation at 22,000 × *g* for 1 h and the pellet was resuspended in DNB and mixed with soft diluted nutrient agar (soft DNAg-0.6% agar) (Appendix A11) containing 300 µl of prey cocktail (10^8 cfu/ml). Then, it was overlaid on diluted nutrient agar (DNAg) (Appendix A3). After incubation at 30°C, the plaque formation was observed within 3 to 7 days indicated the presence of BALOs.

To purify the BALOs, each individual plaque was picked up with sterile pasteur pipette and cultured in DNB containing the prey cocktail (10^8 cfu/ml). After incubation with shaking (200 rpm) at 30°C, it was centrifuged and the supernatant was filtered. Then, soft agar overlay method was performed by mixing 100 µl of the filtrate with 300 µl of the prey cocktail (10^8 cfu/ml) in 4 ml of soft DNAg-0.6% agar and spread on DNAg plate. After 7 days of incubation, an isolated plaque was selected and BALOs purification was repeated twice. At third passages of BALOs purification, an isolated plaque was obtained and cultured in DNB with AHPND *V. parahaemolyticus* PSU5429 to enhance the titer and kept at -80°C with 10% glycerol.

2. Molecular identification

To identify the genus of the BALOs, genomic DNA of BALOs was extracted by boiling method as described above. The 16S rRNA gene was amplified by PCR using primers 63F (5′-GAGGCCTAACACATGCAAGTC-3′) and 842R (5′-CGWCACTGAAGGGGTCAA-3′) specific to the *Bdellovibrio* 16S rRNA gene (800 bp) (Jurkevitch *et al*., 2000) and primers Bac676F (5′-ATTTCGCATGTAGGGGTA-3′) and Bac1442R (5′-GCCACGGTTCAGGTAAG-3′) specific to the *Bacteriovorax* 16S rRNA gene (800 bp) (Davidov *et al*., 2006). The PCR reaction was performed in a reaction mixture consisting of 1.6 µl of 2.5 mM dNTPs, 2µl of each primer (2 µM), 0.1 µl of 5 U/µl Go *Taq* DNA polymerase (Promega, USA), 4.0 µl of 5 x reaction buffer and 2.0 µl of DNA templates in a 20 µl volume. PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) as follows: 5 min for a hot start at 94°C, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 50°C 1 min for the *Bdellovibrio* 16S rRNA gene, 53°C 1 min for the *Bacteriovorax* 16S rRNA gene and extension at 72°C for 1 min and final extension at 72°C for 5 min. Electrophoresis was performed on a 1% agarose gel and the amplicons were detected using a UV transilluminator. To confirm the genus of the BALOs, the PCR products were purified and sequenced. The 16S rRNA sequences of all BALOs isolates were analyzed to compare with known sequences from the NCBI database using the BLAST program.

3. Phylogenetic tree construction

To examine the relationship of BALOs isolates that were obtained in this study, the phylogenetic tree was constructed. 16S rRNA sequences of BALOs were aligned by ClustalW with reference strains obtained from the NCBI database. The aligned sequences were analyzed using MEGA 6.0 software (Tamura *et al*., 2013) and the phylogenetic tree was constructed by neighbor joining using the TN93 model (Tamura and Nei, 1993). The percentages of statistical significant levels of interior nodes were determined by performing bootstrap analysis with 1,000 replications.

4. Evaluation of BALOs as biocontrol agent

4.1 Determination of host range of BALOs

Five AHPND *V. parahaemolyticus* strains (PSU5432, PSU5434, PSU5450, PSU5569 and PSU5581), 2 clinical strains (PSU5666 and PSU5668) and 2 environmental strains (PSU5147 and PSU5150), *Vibrio* spp. including *V. vulnificus*, *V. cholerae* and *V. alginolyticus* and other bacteria including *Staphylococcus aureus* and *E. coli* were used for host range investigation of the BALOs isolates. Briefly, each host bacterium was cultured in 5 ml of LB broth supplement with 1% NaCl and incubated with shaking (160 rpm) for 3 h. Then, the bacterial culture was adjusted to be a concentration of 10^8 cfu/ml and mixed with 4 ml of soft DNAg-0.6% agar before spreading on DNAg to make the bacterial lawn. For BALOs preparation, each pure culture of BALO isolate in DNB was mixed with *V. parahaemolyticus* AHPND host strain (PSU5429) and incubated with shaking (200 rpm) at 30°C for 3 days. After filtration to remove host cell, a 10 µl of the BALO filtrate were spot on the plate containing the bacterial lawn. A clear zone was observed after incubation at 30°C for 3 to 7 days. All experiments were performed in triplicate independently, and at least 1 positive out of the 3 experiments was defined as susceptibility, whereas 3 negative experiments indicated non-susceptibility.

4.2 Investigation of optimal conditions of interaction between BALO and AHPND *V. parahaemolyticus*

To evaluate factors that affect the ability of BALOs against AHPND *V. parahaemolyticus*, the multiplicity of infection (MOI), temperature, salinity and pH of a selected BALO strain and AHPND *V. parahaemolyticus* isolate were investigated. One milliliter of a selected BALO strain at concentrations of 10^3 to 10^6 plaque-forming units per milliliter (pfu/ml) was mixed with 100 ml of DNB containing 1 ml of AHPND *V. parahaemolyticus* PSU5429 suspension at a concentration of 10^7 cfu/ml at the ratio of 1:10, 1:100, 1:1,000 and 1:10,000. The co-culture was incubated at 30°C with shaking (200 rpm) for 7 days and the numbers of AHPND *V. parahaemolyticus* were enumerated daily by the plate count technique. To

investigate the effect of temperature, the co-culture of the BALO and AHPND *V. parahaemolyticus* in 100 ml of DNB was incubated with shaking (200 rpm) at 25°C, 30°C, and 37°C. For salinity evaluation, the co-culture was inoculated in 100 ml of DNB supplemented with 1 to 6% NaCl. In addition, the optimum pH was investigated by culturing the co-culture in 100 ml of DNB at pH 7 to 9. For the control, general shrimp pond conditions were applied, AHPND *V. parahaemolyticus* was inoculated in 100 ml of DNB supplemented with 2% NaCl, pH 7.6 and incubated with shaking (200 rpm) at 30°C. The experiment was performed in triplicate. The reduction of AHPND *V. parahaemolyticus* was calculated as the percentage difference between the initial numbers of the bacterium in the control before and after treatment.

4.3 Evaluation of inhibitory activity of a BALO strain in reducing AHPND *V. parahaemolyticus in vitro*

The co-culture of a BALO strain and AHPND *V. parahaemolyticus* isolate was performed in the optimal conditions described above for determination of inhibitory activity of the BALO against AHPND *V. parahaemolyticus*. The experiment was divided into three groups including AHPND *V. parahaemolyticus* control, BALO control and the co-culture of a BALO strain and *V. parahaemolyticus* AHPND with two replicates per group. In the co-culture, a BALO strain was adjusted to be 10^6 pfu/ml and mixed with 1 ml of AHPND *V. parahaemolyticus* at a concentration of 10^7 cfu/ml in 100 ml of DNB. For control group, one milliliter of each of BALO strain (10^6 pfu/ml) and AHPND *V. parahaemolyticus* (10^7 cfu/ml) was cultured separately in 100 ml of DNB. The numbers of BALO and AHPND *V. parahaemolyticus* were enumerated daily for 7 days using double-layer agar and spread plate techniques, respectively.

4.4 Determination of inhibitory activity of a BALO strain in artificial AHPND *V. parahaemolyticus* infected shrimp larvae

To assess the ability of a BALO strain to reduce mortality in shrimp larvae infected with AHPND *V. parahaemolyticus*, the *Penaeus vannamei* (post larvae at stages PL24) were obtained from a shrimp farm in Songkhla Province, southern Thailand. Larvae were acclimatized in a tank containing 2% artificial sea water (ASW) with aeration at room temperature (30°C) and fed with a commercial diet twice a day. The experiment was divided into six groups and each group was done in duplicate. Three control groups including PL24 in ASW control, AHPND *V. parahaemolyticus* (10^7 cfu/ml) and BALO (10^6 pfu/ml) were set with 20 shrimp larvae in each group. The remaining three groups were the co-culture between AHPND *V. parahaemolyticus* and a BALO strain at various concentrations. In brief, 20 shrimp larvae in each group were incubated into the tanks containing AHPND *V. parahaemolyticus* at the final concentration of 10^7 cfu/ml for 15 min, and then a BALO strain at the final concentrations of 10^2, 10^4 and 10^6 pfu/ml were added into the tanks. The mortality of shrimp larvae was recorded daily for 7 days.

4.5 Evaluate of the ability of a BALO strain to remove biofilm formation by *V. parahaemolyticus*

The assessment of biofilm reduction of AHPND *V. parahaemolyticus*, clinical and environmental *V. parahaemolyticus* isolates by a BALO strain was performed in this study. All tested *V. parahaemolyticus* isolates were subjected to produce biofilm and assessed by quantitative determination (Nesper *et al*., 2001, Chanyi and Koval, 2014). Briefly, 200 µl of *V. parahaemolyticus* culture in LB broth supplemented with 2% NaCl were inoculated into 96-well microtiter plates and incubated at room temperature (30°C) for 24 h to produce biofilms. After washing the wells with water, 200 µl of a BALO strain (10^6 pfu/ml) in DNB were inoculated into the *V. parahaemolyticus* biofilms and incubated at room temperature (30°C) for 24 h. Then, the wells were washed 3 times with water and fixed with 2.5% glutaraldehyde. After washing, the remaining biofilm in each well was stained with 200 µl of crystal

violet (0.4% w/v) and incubated at room temperature (30°C) for 15 min. Then, the well was washed with water and dried for 30-60 min before distaining with ethanol-acetone (80:20) and OD570 of the adherent bacteria was determined using a LUMIstar Omega spectrophotometer (BMG LABTECH, Germany). Biofilm formation of *V. parahaemolyticus* alone was used as a control. In all experiments, the obtained results were an average of 12 wells.

5. Statistical analysis

A potential of a BALO strain to inhibit AHPND *V. parahaemolyticus in vitro* and biofilm assay were carried out using SPSS 11.5 software. All values were subjected to one-way analysis of variance (ANOVA). The data are presented as the mean and standard deviation (SD) of the indicated numbers of each experiment. Means were separated using Duncan's test at $p < 0.05$. The differences observed were considered statistically significant at $p < 0.05$.

CHAPTER 3

RESULTS

Part I Characterization of human pathogenic *V. parahaemolyticus* from clinical samples

V. parahaemolyticus carrying the *trh* gene

In this work, a total of 73 *trh*$^+$ *V. parahaemolyticus* isolates (including 38 *tdh*$^+$ *trh*$^+$ and 35 *tdh*$^-$ *trh*$^+$ isolates) was obtained from clinical samples in ten countries during 1886 to 2012. They were 28 different serotypes consisting of 10 O antigens and 19 K serotypes (Table 8).

Urease production in *trh*$^+$ *V. parahaemolyticus*

Urease is one of the virulence factor detected in various pathogenic bacteria such as *Protease mirabilis* (Sriwanthana *et al*., 1993; 1994), *Klebsiella aerogenes* (Lee *et al*., 1992), *Helicobacter pylori* (Voland *et al*., 2003) and *Yersinia enterocolitica* (Probst *et al*., 1993). In *V. parahaemolyticus*, the rare urease-positive phenotype strongly correlates with the presence of the *trh* gene and can be used as a marker for identification (Suthienkul *et al.*, 1995). In this work, urease production in *trh*$^+$ *V. parahaemolyticus* was evaluated. Previous study, urease production of *trh*$^+$ *V. parahaemolyticus* TH3996 strain was strongly induced by adding 0.1% urea into LB broth (Park *et al*., 2000). Therefore, in this work, all tested isolates were grown in LB broth supplemented with 0.1% urea before evaluation. Urease production of the *tdh*$^+$ *trh*1$^+$, *tdh*$^+$ *trh*2$^+$, *tdh*$^-$ *trh*1$^+$and *tdh*$^-$ *trh*2$^+$*V. parahaemolyticus* isolates varied from 0.46 to 15.54, 1.75 to 21.05, 0.89 to 20.79 and 3.46 to 21.20 μmol NH_3/min/mg protein, respectively. No significant differences were observed in the urease production between *tdh*$^+$ *trh*1$^+$ and *tdh*$^+$ *trh*2$^+$ isolates (p = 0.063) as well as in the *tdh*$^-$ *trh*1$^+$ and *tdh*$^-$ *trh*2$^+$ isolates (p = 0.788) (Figure 18). These indicated that urease production among clinical *trh*$^+$ *V. parahaemolyticus* isolates varied distinctively and it

was not associated with either the *trh* or *tdh* genes. In addition, no correlation between urease production and serotypes of *V. parahaemolyticus* was observed in this study.

Table 8 A total of 73 isolates of *trh*$^{+}$ *V. parahaemolyticus* obtained from clinical samples.

Country	Year	Presence of gene			No. of isolates	O:K serotype (no. of isolates)
		tdh	*trh*1	*trh*2		
Thailand	1991-2012	+	+	-	14	O1:KUT (3), O3:K6 (5), O3:K72 (2), O4:K62 (2), O4:K63 (1), O12:KUT (1)
	1987-2006	-	+	-	10	O1:K48 (1), O1:K56 (1), O1:K69 (1), O3:K6 (3), O3:KUT (2), O4:K53 (1), O5:KUT (1)
	1886-2012	+	-	+	9	O1:K1 (1), O1:KUT (4), O1:K69 (1), O3:K72 (1), O3:KUT (1), O8:K56 (1)
	1999-2012	-	-	+	6	O1:K25 (1), O1:K41 (2), O1:K69 (1), O1:KUT (2)
USA	1990-1996	+	+	-	5	O1:K56 (1), O4:K12 (2), O4:K63 (1), O1:KUT (1)
	1990	-	+	-	1	O4:K12 (1)
	1991-1996	-	-	+	2	O3:K59 (1), O11:K15 (1)
Bangladesh	1994	+	+	-	1	O4:K11 (1)
	1981	-	+	-	1	O4:K11 (1)
	1977-1986	-	-	+	11	O1:K25 (1), O1:K56 (2), O1:KUT (3), O3:K7 (1), O3:KUT (2), O13:KUT (1), O5:KUT (1)
Maldives	1985	-	+	-	1	O3:K6 (1)
Singapore	1985-1992	+	-	+	2	O1:K69 (1), O1:KUT (1)
	1985	-	+	-	1	O4:K11 (1)
Vietnam	2010	-	+	-	1	O1:K1 (1)
India	1994	+	-	+	1	O1:KUT (1)
	1994	-	-	+	1	O1:KUT (1)
Philippines	1983-1987	+	-	+	3	O3:KUT (1), O10:K71 (1), O6:K46 (1)
Hong Kong	1983-1993	+	-	+	2	O1:K1 (1), O1:K69 (1)
Malaysia	1995	+	-	+	1	O4:K12 (1)

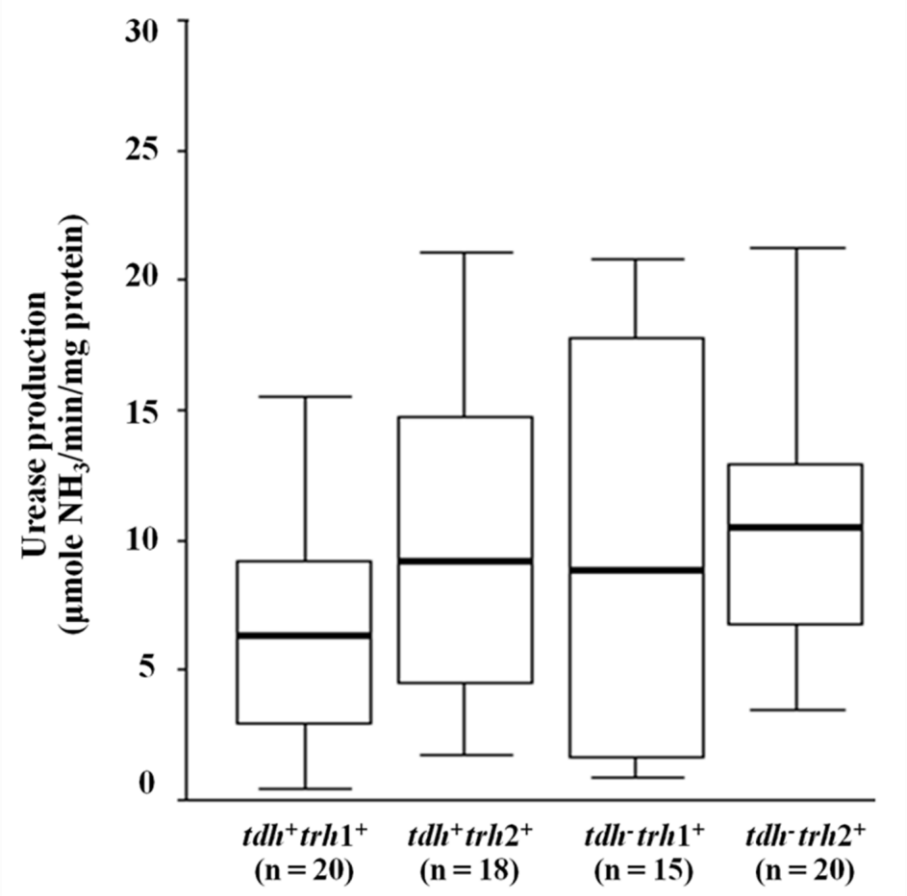

Figure 18 Comparison of urease production between *trh*$^{+}$ *V. parahaemolyticus* isolates according to the presence or absence of *tdh*. Horizontal bar within box represents median values and vertical line out of the box indicates minimum and maximum. The difference in urease production in each group was compared using the independent samples *t*-test analysis.

Haemolytic activity of *trh*$^{+}$ *V. parahaemolyticus*

A total of 15 *trh*1^{+} isolates was determined, 13.3%, 53.3% and 33.3% of the total isolates exhibited high, medium and low haemolytic activity, respectively (Figure 19). For 20 isolates of *V. parahaemolyticus* carrying the *trh*2 gene, 5%, 35% and 60% of the total isolates displayed high, medium and low haemolytic activity. It has been demonstrated that in *V. parahaemolyticus*, the expression of *trh*2 was lower than the *trh*1 (Kishishita *et al*., 1992; Okuda and Nishibuchi, 1998). Therefore, the

results obtained in this study might correlate to the expression of the genes and the sequences variation within the *trh*1 and *trh*2 genes (Kishishita *et al*., 1992).

In this work, correlation between urease production and haemolytic activities of *trh*$^+$ isolates was evaluated. Although the urease production and haemolytic activity ratio of *trh*2$^+$ isolates was higher (1.65 to 21.20 μmol NH_3/min/mg protein) than that of the *trh*1$^+$isolates (0.56 to 19.21 μmol NH_3/min/mg protein), no significant difference was observed (p = 0.683) (Figure 20). This indicated the urease was not involved in the human erythrocyte lysis by TRH hemolysin of *trh*$^+$ *V. parahaemolyticus* strains.

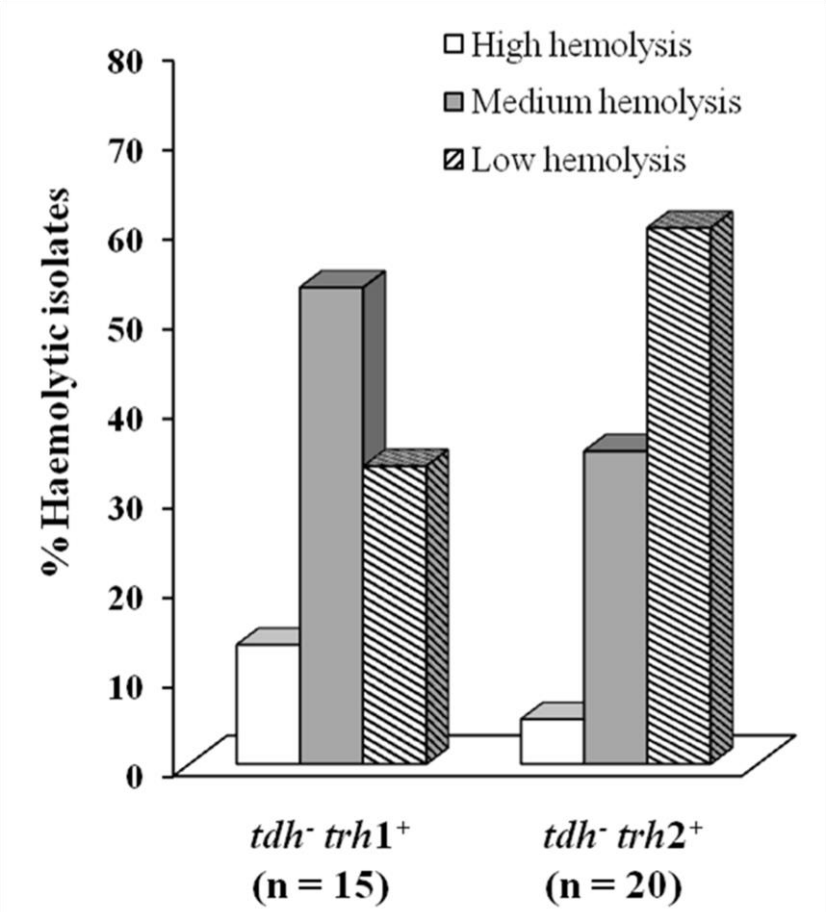

Figure 19 Haemolytic activity of *V. parahaemolyticus* carrying only the *trh* gene using blood agarose assay.

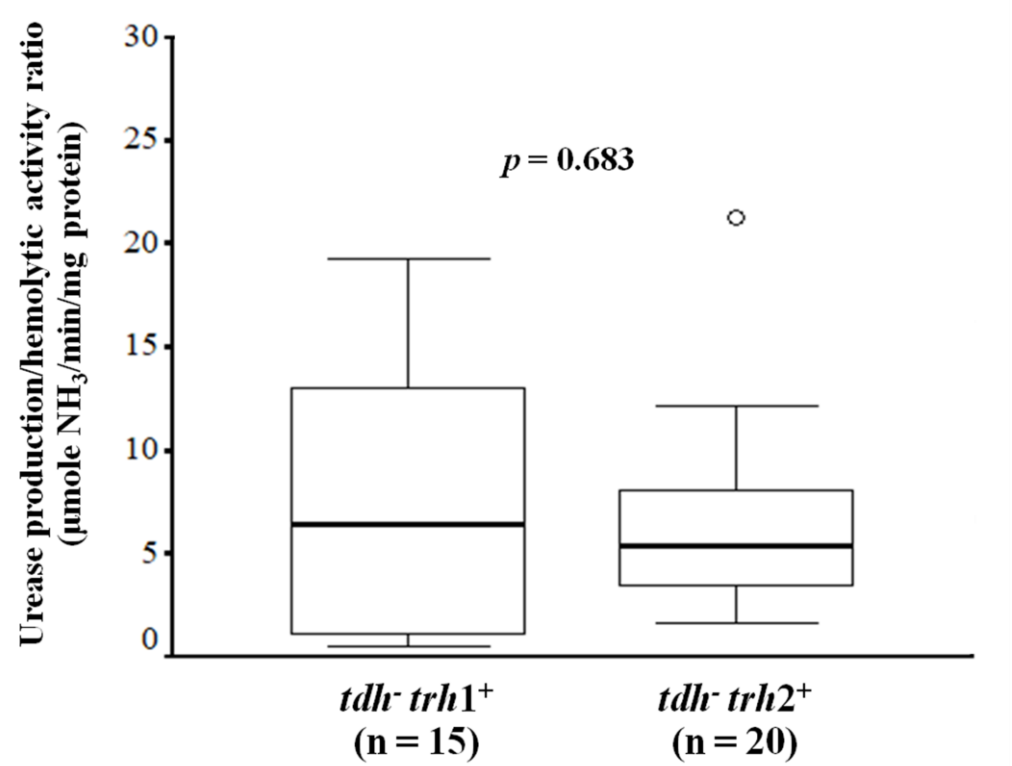

Figure 20 Correlation between urease production and haemolytic activity of *V. parahaemolyticus* isolates carrying only the *trh* gene. Horizontal bar within box represents median values and vertical lines out of the box indicate minimum and maximum. ○ indicates the outlier. No significant difference was observed in the urease production/haemolytic activity between the isolates carrying the *trh*1 and *trh*2 genes (p = 0.683) using the independent samples *t*-test analysis.

Biofilm formation

In order to investigate the involvement of the virulence genes and biofilm production, 4 categories of *V. parahaemolyticus* (*tdh*$^+$ *trh*1$^+$, *tdh*$^+$ *trh*2$^+$, *tdh*$^-$ *trh*1$^+$, and *tdh*$^-$ *trh*2$^+$) were determined. There was no any difference in biofilm formation among 4 categories of *V. parahaemolyticus* because high variation of biofilm formation within the isolates in each category was observed (Figure 21).

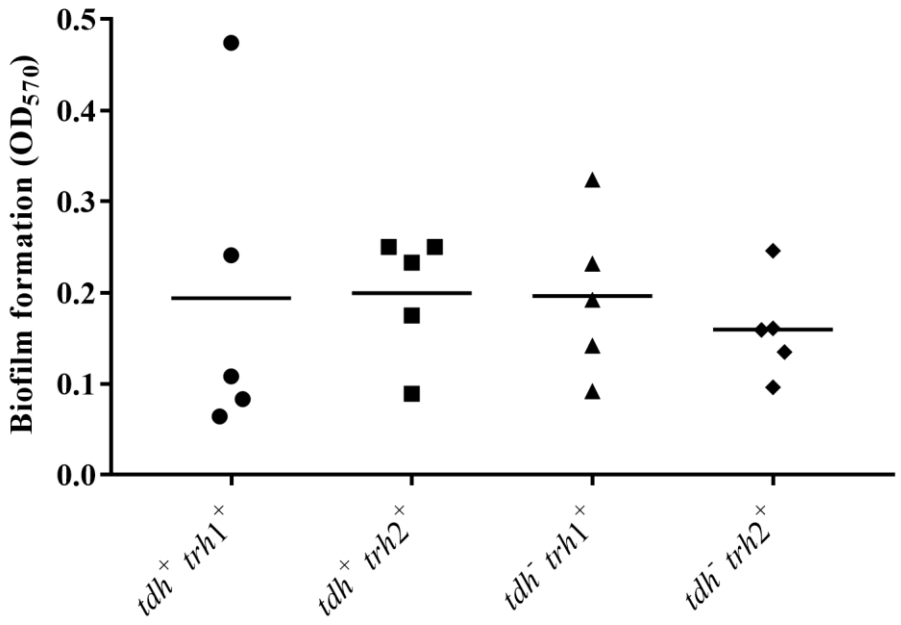

Figure 21 Biofilm formation of *trh*$^{+}$ *V. parahaemolyticus* isolated from clinical samples. Symbols represent mean of biofilm formation of each isolates and horizontal bar indicates mean of biofilm formation of each category.

CRISPR sequences in *trh*$^{+}$ *V. parahaemolyticus*

In the present study, specific primer pairs for detect CRISPR region of *V. parahaemolyticus* were designed and compared to the previously reported by Sun and co-workers (2015). No difference in the specificity detected in the 5 *V. parahaemolyticus* tested isolates after confirmation by sequencing.

Thirty four isolates of *trh*$^{+}$ *V. parahaemolyticus* including 10 isolates of *tdh*$^{+}$ *trh*1^{+} and each 8 isolates of *tdh*$^{+}$ *trh*2^{+}, *tdh*$^{-}$ *trh*1^{+} and *tdh*$^{-}$ *trh*2^{+} were selected for CRISPR sequences analysis. Sixteen isolates (47.0%) were positive for CRISPR, they were 8, 5 and 3 from *tdh*$^{+}$ *trh*1^{+}, *tdh*$^{+}$ *trh*2^{+}, and *tdh*$^{-}$ *trh*2^{+} isolates, respectively (Table 9). None of the isolate carrying *tdh*$^{-}$ *trh*1^{+} was positive for CRISPR. The result obtained in this study was higher than that of reported from the previous study in which only 2 of 6 (33.3 %) isolates were positive for CRISPR (Sun *et al*., 2015).

The numbers of DRs detected in CRISPR region were between 25 and 28 bp nucleotides in lengths and the DR unique sequences of all CRISPR-positive isolates were GTGAACTGCCGAATAGGTAGCTGAT (Table 9). A total of 28

spacers were obtained and the number of spacer detected in each isolate was between 1 or 2 with 30 to 33 bp nucleotides in lengths (Table 10). Spacer analysis using the CRISPRTarget and the BLAST databases from NCBI revealed that most of them showed 87-100% similarity to *Vibrio alginolyticus* plasmids except one spacer of *V. parahaemolyticus* PSU5256 suggesting the possibility of horizontal genetic transfer between *V. alginolyticus* and *V. parahaemolyticus* (Table 10). Phylogenetic tree of all 28 spacers was generated and 6 different spacer patterns designated as SP1 to SP6 were classified using maximum likelihood method (Figure 22).

CRISPR detection in 16 *trh*$^+$ *V. parahaemolyticus* isolates was classified into 5 CRISPR types (CTs) based on CRISPR spacer patterns (Figure 23). All isolates in CT1 type were *trh*1$^+$ isolates carrying spacers SP4 and SP6. CT2 type detected in both the *trh*1$^+$ and *trh*2$^+$ isolates possessing spacers SP1 and SP6 (Figure 23). One isolate of *trh*2$^+$ *V. parahaemolyticus* was classified into CT3 containing spacers SP1 and SP5. CT4 and CT5 contained only one spacer (SP3 in CT4; SP2 in CT5) (Figure 23). Using the CPISPR-based typing, all tested *V. parahaemolyticus* isolates could not be grouped according to *trh* gene subtype (*trh*1 or *trh*2).

In this work, a combination of CRISPR spacer sequences with virulence genes (*tdh*, *trh*1 and *trh*2 genes) of *V. parahaemolyticus* isolates was analyzed. CRISPR-virulence typing profiles obtained from 34 isolates of *trh*$^+$ *V. parahaemolyticus* were organized into 7 clusters with 12 different profiles at 75% similarity level (Figure 24). The isolates within the same cluster possessed the identical subtype of the *trh* gene (either *trh*1 or *trh*2). All *trh*1$^+$ isolates were classified in the CV1, CV2 and CV3 clusters of CRISPR-virulence typing, the remaining four clusters (CV4 to CV7) were *trh*2$^+$ isolates. The isolates in clusters CV1, CV2, CV4 and CV6 possessed the *tdh* gene, but not all of them were positive for CRISPR detection. The CV3 and CV5 clusters were negative for CRISPR region (Figure 24). Identical CRISPR-virulence typing profiles were detected in clusters CV1 (PSU5105 and PSU 5106; PSU4921, PSU5107, PSU5296 and PSU5322), CV2 (PSU5069 and PSU5305; PSU5264 and 1884) and CV6 (PSU5323 and PSU5331; 2435, 2443 and 2463) (Figure 24). It was of interest that the spacers SP2 and SP5 were not detected in the *trh*1$^+$ isolates and SP4 was not detected in the *trh*2$^+$ isolates (Figure 24). Therefore, the unique CRISPR spacers detected in *trh*1$^+$ (SP4) or *trh*2$^+$ (SP2 and SP5)

isolates may be used as spacer marker to differentiate *trh*$^{+}$ *V. parahaemolyticus* isolates.

The discriminatory power index (DI) of both CRISPR analysis and CRISPR-virulence typing were evaluated. DI of CRISPR-virulence typing (0.90) was higher than that of CRISPR typing (0.67). This was in agreement of a recent study that DI of CRISPR-virulence typing for *Helicobacter pylori* was higher than the DI of CRISPR typing alone (Bangpanwimon e*t al*., 2017).

Table 9 Characteristics of CRISPR loci in all 16 CRISPR-positive *V. parahaemolyticus* isolates.

Isolate assigned	Gene harboring	Consensus direct repeats (CDRs) sequences[a]	No. of DRs	No. of spacers	CRISPR locus pattern (bp)[b]
PSU4921	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28 -**31**-28-**31**-28
PSU5105	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**31**-28-**31**-28
PSU5106	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**31**-28-**31**-28
PSU5107	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**31**-28-**31**-28
PSU5264	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	2	1	28-**33**-28
PSU5296	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**31**-28-**31**-28
PSU5322	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**30**-28-**31**-28
1884	*tdh*$^+$ *trh*1$^+$	GTGAACTGCCGAATAGGTAGCTGATA	2	1	26-**31**-26
1990	*tdh*$^+$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	2	1	28-**31**-28
2475	*tdh*$^+$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	2	1	28-**32**-28
2435	*tdh*$^+$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**32**-28-**31**-28
2443	*tdh*$^+$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**31**-28-**31**-28
2463	*tdh*$^+$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGAT	3	2	25-**31**-25-**31**-25
PSU5256	*tdh*$^-$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**32**-28-**31**-28
PSU5323	*tdh*$^-$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**30**-28-**31**-28
PSU5331	*tdh*$^-$ *trh*2$^+$	GTGAACTGCCGAATAGGTAGCTGATAAT	3	2	28-**30**-28-**31**-28

[a] Underline indicates the consensus sequence

[b] Underline indicates the direct repeat length and bold indicates the spacer length

Table 10 Foreign genetic element similar to spacers using CRISPR targets analysis.

Spacer groups	Spacers	Nucleotide sequence of spacers (bp)	Similar genetic elements/ hypothetical proteins	Accession number
SP1	PSU5323-s1	TCATTCTCACGATCTAATTACAGTTGGCAC (30)	*Vibrio alginolyticus* strain K06K5 plasmid	NZ_CP017898.1
	PSU5256-s1	TCATTCTCACGGATCTAATAACAGTTGGTCAC (32)	-*Escherichia coli* strain Ecol_AZ159 and EQ011 plasmid**s** -*Enterobacter cloacae* strain 704SK10 and R11 plasmid**s** -*Klebsiella pneumoniae* strain CAV1193-258 and CAV1344-250 plasmids	NZ_CP019007.1 and NC_023315.1 NZ_CP022149.1 and NZ_CP019840.1 NZ_CP013323.1 and NZ_CP011622.1 and NZ_CP011623.1
	PSU4921-s1, PSU5107-s1, PSU5296-s1, 2443-s1, 2463-s1	TCATTCTCACGATCTAATTACAGTTGGTCAC (31)	*Vibrio alginolyticus* strain K06K5 and K04M3 plasmids	NZ_CP017898.1 and NZ_CP017909.1
	PSU5322-s1, PSU5331-s1	TCATTCTCACGATCTAATTACAGTTGGCAC (30)	-	-
	2435-s1	TCATTCTCACGGATCTAATTACAGTTGGTCAC (32)	-	-
SP2	2475-s1	CTCCAACTAAGTTTTCTGTAGCTTCATCATT (32)	*Vibrio alginolyticus* strain K04M3, K08M3 and K04M5 plasmids	NZ_CP017898.1, NZ_CP 017915.1 and NZ_CP017901.1
SP3	PSU5264-s1	TGCAGACAAACAAAGAGGCATCGACGAGTGCAT (33)	*Vibrio alginolyticus* strain K04M3 and K04M5 plasmids	NZ_CP017898.1 and NZ_CP017901.1
	1884-s1, 1990-s1	TGCAGACAAACAAAGAGCCATCGACGAGTGC (31)	*Vibrio alginolyticus* strain K04M3, K08M3 and K04M5 plasmids	NZ_CP017898.1, NZ_CP 017915.1 and NZ_CP017901.1

Table 10 Foreign genetic element similar to spacers using CRISPR targets analysis (continued).

Spacer groups	Spacers	Nucleotide sequence of spacers (bp)	Similar genetic elements/ hypothetical proteins	Accession number
SP4	PSU5105-s1, PSU5106-s1	GAGATACCACAGGCTCAAGCAGATGCTAACAG (31)	*Vibrio alginolyticus* strain K06K5 and K04M3 plasmids	NZ_CP017898.1 and NZ_CP017909.1
SP5	PSU5256-s2	AGTCGGTCAACTGAGAATACGTTGTTGCCAA (31)	-	-
SP6	PSU4921-s2, PSU5105-s2, PSU5106-s2, PSU5107-s2, PSU5296-s2, PSU5322-s2, 2443-s2, 2463-s2	TCATTCTCACGATCTAATTACAGTTGGTCAC (31)	*Vibrio alginolyticus* strain K06K5 and K04M3 plasmids	NZ_CP017898.1 and NZ_CP017909.1
	PSU5323-s2, PSU5331-s2, 2435-s2	AGTCGGTCAACTGAGAATCGTTTGTTGCCAA (31)	-	-

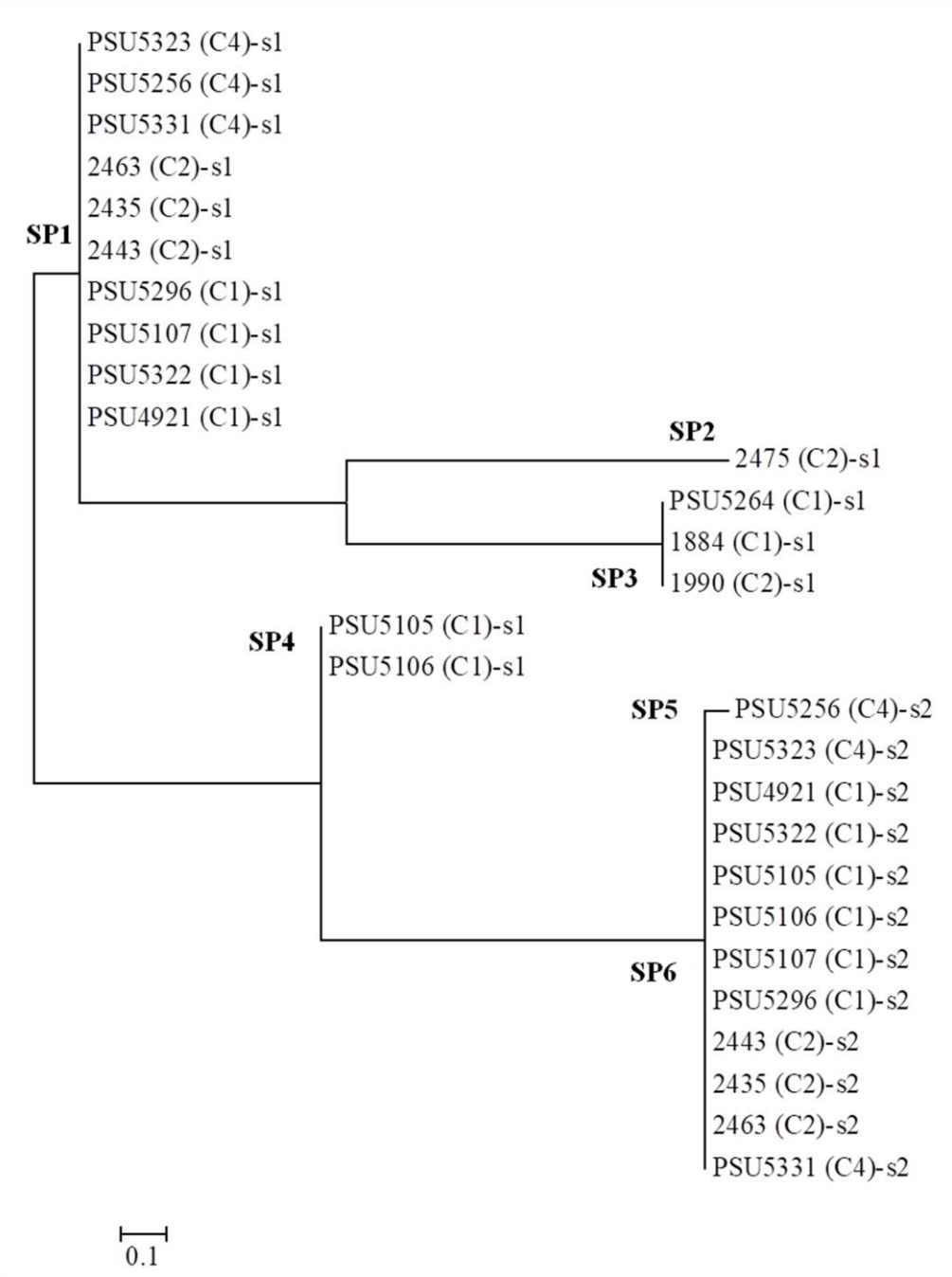

Figure 22 Phylogenetic relationship of all 28 spacer sequences detected in 16 CRISPR-positive *V. parahaemolyticus* isolates. The tree was constructed using maximum likelihood method. Numbers at branch-points represent confidence values obtained after bootstrap analysis of the maximum likelihood tree using 1000 replicates. The scale bar represents 0.1 substitutions per nucleotide position. PSU5256-s2, spacer no.2 of *V. parahaemolyticus* PSU5256; C1, *tdh*$^+$ *trh*1$^+$; C2, *tdh*$^+$ *trh*2$^+$; C3, *tdh*$^-$ *trh*1$^+$; C4, *tdh*$^-$ *trh*2$^+$.

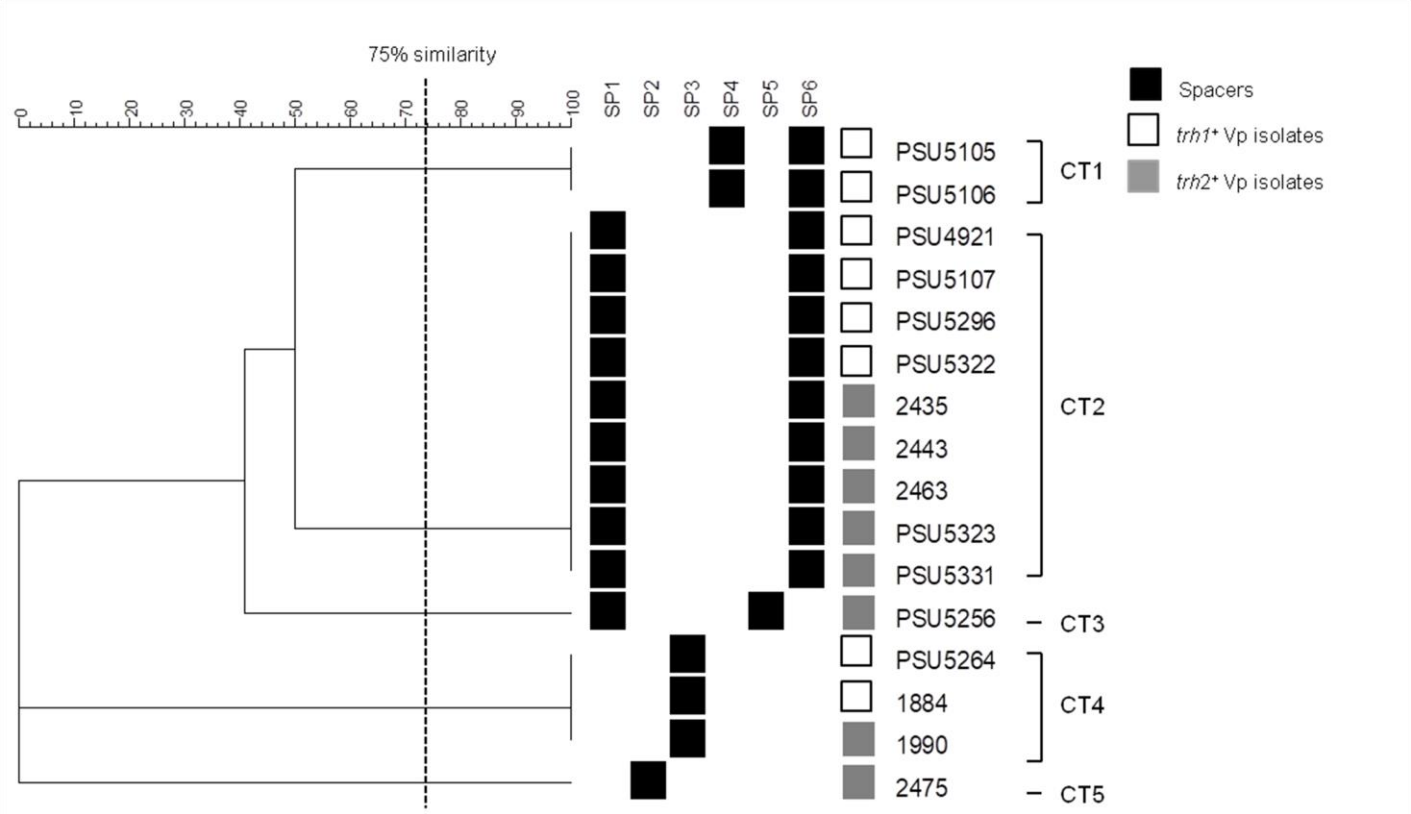

Figure 23 CRISPR-based typing of 16 *V. parahaemolyticus* isolates. Dendrogram was constructed based on binary matrix using BioNumerrics 7.0. Similarity (%) between patterns was calculated using the Dice index. The data were sorted using the UPGMA method.

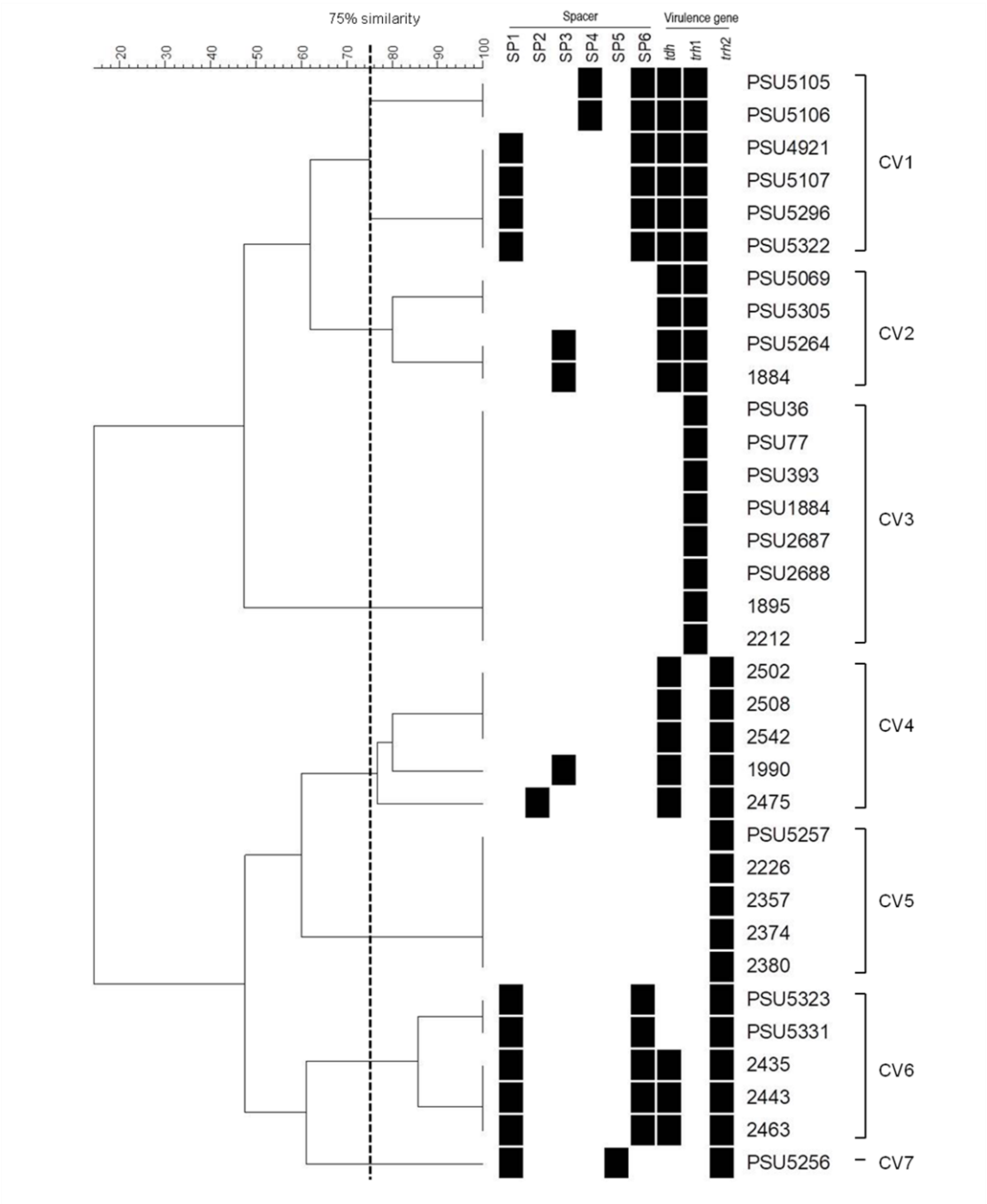

Figure 24 CRISPR-virulence analysis of 34 *trh*$^{+}$ *V. parahaemolyticus*. Dendrogram was constructed based on binary matrix using BioNumerrics 7.0. Similarity (%) between patterns was calculated using the Dice index. The data were sorted using the UPGMA method.

Part II Characterization of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) and rapid technique to detect *V. parahaemolyticus* causing AHPND in shrimp

Part IIA Characterization of shrimp pathogenic *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND)

Isolation and identification of AHPND *V. parahaemolyticus*

In this study, eleven shrimp ponds of four shrimp farms located in Pattani and one farm located in Songkhla provinces in southern Thailand were investigated. All 108 suspected *V. parahaemolyticus* isolates were isolated from hepatopancrease (73 isolates) and intestine (35 isolates) of AHPND shrimps using CHROMagar Vibrio. All isolates were positive for the *toxR* gene by PCR and were subjected to PCR using the specific primer pairs AP2 and AP3 for investigation of AHPND *V. parahaemolyticus*. In addition, *V. parahaemolyticus* isolated from clinical and environmental samples which obtained during 2008 to 2014 from the same area were also used for comparison in this study. AHPND *V. parahaemolyticus* isolates were isolated from 8 of 11 shrimp ponds and a total of 33 of the 108 isolates were PCR positive for AHPND *V. parahaemolyticus* (positive for both the AP2 and AP3 primers). The highest numbers of AHPND *V. parahaemolyticus* (42.9%) were detected in the intestine samples, whereas 24.7% of these bacteria were obtained from hepatopancrease samples (Table 11). All clinical and environmental *V. parahaemolyticus* were negative for both the AP2 and AP3 primers.

Table 11 Isolation of AHPND *V. parahaemolyticus* isolated from shrimp.

No. of farms	Locations	Pond number	No. of AHPND isolates from		Serotypes (no. of isolate)	Isolate number	DNA profiles
			hepatopancrease	intestine			
1	Pattani	1	0/3[a]	0/4	-[b]	-	-
		2	0/4	0/4	-	-	-
2	Pattani	3	1/4	0/4	O1:KUT (1)[c]	1	A1
		4	0/4	2/5	O1:KUT (2)	2, 3	A1
		5	2/5	1/3	O1:KUT (3)	4, 5, 6	A1, A2
		6	3/3	3/3	O1:KUT (6)	7, 8, 9, 10, 11, 12	A2
		7	3/3	2/3	O1:KUT (5)	13, 14, 15, 16, 17	A2
3	Pattani	8	1/5	3/4	O1:K33 (4)	18, 19, 20, 21	B
4	Pattani	9	1/5	4/5	O1:KUT (1), O1:K68 (4)	22, 23, 24, 25, 26	A2, C
5	Songkhla	10	7/19	0	O1:KUT (7)	27, 28, 29, 30, 31, 32, 33	A3
		11	0/18	0	-	-	-
Total (%)			18/73 (24.7)	15/35 (42.9)	-	-	-

[a] No. of selected colonies

[b] Not determined

[c] No. of isolate(s)

Determination of haemolytic activity and investigation of virulence genes

The virulence factors including haemolytic activity and virulence genes (*tdh*, *trh*, T3SS1, T3SS2, T6SS1 and T6SS2) that may be involved in the pathogenesis of AHPND were determined in all 33 isolates of AHPND *V. parahaemolyticus*. The results showed that all the AHPND isolates and all five environmental *V. parahaemolyticus* (control group) exhibited haemolytic activity on PBA but not on SBA (Table 12).

In this study, all AHPND isolates were negative for both the *tdh* and *trh* genes, and were also negative for *vcrD*2 gene (T3SS2) that associated with human pathogenic *V. parahaemolyticus* possessing the *tdh* and/or *trh* genes (Park *et al*., 2004; Okada *et al*., 2009). All of them were positive for *vcrD*1 gene (T3SS1) which was detected in all *V. parahaemolyticus* isolates (Table 12). Detection of those genes in the control groups of *V. parahaemolyticus* from clinical and environment isolates revealed all the isolates were positive for T3SS1, but only the isolates that carrying only the *tdh* or *trh* or both genes were positive for T3SS2 (Table 12).

For determination of the T6SS1 and T6SS2 in AHPND *V. parahaemolyticus*, the detection of the *vipA*1 (T6SS1) and *vipA*2 (T6SS2) genes in all AHPND isolates were investigated. All of them were positive for both the *vipA*1 and *vipA*2 genes, whereas in *V. parahaemolyticus* control groups, the 74.2% of environmental and 84.1% of clinical isolates were positive for *vipA*1 gene, and the 63.6% and 92.1% of environmental and clinical isolates, respectively, were positive for *vipA*2 gene (Table 12).

Table 12 Investigation of haemolytic activity and virulence genes of AHPND *V. parahaemolyticus* isolates.

Tests	AHPND Vp positive/ total (%)	Environmental Vp positive/ total (%)	Clinical Vp positive/total (%)
Hemolysis on SBA[a]	0/33 (0)	0/5 (0)	ND[c]
Hemolysis on PBA[b]	33/33 (100)	5/5 (100)	ND
tdh	0/33 (0)	0/66 (0)	50/63 (79.4)
trh	0/33 (0)	0/66 (0)	5/63 (7.9)
*vcrD*1 (T3SS1)	33/33 (100)	66/66 (100)	63/63 (100)
*vcrD*2 (T3SS2)	0/33 (0)	0/66 (0)	51/63 (81.0)
*vipA*1 (T6SS1)	33/33 (100)	49/66 (74.2)	53/63 (84.1)
*vipA*2 (T6SS2)	33/33 (100)	42/66 (63.6)	58/63 (92.1)

[a] Sheep blood agar

[b] Prawn blood agar

[c] Not determined

Diversity of serotype and DNA profiles

The O:K serotype of all 33 AHPND *V. parahaemolyticus* isolates were determined by agglutination with anti-O and anti-K antibodies. All isolates had the same O1 antigen but different K antigens (KUT or K untypeable, K33 and K68) (Table 11). Most of the isolates were KUT and identical serotypes were detected with the isolates obtained from the same ponds except pond no. 9 that O1:KUT (isolate no. 22 only) and O1:K68 were observed (Table 11). Using PFGE technique, DNA profiles obtained with *Not*I enzyme digestion of all 33 AHPND *V. parahaemolyticus* isolates including the 17 clinical and 14 environmental *V. parahaemolyticus* isolates were demonstrated (Figure 25). DNA profiles obtained from AHPND *V. parahaemolyticus* isolates were organized into groups A (including A1, A2 and A3), B and C, whereas the DNA profiles of clinical and environmental *V. parahaemolyticus* isolates were classified into group D and E, respectively (Figure 26 and Table 11). All the isolates in group A1 to A3 were O1:KUT serotype, and the isolates in group B and C were O1:K33 and O1:K68, respectively (Table 11). Most of the isolates that obtained from the same pond and possessed the same O1:KUT serotype exhibited similar DNA profiles, but were distinct from the DNA profiles

acquired from clinical and environmental isolates (Figure 25). The DNA profile of isolate no.3 obtained from pond no. 4 was not included because it was degraded (Figure 25, lane 3), although its DNA profile was repeated, suggesting that autolysis might occur in this isolate.

Dendrogram analysis indicated that the similarity of the DNA profile of isolates in the group A2 was 95%, and those isolates were slightly different from the isolates in group A1 The DNA profiles of the isolates in groups A1 and A2 were 16% different from the profiles obtained from the isolates in group A3 (from Songkhla farm) (Figure 25 and 26). The DNA profiles of isolates in group B that possessed O1:K33 were identical. In addition, the DNA profiles of O1:K68 belonging to the isolates in group C were indistinguishable (Figure 25). However, the DNA profiles of the isolates in group B and C were, respectively, 8% and 23% different from the DNA profile of the isolates in group A (A1 to A3) (Figure 26). All AHPND *V. parahaemolyticus* isolates seemed to share a similar DNA profile, whereas those from the clinical and environmental isolates appeared to be more diverse (Figure 25). The similarity of the DNA profiles of the clinical isolates (group D) was 30%, whereas that from the environmental isolates (group E) was 24%. However, the DNA profiles of the clinical and environmental isolates were between 84% and 88% different from those of the AHPND isolates *V. parahaemolyticus* (Figure 26).

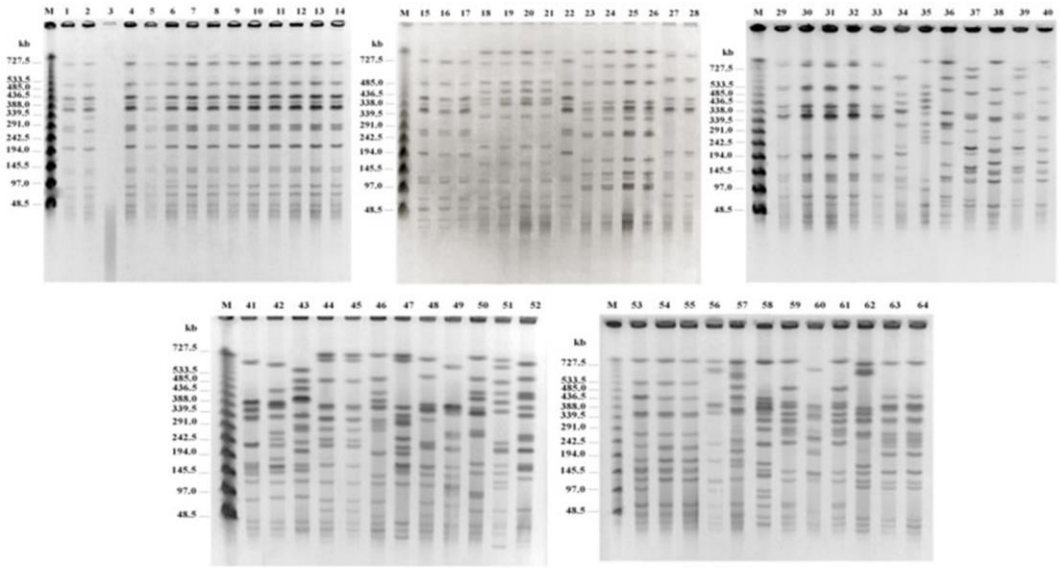

Figure 25 PFGE profiles of *Not*I-digested DNA of AHPND *V. parahaemolyticus* including clinical and environmental *V. parahaemolyticus* isolates.

M: lambda ladder PFG marker (Bio-Rad Laboratories, MA, USA)

Lane 1: AHPND Vp isolate no.1, O1:KUT, pond no. 3
Lane 2: AHPND Vp isolate no.2, O1:KUT, pond no. 4
Lane 3: AHPND Vp isolate no.3, O1:KUT, pond no. 4 (autolysis)
Lane 4-6: AHPND Vp isolate no.4-6, O1:KUT, pond no. 5
Lane 7-12: AHPND Vp isolate no.7-12, O1:KUT, pond no. 6
Lane 13-17: AHPND Vp isolate no.13-17, O1:KUT, pond no. 7
Lane 18-21: AHPND Vp isolate no.18-21, O1:K33, pond no. 8
Lane 22: AHPND Vp isolate no.22, O1:KUT, pond no. 9
Lane 23-26: AHPND Vp isolate no.23-26, O1:K68, pond no. 9
Lane 27-33: AHPND Vp isolate no.27-33, O1:KUT, pond no. 10
Lane 34: Vp PSU4051, environmental
Lane 35: Vp PSU4054, environmental
Lane 36: Vp PSU3934, clinical
Lane 37: Vp PSU3953, clinical
Lane 38: Vp PSU3955, clinical
Lane 39: Vp PSU3958, clinical
Lane 40: Vp PSU3960, clinical
Lane 41: Vp PSU4058, environmental
Lane 42: Vp PSU4064, environmental
Lane 43: Vp PSU4065, environmental
Lane 44: Vp PSU4081, environmental
Lane 45: Vp PSU4091, environmental
Lane 46: Vp PSU4092, environmental
Lane 47: Vp PSU4093, environmental
Lane 48: Vp PSU4096, environmental
Lane 49: Vp PSU4415, environmental
Lane 50: Vp PSU4416, environmental
Lane 51: Vp PSU4421, environmental
Lane 52: Vp PSU4423, environmental
Lane 53: Vp PSU3961, clinical
Lane 54: Vp PSU3963, clinical
Lane 55: Vp PSU3975, clinical
Lane 56: Vp PSU3977, clinical
Lane 57: Vp PSU3981, clinical
Lane 58: Vp PSU4793, clinical
Lane 59: Vp PSU4795, clinical
Lane 60: Vp PSU4796, clinical
Lane 61: Vp PSU4797, clinical
Lane 62: Vp PSU4800, clinical
Lane 63: Vp PSU4803, clinical
Lane 64: Vp PSU4811, clinical

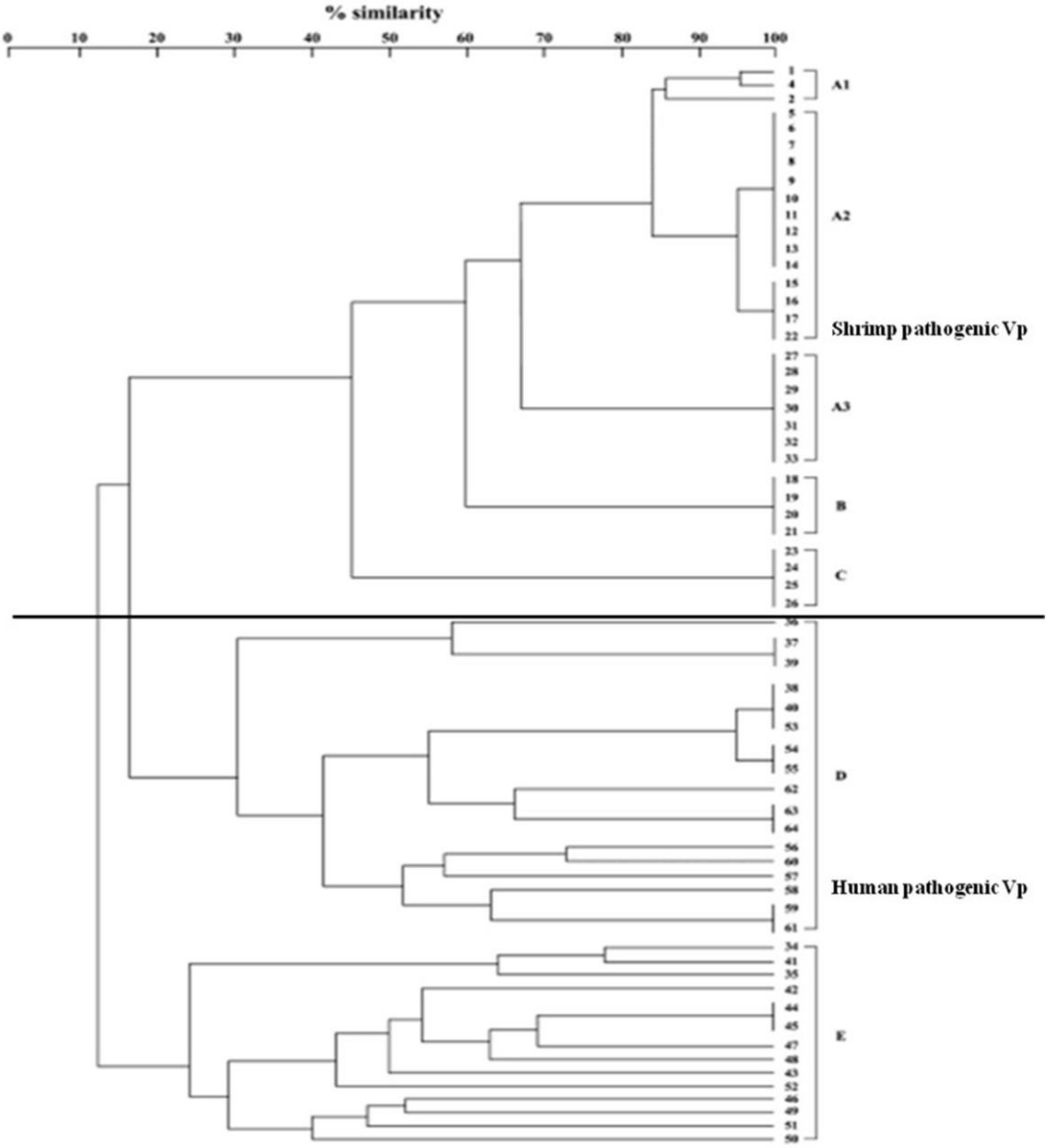

Figure 26 A dendrogram constructed from PFGE of AHPND, clinical and environmental *V. parahaemolyticus* isolates. Numbers 1 to 33 are AHPND *V. parahaemolyticus* isolates, numbers 34-35 and 41-52 are environmental isolates, and numbers of 36-40 and 53-64 are clinical isolates, each number is correlated to the isolate number listed in Figure 24. A1 to E indicate the groups of *V. parahaemolyicus*.

Antibiogram patterns

In this study, the antibiotic susceptibility of the 33 AHPND *V. parahaemolyticus* isolates and 16 environmental *V. parahaemolyticus* isolates was evaluated. All AHPND *V. parahaemolyticus* isolates were resistant to ampicillin (Amp) and erythromycin (E), whereas they were susceptible to tetracycline (T), chloramphenicol (C), sulphamethoxazole/trimethoprim (SXT), gentamycin (CN) and norfloxacin (NOR) (Figure 27). It was of interest that the antibiogram pattern of AHPND *V. parahaemolyticus* to gentamycin was different from the control isolates.

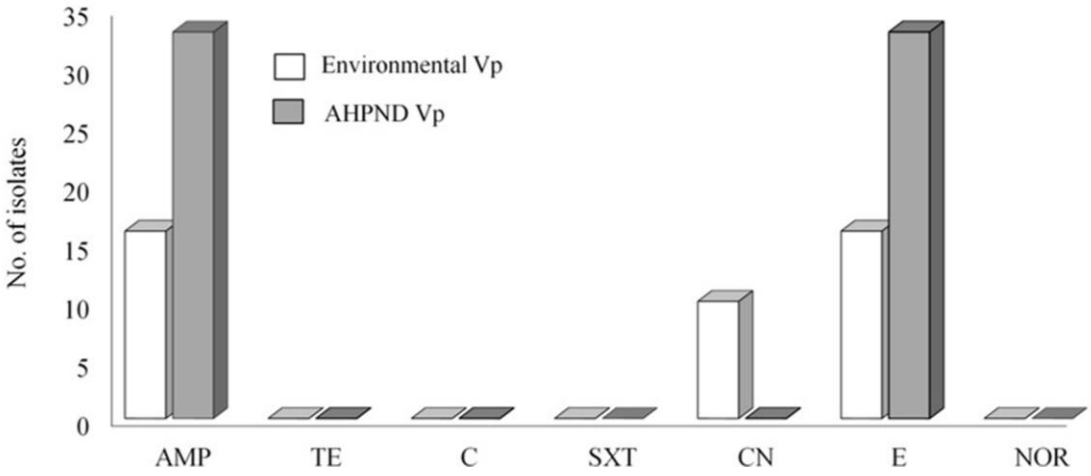

Figure 27 Antibiotic resistances of 33 AHPND *V. parahaemolyticus* isolates (AHPND Vp) and 16 environmental isolates (Control Vp). Amp (ampicillin), TE (tetracycline), C (chloramphenicol), SXT (sulphamethoxazole/trimethoprim), CN (gentamycin), E (erythromycin) and NOR (norfloxacin).

Part IIB. Rapid technique to detect AHPND *V. parahaemolyticus*

Specificity and sensitivity of LAMP assay in pure cultures

In this study, LAMP assay was established and LAMP-A2 and LAMP-A3 primers were designed and were evaluated for specificity and sensitivity. Their specificities were evaluated using 14 *Vibrio* spp., 6 non-*Vibrio* spp. and 33 AHPND *V. parahaemolyticus* isolates (Table 13). Two sets of LAMP primers amplified only the genomic DNA of all AHPND *V. parahaemolyticus* isolates. In contrast, no DNA amplification was observed with other bacteria and 10 environmental *V. parahaemolyticus* isolates (Table 13).

The sensitivity of the LAMP-A3 assay in pure culture was better than that of the LAMP-A2 assay. The detection limit of LAMP-A3 assay was found to be as little as 0.1 cfu/reaction or 53 cfu/ml, whereas the detection limit of LAMP-A2 assay was 1.1 cfu/reaction or 5.3×10^2 cfu/ml) (Table 14). Comparison of the sensitivity of the LAMP assays with PCR, both LAMP-A2 and LAMP-A3 were able to detect 1.1 cfu of AHPND *V. parahaemolyticus* per reaction (or 5.3×10^2 cfu/ml) in contrast to the 10.6 cfu/reaction (or 5.3×10^3 cfu/ml) detection limit using PCR in which only two of three experiments were positive (Table 14).

Sensitivity in spiked shrimp experiment

In spiked shrimp experiments, the sensitivity of AHPND *V. parahaemolyticus* detection was lower than that of pure culture. Both LAMP-A2 and LAMP-A3 assays could detect 8.8×10^2 cfu/reaction or 4.4×10^5 cfu/ml in all triplicate experiments and 88 cfu/reaction or 4.4×10^4 cfu/ml in one out of three experiments using the LAMP-A3 assay (Table 14). In this study, PCR was 10-fold less sensitive for the detection of AHPND *V. parahaemolyticus* in spiked shrimp than LAMP-A3 assay because it could detect 8.8×10^2 cfu/reaction or 4.4×10^5 cfu/ml in two out of three experiments (Table 14).

Table 13 Primer specificity for the detection of AHPND *V. parahaemolyticus* via LAMP assay.

Bacteria	No. of tested isolates	No. of positive identifications	
		LAMP-A2	LAMP-A3
V. parahaemolyticus (environmental isolates)	10	0	0
V. furnissii	1	0	0
V. fluvialis	1	0	0
V. vulnificus	1	0	0
V. mimicus	1	0	0
V. alginolyticus	2	0	0
V. harveyi	2	0	0
V. metschnikovii	1	0	0
V. cholerae	2	0	0
V. campbellii	1	0	0
V. cincinatiensis	1	0	0
V. splendidus	1	0	0
V. gazogenes	1	0	0
V. mytili	1	0	0
AHPND *V. parahaemolyticus*	33	33	33
Grimontia hollisae	1	0	0
Photobacterium damselae	1	0	0
Pseudomonas sp.	1	0	0
Escherichia coli	1	0	0
Plesiomonas sp.	1	0	0
Listeria sp.	1	0	0

Table 14 Sensitivity of the LAMP-A2 and LAMP-A3 primers for the detection of AHPND *V. parahaemolyticus*.

Samples	Assays	No. of isolates	Dilutions				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Pure culture		cfu/ml	5.3×10^4	5.3×10^3	5.3×10^2	53	5.3
		cfu /reaction	106	10.6	1.1	0.1	0.01
	LAMP-A2		+	+	+[a]	-[b]	-
	LAMP-A3		+	+	+	+	-
	PCR		+	±[c] (2/3)	-	-	-
Spiked shrimp		cfu /ml	4.4×10^7	4.4×10^6	4.4×10^5	4.4×10^4	4.4×10^3
experiment		cfu /reaction	8.8×10^4	8.8×10^3	8.8×10^2	88	8.8
	LAMP-A2		+	+	+	-	-
	LAMP-A3		+	+	+	± (1/3)	-
	PCR		+	+	± (2/3)	-	-

[a] Positive for all triplicate experiments

[b] Negative for all triplicate experiments

[c] Variable detection in triplicate experiments (positive identifications/number tested)

Specificity and sensitivity in samples derived from shrimp farms

Comparison of the efficacy of the LAMP-A2, LAMP-A3 and PCR assays using shrimp aquaculture environmental samples including 3 samples of post-larvae and each 7 samples of shrimp, sediment and water. All positive samples were confirmed by *tlh* gene sequencing after the LAMP amplification product was amplified using primers B3 and F3 of LAMP-A2 and LAMP-A3. All post-larvae samples were negative for AHPND *V. parahaemolyticus* with both LAMP assays and PCR, whereas two out of seven water samples were positive for AHPND *V. parahaemolyticus* for both the LAMP assay and PCR (Table 15). The LAMP-A3 assay is better than the LAMP-A2 assay and PCR in detection of this organism in shrimp samples because no false positive was detected. However, using LAMP-A2, one shrimp sample collected from pond number 10/5 was AHPND *V. parahaemolyticus* positive, but its product could not be amplified with the B3 and F3 primers of LAMP-A2 (although the LAMP assay targeting the *tlh* gene was positive) (Table 15). Using the LAMP-A3 assay, we found that all of the sediments collected from every shrimp pond tested were positive for AHPND *V. parahaemolyticus*. All of the LAMP amplification products were confirmed by DNA sequencing. Thus, the presence of AHPND *V. parahaemolyticus* in all sediment samples indicated that the organisms already exist in the shrimp farm environment.

The sensitivity and specificity of the LAMP assays for the detection of AHPND *V. parahaemolyticus* were compared to PCR. A total of 24 samples were analyzed, 10 and 13 samples were positive by LAMP-A2 and LAMP-A3, respectively, whereas only four samples were positive by PCR (Table 16). Therefore, sensitivity of both LAMP assays was 100 %. Conventional PCR indicated 20 negative results, while LAMP-A2 and LAMP-A3 revealed six and nine positive results, respectively. LAMP-A2 and PCR shared up to 14 matched negative results indicating higher specificity (70%) whereas, LAMP-A3 and PCR shared only 11 matched negative results thus indicating lower specificity (55%) (Table16).

Table 15 Comparison of LAMP and PCR for the detection of AHPND *V. parahaemolyticus* in shrimp culture.

Pond number	Post-larva			Shrimp			Sediment			Water		
	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR
2/2	-	-	-	-	-	-	-	+	-	-	-	-
3/1	-	-	-	-	-	-	+	+	-	-	-	-
3/2	ND[a]	ND	ND	+	+	-	+	+	-	-	-	-
4/1	ND	ND	ND	+	+	+	+	+	-	-	-	-
7/5	ND	ND	ND	+	+	+	+	+	-	+	+	+
10/5	-	-	-	-[b]	-	-	+	+	-	-	-	-
11/4	ND	ND	ND	-	+	-	-	+	-	+	+	+
Total	0	0	0	3	4	2	5	7	0	2	2	2

[a] Not determined

[b] False positive detected but the product could not be amplified with F3 and B3 primers of LAMP-A2

Table 16 Specificity and sensitivity of LAMP compared to PCR for the detection of AHPND *V. parahaemolyticus* in shrimp culture.

Tests		PCR			Specificity (%)	Sensitivity (%)
		Positive	Negative	Total		
LAMP-A2	Positive	4	6	10	70	100
	Negative	0	14	14		
	Total	4	20	24		
LAMP-A3	Positive	4	9	13	55	100
	Negative	0	11	11		
	Total	4	20	24		

Part III. Isolation and identification of *Bdellovibrio* and like organisms (BALOs) against AHPND *V. parahaemolyticus*

Isolation and identification of BALOs

A total of 25 samples including 13 water and 12 sediment samples were collected from Yor Island and shrimp farms. Nine isolates of BALOs were obtained from the water (3 isolates) and from the sediment (6 isolates) samples (Table 17). Confirmation by 16S rRNA sequencing revealed that all of them were *Bacteriovorax* spp. with 89 to 100% homology to the reference strains in GenBank. A comparison of the phylogenetic tree analysis with 11 *Bacteriovorax* spp. reference strains was illustrated in Figure 28. *Bacteriovorax* NBV2-5 were 89 to 99% similar in sequences to the NE1, DA5, DD1, and NB2 reference strains. *Bacterivorax* MBV 5-6 showed 100% homology to the RM2T4-S, RM3S1-S, RM2B2-S, RL1T4-S, RM3T4-S, and RM3S3-S reference strains, whereas *Bacterivorax* BV-A was 91% homologous to those reference strains. *Bacterivorax* MBV 4 and NBV1 displayed around 94 to 100% similarity to BV-A and the rest of the *Bacteriovorax* obtained in this study (Table 17 and Figure 28).

Table 17 Isolation of *Bdellovibrio* and like organisms (BALOs) from environmental samples and shrimp farms.

Source	Sample type	No. of positive isolates	Isolates code	NCBI Description	Homology (%)
Yor island	Water	3	MBV4	*Bacteriovorax* sp. B3S2-S	94
			MBV5	*Bacteriovorax* sp. RM3T4-S	100
			MBV6	*Bacteriovorax* sp. RM3T4-S	100
	Sediment	2	NBV1	*Bacteriovorax* sp. NE1	100
			NBV2	*Bacteriovorax* sp. NE1	89
Trang farm	Sediment	1	BV-A	*Bacteriovorax* sp. DA5	91
Samutsongkram farm	Sediment	2	NBV3	*Bacteriovorax* sp. NE1	99
			NBV4	*Bacteriovorax* sp. NE1	99
Songkhla farm	Sediment	1	NBV5	*Bacteriovorax* sp. NE1	98

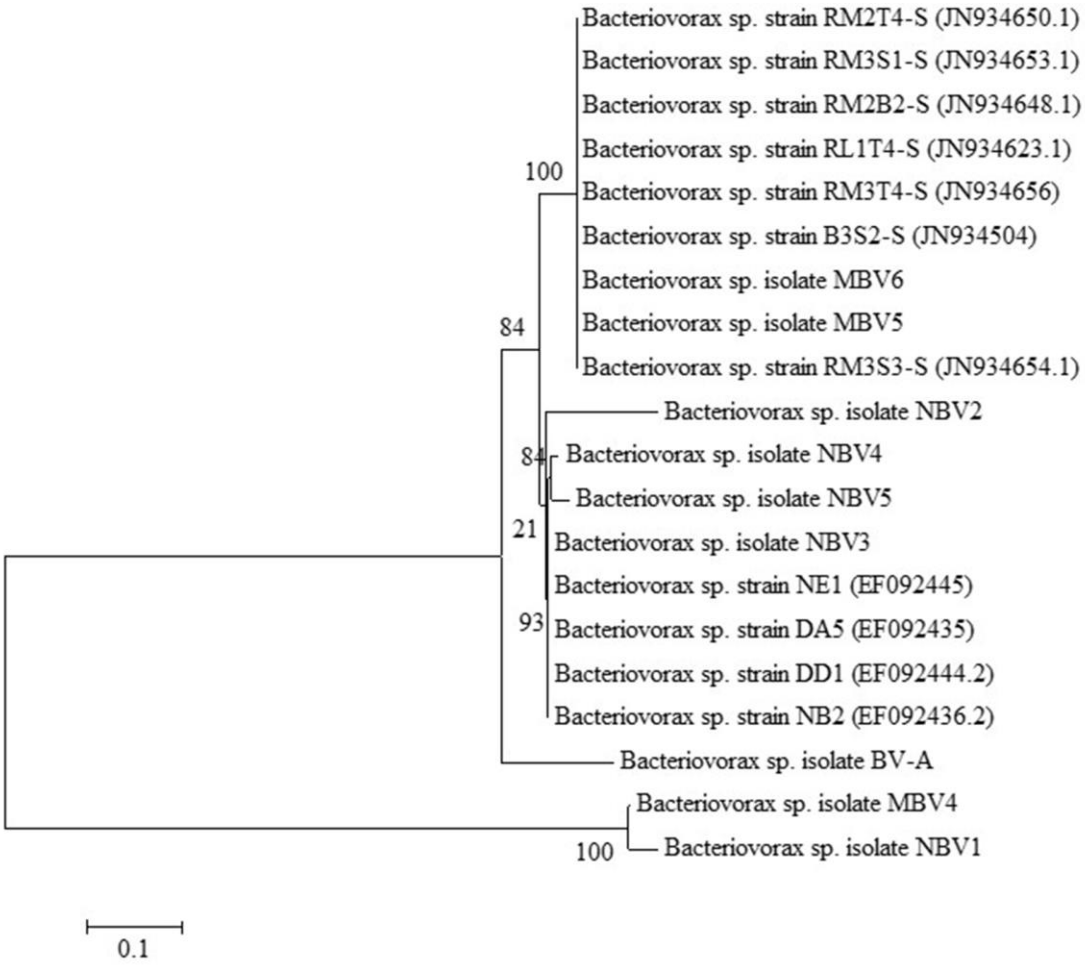

Figure 28 Phylogenetic relationship between *Bacteriovorax* isolates obtained in this study and 11 reference strains. The tree was constracted using neighbor-joining method based on 16S rRNA sequences. Numbers at branch-points represent confidence values obtained after bootstrap analysis based on 1000 replicates. The scale bar represents 0.1 substitutions per nucleotide position.

Susceptibility of Gram-positive and Gram-negative bacteria to BALOs

The ability of 9 isolates of *Bacteriovorax* spp. to lyse Gram-positive and Gram-negative bacteria, including *Vibrio* spp., was evaluated. Two isolates of *Bacteriovorax* spp. could attack *Staphylococcus aureus*, and 4 isolates could predate *E. coli*, whereas 5 to 6 isolates possessed the ability to lyse *Vibrio vulnificus*, *V. cholerae*, and *V. alginolyticus* (Table 18). All of them could lyse all 5 AHPND *V. parahaemolyticus* tested isolates, however, only 2 to 5 isolates of *Bacteriovorax* spp. could attack clinical and environmental *V. parahaemolyticus* (Table 18).

Table 18 Susceptibility of other bacteria and *Vibrio* spp. to *Bacteriovorax* spp.

Bacteriovorax isolates	Hosts													
	Other bacteria		*Vibrio* spp.			AHPND Vp					Vp			
	S. aureus	*E. coli*	Vv	Vc	Va	EMS_1S_2	VP12	7.2L3	PeP_{16}	6.1L3	Clinical		Environmental	
											PSU 5666	PSU 5668	PSU 5147	PSU 5150
BV-A	+3[a]	+3	+3	+3	+3	+3	+3	+3	+3	3	+3	-	-	+3
MBV4	-[b]	-	+2	-	-	+3	+3	+3	+3	+3	+3	-	+3	+3
MBV5	+3	-	-	+2	+2	+3	+3	+3	+3	+3	-	-	-	-
MBV6	-	-	-	-	-	+3	+3	+3	+3	+3	-	-	-	-
NBV1	-	-	+2	-	+3	+3	+3	+3	+3	+3	-	-	-	-
NBV2	-	+2	+3	+3	+3	+3	+3	+3	+3	+3	+3	-	-	-
NBV3	-	+3	+3	+3	+2	+3	+3	+3	+3	+3	+3	+3	+3	+1
NBV4	-	+3	-	+2	+3	+3	+3	+3	+3	+3	+3	-	-	-
NBV5	-	-	-	+3	-	+3	+3	+3	+3	+3	-	+1	-	-
Total	2	4	5	6	6	9	9	9	9	9	5	2	2	3

[a] The numbers indicated positive out of three experiments

[b] All three experiments were negative

Vv, *Vibrio vulnificus*; Vc, *V. cholerae*; Va, *V. alginolyticus*; Vp, *V. parahaemolyticus*; AHPND Vp, AHPND *V. parahaemolyticus*

Factors involved in interaction between a BALO strain and AHPND *V. parahaemolyticus*

To investigate the optimal ratio for the interaction of BALOs with AHPND *V. parahaemolyticus*, *Bacteriovorax* BV-A was selected because it was isolated from a shrimp farm that was less severely affected by AHPND. In addition, it was able to attack many bacterial strains (Table 18). BV-A was incubated with AHPND *V. parahaemolyticus* PSU5429 at a ratio between 1:10 and 1:10000. Although the numbers of AHPND *V. parahaemolyticus* continuously decreased in every interaction ratio after 1 day of incubation, the highest reduction in numbers of AHPND *V. parahaemolyticus* (91.1%) was detected at a ratio of 1:10 (Figure 29). Thus, this ratio was used for interaction evaluation between these organisms throughout this study.

Conditions involved in interaction between *Bacteriovorax* BV-A and AHPND *V. parahaemolyticus* were determined using co-culture at different temperature, NaCl concentration and pH. Reduction in numbers of AHPND *V. parahaemolyticus* in co-culture between BV-A and AHPND *V. parahaemolyticus* in the whole period of time was greater at 30°C than at 25°C or 37°C (Figure 30A). In addition, 90.8 to 95.3% reduction in numbers of AHPND *V. parahaemolyticus* was detected in the co-culture at NaCl concentrations between 1 and 3% (Figure 30B). No significant difference in the reduction of AHPND *V. parahaemolyticus* isolate was observed after co-culture with BV-A at pH between 7 and 8 (Figure 30C). In Thailand, the level of salinity in shrimp farms is between 1 and 3% (Flaherty *et al*., 2000), and the ambient temperature is 30°C. Therefore, 2% NaCl and 30°C were applied for subsequent co-culture of *Bacteriovorax* and AHPND *V. parahaemolyticus* in DNB at pH 7.6. This pH was selected because the typical pH of seawater was between 7.5 and 8.4, and the appropriate pH of water in shrimp ponds should be maintained between 7.5 and 8.5 (Anh *et al*., 2010).

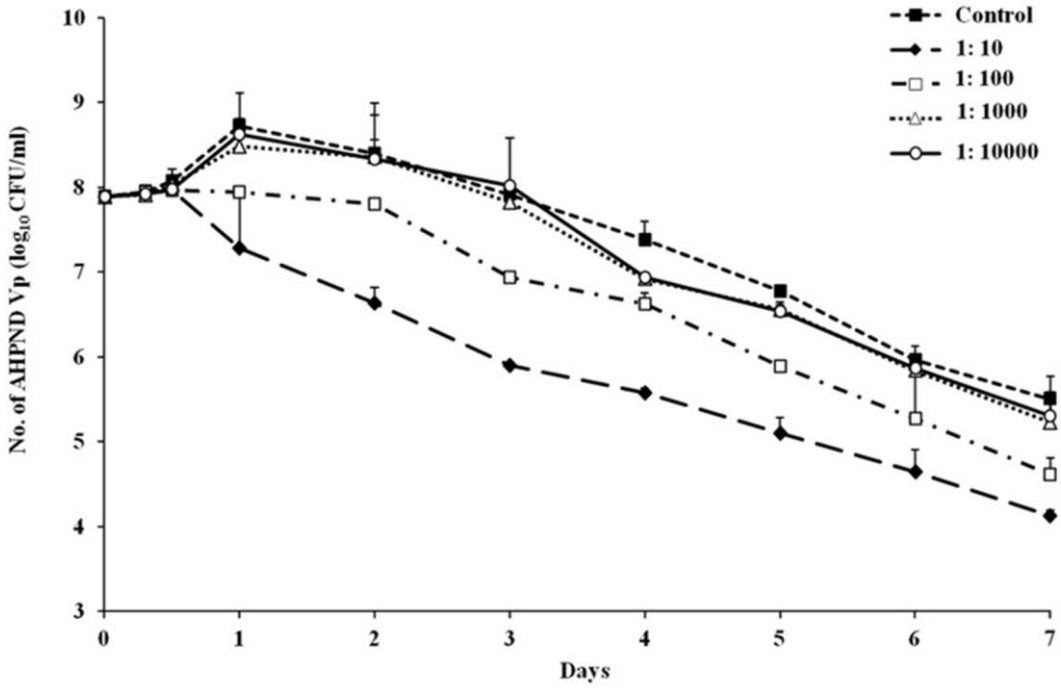

Figure 29 Determination of the optimal ratio for interaction between *Bacteriovorax* BV-A and AHPND *V. parahaemolyticus*. BV-A was incubated with AHPND *V. parahaemolyticus* at ratios between 1:10 and 1:10000 for 7 days, and the numbers of AHPND *V. parahaemolyticus* were enumerated daily. The control contained only AHPND *V. parahaemolyticus*. Values are mean ± SD of 2 experiments; each experiment was performed in triplicate.

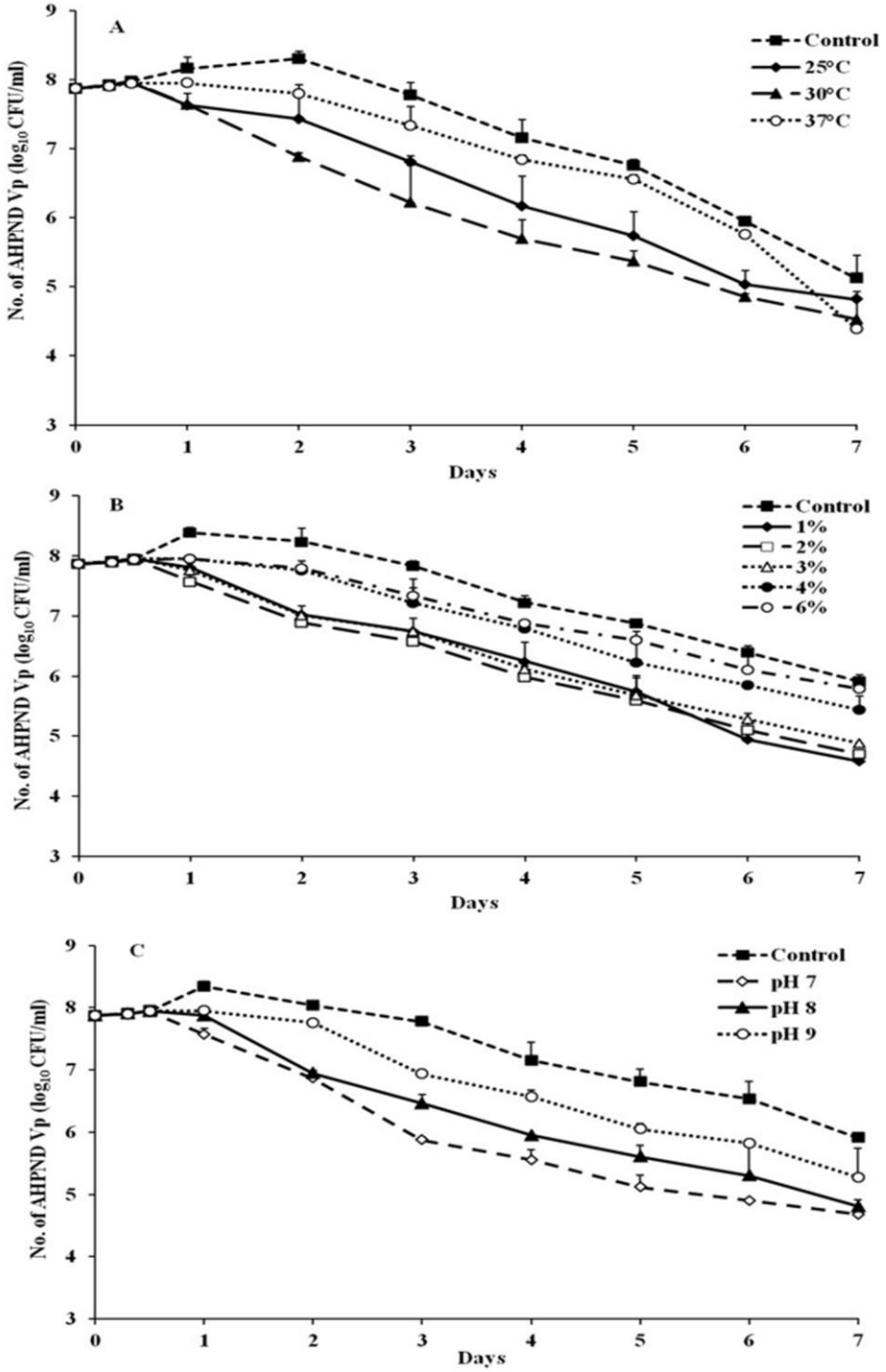

Figure 30 Conditions involved in interaction between *Bacteriovorax* BV-A and AHPND *V. parahaemolyticus*. Co-culture of BV-A and AHPND *V. parahaemolyticus* was performed at 25°C, 30°C and 37°C (A), in salinity between 1and 6% (B) and in

different pH of 7 to 9 (C). General shrimp pond conditions, 30°C, 2% salinity and pH 7.6 were applied for AHPND *V. parahaemolyticus* control.

In vitro interaction between a BALO strain and AHPND *V. parahaemolyticus*

One milliliter each of BV-A (10^6 pfu/ml) and AHPND *V. parahaemolyticus* PSU5429 (10^7 cfu/ml) were mixed in 100 ml of DNB containing 2% NaCl, pH 7.6, and incubated at 30°C. After 2 days of incubation, the numbers of AHPND *V. parahaemolyticus* were significantly lower than in the AHPND *V. parahaemolyticus* control and continuously decreased over the following days (Figure 31). However, the numbers of BV-A significantly increased after 3 days of incubation and continued until Day 5 of incubation, before decreasing until Day 7. In the AHPND *V. parahaemolyticus* control group, the bacterial numbers significantly increased during Day 1 and 3 of incubation and subsequently decreased from Days 5 to 7, whereas a slight decrease in numbers of the BVA control was detected on Day 1 and carried on to Day 7.

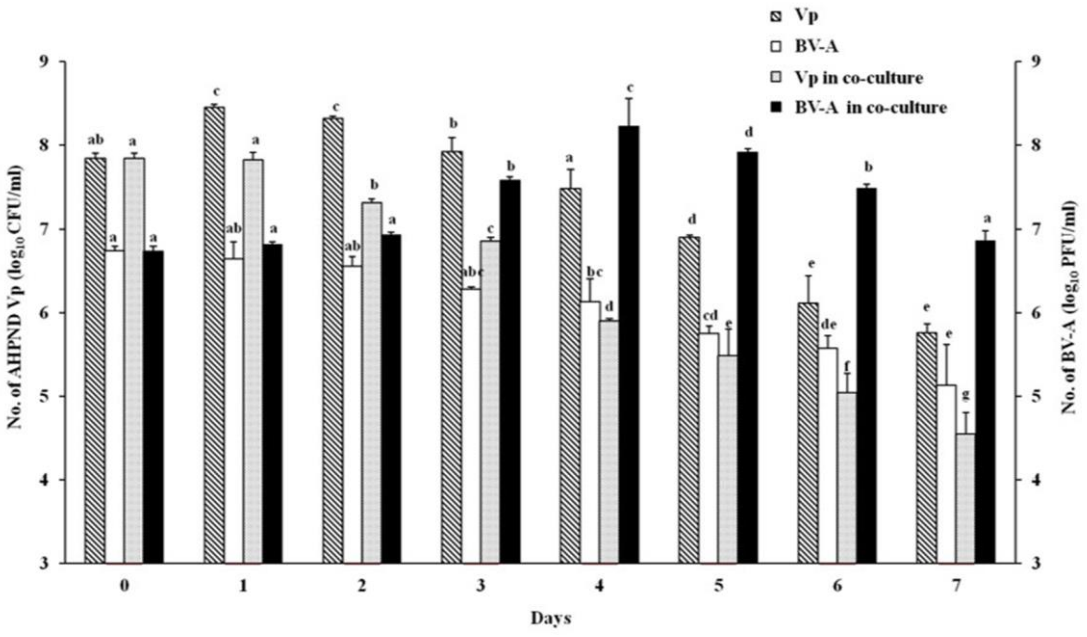

Figure 31 Potential of *Bacteriovorax* BV-A to inhibit AHPND *V. parahaemolyticus in vitro*. BV-A was co-cultured with AHPND *V. parahaemolyticus* for 7 days, and each organism was enumerated daily and compared to the BV-A and AHPND *V. parahaemolyticus* controls. Values are mean ±

SD of two experiments. Different letters above bars show values that are significantly different ($p < 0.05$). Each value is compared within each category.

***In vivo* reduction of shrimp larval mortality by BV-A**

Shrimp post-larvae (PL24) were inoculated with AHPND *V. parahaemolyticus* PSU 5429 at a final concentration of 10^7 cfu/ml, and various final concentrations of BV-A between 10^2 and 10^6 pfu/ml were added into the shrimp tanks. The mortality of the post-larvae was determined daily for 7 days. On Day 1, approximately 7.5, 2.5, 2.5, and 0% of PL24 were dead in the AHPND *V. parahaemolyticus* control and in the infected post-larval groups treated with 10^2, 10^4, and 10^6 pfu/ml of BV-A, respectively. The numbers of dead post-larvae in those groups increased to 50.0, 30.0, 20.0, and 17.5% on Day 3 (Figure 32). At the end of treatment, more than 90% of post-larvae died in the AHPND *V. parahaemolyticus* control, whereas in the infected groups containing BV-A at final concentrations of 10^2, 10^4, and 10^6 pfu/ml, mortality was 72.5, 62.5, and 47.5%, respectively. No post-larvae died in the post-larvae and BV-A control groups.

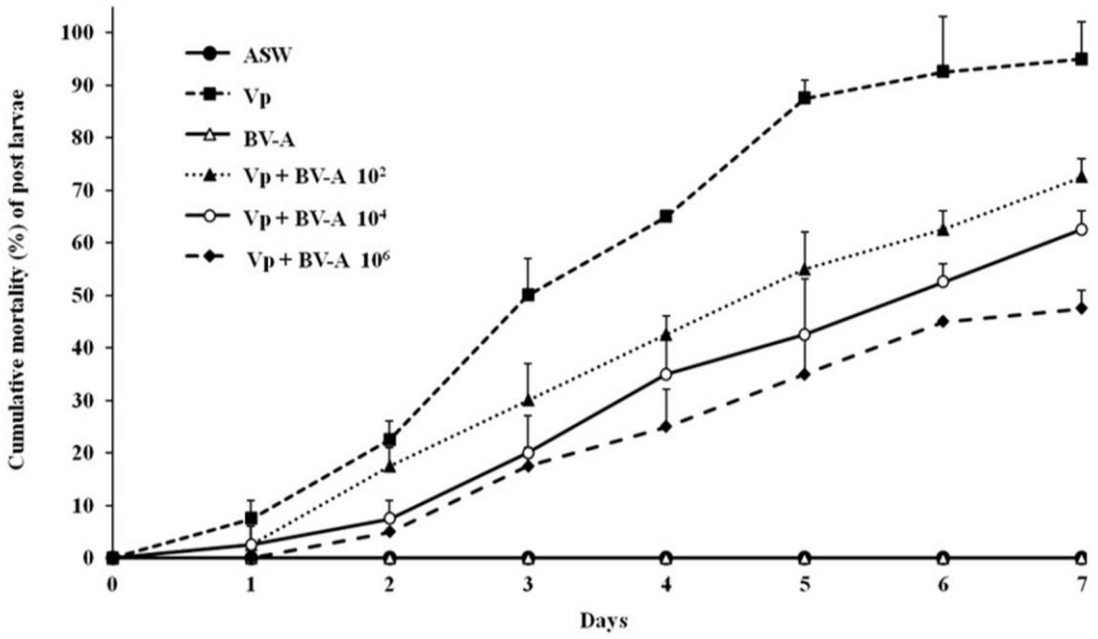

Figure 32 Efficacy of *Bacteriovorax* BV-A to decrease shrimp post-larval mortality after treatment with AHPND *V. parahaemolyticus*. *L. vannamei* post-larvae (PL24) were exposed to AHPND *V. parahaemolyticus* and subsequently treated with

Bacteriovorax BV-A at concentrations of 10^2, 10^4, and 10^6 pfu/ml. Dead post-larvae were counted daily. PL24 treated with artificial seawater (ASW), AHPND *V. parahaemolyticus* (10^7 cfu/ml), or BV-A (10^6 pfu/ml) alone were used as controls.

Reducing *V. parahaemolyticus* biofilm formation by BV-A

The effect of BV-A on biofilm formation of AHPND *V. parahaemolyticus*, clinical and environmental *V. parahaemolyticus* isolates were investigated using 96-well microtiter plates. Biofilm formation of all 3 categories of *V. parahaemolyticus* was significantly reduced by BV-A (Figure 33).

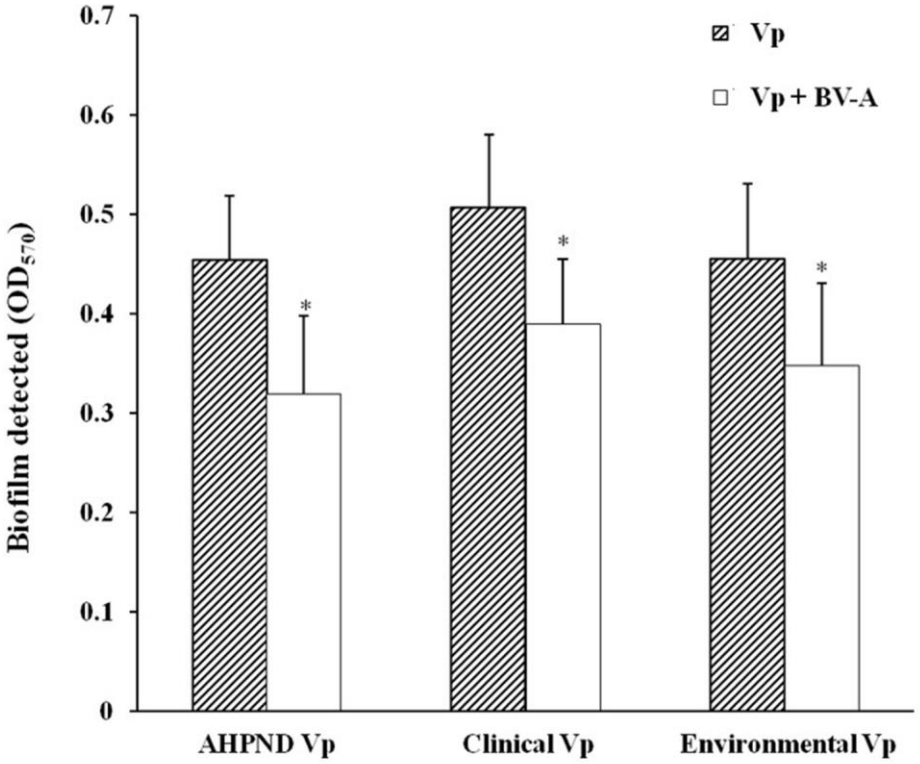

Figure 33 Capability of *Bacteriovorax* BV-A to decrease biofilm formation of AHPND *V. parahaemolyticus*, clinical and environmental isolates of *V. parahaemolyticus*. *V. parahaemolyticus* biofilms were preformed in 96-well microtiter plates for 24 h, and BV-A suspension was added after removal of planktonic cells. Biofilm was quantitated using crystal violet staining. * indicates significant differences ($p < 0.05$).

CHAPTER 4

DISSCUSSIONS

Part I Characterization of human pathogenic *V. parahaemolyticus* from clinical samples

In this study, 73 isolates of *V. parahaemolyticus* carrying *trh* gene obtained previously from various countries were characterized. No significant differences were observed in the urease production between *tdh*$^+$ *trh1*$^+$ and *tdh*$^+$ *trh2*$^+$ ($p = 0.063$) and between the *tdh*$^-$ *trh1*$^+$ and *tdh*$^-$ *trh2*$^+$ isolates ($p = 0.788$) (Figure 18). These indicated that urease production among clinical *trh*$^+$ *V. parahaemolyticus* isolates was not associated with either the *trh* or *tdh* genes. Although the presence of gene encoded for urease is associated with the existence of the *trh* gene, acquirement of those genes might occur at different period of times by horizontal gene transfer from different bacteria without any connection.

For haemolysis activity, a total of 15 and 20 *V. parahaemolyticus* isolates carrying only the *trh*1 or *trh*2 gene were determined. The results demonstrated that the haemolytic activity of the isolates carrying only the *trh*1 gene was higher than the *trh*2$^+$ isolates (Figure 19). Previous studies reported the haemolytic activity of *trh*$^+$ *V. parahaemolyticus* strains correlated with the sequence variation in *trh*1 and *trh*2 genes (Kishishita *et al.*, 1992; Okuda and Nishibuchi 1998). In this work, correlation between urease production and haemolytic activities of *trh*$^+$ isolates was evaluated. No significant difference was observed in the urease production and haemolytic activity ratio between the *trh*1$^+$ and *trh*2$^+$ *V. parahaemolyticus* isolates (Figure 20). This indicated the urease was not involved in the human erythrocyte lysis by TRH haemolysin of *trh*$^+$ *V. parahaemolyticus* strains.

Yildiz and Visick (2009) demonstrated that biofilm formation of vibrios was a survival mechanism associated with their pathogenesis and stress tolerance. In this work, the involvement of the virulence genes and biofilm production of 4 categories of *V. parahaemolyticus* (*tdh*$^+$ *trh*1$^+$, *tdh*$^+$ *trh*2$^+$, *tdh*$^-$ *trh*1$^+$, and *tdh*$^-$ *trh*2$^+$) were investigated. *V. parahaemolyticus* in each category displayed high variation of biofilm

formation, and no significant difference in biofilm formation among 4 categories was observed (Figure 21). This implied that biofilm production of *V. parahaemolyticus* was not associated with the virulence genes (*tdh* and *trh* genes). High variability in biofilm formation of 34 strains of *Acinetobacter baumannii* isolated from hospitalized patients was observed without correlation to molecular types and antimicrobial resistance (Wroblewska *et* al. (2008). Ninety-eight strains with the same serotype of Listeria monocytogenes displayed different biofilm formation (Doijad et al., 2015). Additionally, quantitative biofilm assay of Vibrio cholerae isolates in Thailand demonstrated that those isolates possessed a wide range of biofilm production (Preeprem et al., 2014).

The number of CRISPR-positive *trh*$^{+}$ *V. parahaemolyticus* isolates obtained in this study (47.0%) was higher than that of reported from the previous study (33.3 %) (Sun *et al*., 2015). They were 8, 5 and 3 from isolates belonging to *tdh*$^{+}$ *trh*1^{+}, *tdh*$^{+}$ *trh*2^{+}, and *tdh*$^{-}$ *trh*2^{+} groups, respectively (Table 9). It is of interest that CRISPR was mostly detected in the isolates carrying the *trh* gene together with the *tdh* gene. Association of *tdh* and the presence of CRISPR was observed in 97.4 % of *V. parahaemolyticus* isolates (Sun *et al*., 2015). This confirms the role of bacteriophages in horizontal gene transfer of virulence gene among *V. parahaemolyticus*. In this work, all *V. parahaemolyticus* isolates carrying only *trh*1gene were not positive for CRISPR.

A total of 28 CRISPR spacers obtained in this work were classified into 6 different spacer patterns (SP1 to SP6) (Figure 22). CRISPR analysis based on the CRISPR spacer sequences have been utilized for bacterial subtyping such as *Campylobacter jejuni*, *Mycobacterium tuberculosis*, *Salmonella enterica* and *Yersinia pestis* (Shariat and Dudley, 2014; Calleros *et al.*, 2017; Fricke *et al.*, 2011; Pourcel *et al*., 2005). In this work, all 16 CRISPR-positive *trh*$^{+}$ *V. parahaemolyticus* isolates were classified into 5 CRISPR types (CT1 to CT5) based on CRISPR spacer patterns at 75% similarity level (Figure 23). Previous study demonstrated that a combination of CRISPR and virulence genes significantly increased the discriminatory power and could be a useful subtyping method for investigation of *Salmonella* outbreaks (Liu *et al*. 2011). In this work, a combination of CRISPR spacer sequences with virulence genes (*tdh*, *trh*1 and *trh*2 genes) of *V. parahaemolyticus* isolates was investigated. The profiles of 34 *trh*$^{+}$ *V. parahaemolyticus* isolates were organized into 7 clusters (CV1 to CV7) using

CRISPR-virulence typing at 75% similarity level, and the same subtype of the *trh*$^+$ isolates (either *trh*1 or *trh*2) was classified in the same cluster (Figure 24). Identical CRISPR-virulence typing profiles were detected in clusters CV1, CV2 and CV6 (Figure 24). It is postulated that these bacteria might obtain the plasmids or exogenous genetic elements derived from the same origin. In this work, the CRISPR spacers detected in only *trh*1$^+$ isolates was SP4 whereas they were SP2 and SP5 in *trh*2$^+$ isolates which may be used as spacer marker to differentiate *trh*1$^+$ and *trh*2$^+$ *V. parahaemolyticus* isolates. The spacer SP2 and SP4 displayed homology to *V. alginolyticus* plasmids, whereas spacer SP5 showed no sequence homology with any bacteriophages or plasmids (Table 10). It is possible that this spacer is self-targeting spacer that is a form of autoimmunity of bacteria (Stern *et al*., 2010; Shmakov *et al*., 2017). Moreover, self-derived spacers have been detected in some microbial genomes (Stern *et al.*, 2010; Hooton and Connerton, 2014; Hooton *et al*., 2016).

The discriminatory power index (DI) of CRISPR typing alone and CRISPR-virulence typing indicated that combining CRISPR spacers with virulence genes for differentiating the *trh*$^+$ *V. parahaemolyticus* strains was superior to CRISPR typing alone. Although, pulse field gel electrophoresis (PFGE) is a good discriminatory power technique for genotyping of *V. parahaemolyticus*, it is laborious, time consuming and requires complicated instrumentation (Martinez-Urtaza *et al.*, 2004; Kam *et al.*, 2008). In this study, CRISPR-virulence gene typing is based on PCR technique providing high DI (0.90). It is easy to perform and can be a useful method for typing *trh*$^+$ *V. parahaemolyticus*

Part II Characterization of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) and rapid technique to detect *V. parahaemolyticus* causing AHPND in shrimp

Part IIA Characterization of shrimp pathogenic *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND)

A total of 108 *V. parahaemolyticus* isolates were obtained from 11 shrimp ponds of 5 shrimp farms located in Pattani and Songkhla provinces in southern Thailand (Table 11). AHPND *V. parahaemolyticus* isolates were detected in 8 out of 11 shrimp ponds and a total of 33 of the 108 isolates were PCR positive for AHPND *V. parahaemolyticus*. The negative shrimp ponds might be due to low or absence of AHPND *V. parahaemolyticus*. The highest numbers of these bacteria detected (42.9%) were obtained from the shrimp intestine, whereas 24.7% of these bacteria were obtained from hepatopancrease samples (Table 11). It was of interest that all clinical and environmental *V. parahaemolyticus* obtained previously from 2008 to 2014 from the same area were negative for AHPND detection. This indicated that the AHPND *V. parahaemolyticus* may be an emerging strain in this area. This disease was first reported in China and has subsequently spread to many countries in Asia. Thus, spreading of AHPND *V. parahaemolyticus* to Thailand via sea water or some other vehicle needs to be clarified.

Tran and co-worker (2013) indicated that the AHPND affected the shrimp hepatopancreas characterized by tubule epithelial cells sloughing into the hepatopancreas tubule lumens. To determine the virulence factors involved in the pathogenesis of AHPND, in this study, all 33 AHPND *V. parahaemolyticus* isolates were subjected to determine haemolytic activity and virulence genes (Table 12). All the AHPND isolates showed haemolytic activity on PBA but not on SBA which correlated to the work of Chang *et al*. (2000) who demonstrated that the highest percentage of bacteria isolated from the hepatopancreas of prawn (90%) caused haemolysis on PBA. In addition, haemolytic activity on PBA was correlated to the mortality rate of the shrimp (Chang *et al*., 2000; Rattanama *et al*., 2009). Therefore, this haemolytic activity may be one factor involved in shrimp pathogenesis.

All isolates of AHPND *V. parahaemolyticus* obtain in this study were negative for the *tdh* and *trh* genes that are the important virulence genes detected in human pathogenic *V. parahaemolyticus.* They were positive for *vcrD*1 (T3SS1) but were negative for *vcrD*2 (T3SS2) (Table 12). T3SS1 has been detected in all *V. parahaemolyticus* isolates, whereas T3SS2 has been identified only in human pathogenic *V. parahaemolyticus* harboring the *tdh* and/or *trh* genes (Park *et al.*, 2004; Okada *et al*., 2009). The results indicated that the clone of AHPND *V. parahaemolyticus* was different from the human pathogenic *V. parahaemolyticus* clone. In addition, it has not been reported to associate with human disease. *vipA*1 (T6SS1) and *vipA*2 (T6SS2) genes were determined. All AHPND *V. parahaemolyticus* isolates were positive for both the *vipA*1 and *vipA*2 genes, whereas 84.1% and 74.2% of clinical and environmental isolates, respectively, were positive for *vipA*1, and 92.1% and 63.6% of clinical and environmental isolates, respectively, were positive for *vipA*2 (Table 12). T6SS1 and T6SS2 involved the regulated systems for adhesion to host cell (Bingle *et al*., 2008). T6SS1 of *V. parahaemolyticus* contributed to interbacterial competition and was shown to kill several Gram-negative bacteria such as *Escherichia coli, Vibrio cholerae, Yersinia pseudotuberculosis* and *Vibrio natriegens* (Salomon *et al.*, 2013). Thus, two types of T6SS detected in all AHPND *V. parahaemolyticus* isolates indicated their abilities to adhere to host cells as well as to inhibit various other bacteria in the environment.

In this study, serotypes of all 33 AHPND *V. parahaemolyticus* isolates were O1 with different K antigens including KUT, K33 and K68 (Table 11). Most of the isolates were KUT serotype. It was previously demonstrated that most of the isolates of *V. parahaemolyticus* obtained from the environment are KUT (Thongchan *et al.*, 2013). This may be due to the schematic serotyping of *V. parahaemolyticus* has been established from the clinical isolates. In this work, it is postulated that the AHPND isolates might originated from one clone of *V. parahaemolyticus*, and subsequently new serovarients have been developed gradually.

Using PFGE, DNA profiles of all 33 AHPND *V. parahaemolyticus* isolates were organized into groups A1, A2, A3, B and C, whereas the DNA profiles of clinical and environmental *V. parahaemolyticus* isolates were classified into group D and E, respectively (Figure 26 and Table 11). Most of the isolates that originated from

the same pond and possessed the same O1: KUT serotype exhibited similar DNA profiles (Figure 25 and 26). Dendrogram of PFGE patterns indicated that the DNA profile of isolates in the group A1 were slightly different from the isolates in group A2 and the DNA profiles of the isolates in group A1 and A2 were 16% different from the profiles obtained from the isolates in group A3 (Figure 26). In addition, the DNA profiles of the isolates in group B and C were, respectively, 8% and 23% different from the DNA profile of the isolates in group A (Figure 26). However, the DNA profiles of the clinical and environmental isolates were between 84% and 88% different from those of the AHPND *V. parahaemolyticus* isolates (Figure 26). All AHPND *V. parahaemolyticus* isolates seemed to share a similar DNA profile, whereas those from the clinical and environmental isolates appeared to be more diverse (Figure 25). Thus, the causative agent of AHPND might have originated from the same clone, and variant strains may have subsequently developed.

Antibiotics are frequently used in aquaculture, and antibiogram patterns can be used to evaluate different populations of bacteria. In this study, all AHPND and environmental *V. parahaemolyticus* isolates showed identical antibiogram patterns to ampicillin, erythromycin, tetracycline, chloramphenicol, sulphamethoxazole/trimethoprim and norfloxacin (Figure 27). It was of interest that the antibiogram pattern of the AHPND *V. parahaemolyticus* to gentamycin was different from the environmental isolates (Figure 27). Thus, it implies that AHPND *V. parahaemolyticus* isolates are different from the *V. parahaemolyticus* isolates existing in this environment.

Part IIB. Rapid technique to detect *V. parahaemolyticus* causing AHPND

LAMP assay was established to detect AHPND *V. parahaemolyticus*. The specificity of both LAMP-A2 and LAMP-A3 primers were evaluated using 14 *Vibrio* spp., 6 non-*Vibrio* spp. and 33 AHPND *V. parahaemolyticus* isolates. Thirty-three of AHPND *V. parahaemolyticus* isolates were positive by both LAMP-A2 and LAMP-A3 assay, whereas other bacteria were negative (Table 13). The results demonstrated that two sets of LAMP primers had a high specificity for detection of AHPND *V. parahaemolyticus*.

The sensitivity of the LAMP-A3 assay in pure culture was better than that of the LAMP-A2 assay and PCR (Table 14). Both LAMP-A2 and LAMP-A3 were able to detect 1.1 CFU per reaction of AHPND *V. parahaemolyticus* in contrast to the 10.6 CFU per reaction detection limit using PCR and only two of three experiments were positive (Table 14). Previous study demonstrated that the sensitivity of the LAMP assay for direct detection of *V. parahaemolyticus* in pure cultures targeted to the *tlh* gene was 10-fold more sensitive than that of the conventional PCR (Yamazaki *et al.*, 2008).

In spiked shrimp experiments, the sensitivity to detect AHPND *V. parahaemolyticus* was lower because both LAMP-A2 and LAMP-A3 assays could detect 8.8×10^2 CFU per reaction in all triplicate experiments and 8.8 CFU per reaction in one out of three experiments using the LAMP-A3 assay (Table 14). This is not particularly surprising for spiked shrimp experiments as a variety of chemicals and particles in complex matrices (such as shrimp tissue) may interfere with the amplification reaction. However, better sensitivity of LAMP assay (between 0.8 and 21.3 CFU per reaction) have been observed in a previous study that detected human pathogenic *V. parahaemolyticus* harboring the *tdh*, *trh1* and *trh2* genes in spiked shrimp samples (Yamazaki *et al*., 2010). In this study, PCR was ten times less sensitive for the detection of AHPND *V. parahaemolyticus* in spiked shrimp samples (Table 14). The LAMP assay does not require expensive instruments or sophisticated techniques. It is easy to be performed, and the results obtained are more rapid than conventional PCR. In addition, the LAMP results could be interpreted by eye without the requirement of gel electrophoresis for the detection of DNA amplicons.

In this work, both LAMP-A2 and LAMP-A3 assay were compared using samples collected from the shrimp farms. The LAMP-A3 assay is better than the LAMP-A2 and PCR assays in detection of AHPND *V. parahaemolyticus* in shrimp samples because no false positive was detected (Table 15). All post-larvae samples were negative for AHPND *V. parahaemolyticus* with both LAMP assays and PCR. It has previously been demonstrated that in shrimp farming, the numbers of total and human pathogenic *V. parahaemolyticus* are higher in sediment than those collected from overlying water (Yingkajorn *et al.*, 2014). In this study, a total of 7 sediment samples collected from shrimp ponds were positive for AHPND *V. parahaemolyticus* using the LAMP-A3 detection (Table 15). Thus, this indicated that AHPND *V. parahaemolyticus* already exist in the shrimp farm environment. Therefore, an examination of post-larvae, prior to their release into the earthen pond, does not guarantee that shrimp in the pond will not be infected by AHPND *V. parahaemolyticus*. In this case, decontamination of AHPND *V. parahaemolyticus* in shrimp pond sediment and subsequent confirmation of this decontamination by LAMP assay may be the important activity to reduce the risk of infection.

Part III. Isolation and identification of *Bdellovibrio* and like organisms (BALOs) against AHPND *V. parahaemolyticus*

In the current study, BALOs were evaluated for control AHPND *V. parahaemolyticus*. They were predominantly isolated from the sediment than water samples because 6 out of 12 sediments were positive (50%) whereas they were only detected in 3 out of 13 water samples (23%) (Table 17). 16S rRNA sequencing demonstrated that all of them were *Bacteriovorax* spp., and phylogenetic analysis revealed 100% confidence of BALOS designated as MBV4 and NBV1 (Figure 28). Previously, there had been no evidence that Gram-positive bacteria were susceptible to BALOs; recently, however, *Bdellovibrio bacteriovorus* HD 100 has been demonstrated to decrease numbers of *Staphylococcus aureus* in broth culture (Iebba *et al.*, 2014). In the present study, only 2 out of 9 isolates of *Bacteriovorax* spp. (BV-A and MBV-5) were able to attack *S. aureus* (Table 18). All *Bacteriovorax* spp. obtained in this study could lyse all AHPND *V. parahaemolyticus* tested isolates, whereas their capabilities to attack clinical and environmental *V. parahaemolyticus* were lower. This may be due to most of the *Bacteriovorax* spp. were isolated from AHPND affected shrimp ponds, and AHPND *V. parahaemolyticus* PSU5429 was used as a host for their propagation. In this work, although MBV-5 and MBV-6 showed 100% homology to 7 *Bacteriovorax* reference strains (Figure 28), their bacteriolytic activities toward prey bacteria were slightly different (Table 18). The information of 16S rRNA sequencing might not be enough to differentiate some BALO strains. The gene encoding the β-subunit of RNA polymerase (*rpoB*) is more discriminating than the 16S rRNA gene for differentiation of saltwater members of the genus *Bacteriovorax* (Piñeiro *et al.*, 2008). Additionally, the mechanism of BALOs to select their prey is not clearly understood, and variability in prey range between 2 closely related BALO strains has been documented (Chanyi *et al*., 2013).

The optimal ratio for interaction between *Bacteriovorax* BV-A and AHPND *V. parahaemolyticus* was evaluated. In this work, AHPND *V. parahaemolyticus* PSU 5429 at a concentration of 10^7 cfu/ml was selected for the assay because investigation of AHPND in a shrimp farm in Mexico revealed that different virulence of *V. parahaemolyticus* depended on bacterial density, and the minimum infective density was 10^4 cfu/ml (Soto-Rodriguez *et al.*, 2015). In addition,

no shrimp mortality was observed at density below this value. In our study, the reduction of AHPND *V. parahaemolyticus* isolates was highest at a ratio of 1:10 of BV-A and AHPND *V. parahaemolyticus* (Figure 29). Thus, this ratio was used for the consecutive assays.

To evaluate the capability of *Bacteriovorax* BV-A to decrease numbers of AHPND *V. parahaemolyticus in vitro,* co-culture of those organisms was conducted. At the end of incubation (Day 7), the numbers of both control organisms decreased (Figure 31). This might be due to the depletion of nutrients in DNB during the growth of AHPND *V. parahaemolyticus* approached the decline phase and the reduction of BV-A was due to the lack of prey (AHPND *V. parahaemolyticus*). In co-culture, the numbers of AHPND *V. parahaemolyticus* decreased significantly more than that of the control group, indicating the potential of BV-A to eliminate this bacterium. This was clearly detected on Day 2 of incubation, and the numbers of BV-A started increasing in the subsequent days. On Day 5, the numbers of BV-A in co-culture started decreasing, and this continued until the end of the incubation period, suggesting that this reduction might be due to the decrease in numbers of prey (Figure 31).

To evaluate the effectiveness of BALOs in the prevention of AHPND *V. parahaemolyticus* infection in shrimp, a different concentration of BV-A was applied to AHPND *V. parahaemolyticus*-infected PL24. Post-larval mortality was decreased after treatment with the high concentration of BV-A (Figure 32). This indicates the protective efficacy of BALO for post-larval shrimp. However, in this study, the mortality of post-larvae was reduced by approximately 50% with a 1:10 ratio of BV-A to AHPND *V. parahaemolyticus*. Therefore, to improve the effectiveness of the treatment, a higher concentration of BALO is suggested.

In the current work, we demonstrated the potential of BV-A to significantly diminish biofilm formed by AHPND *V. parahaemolyticus* and clinical and environmental *V. parahaemolyticus* isolates (Figure 33). *V. parahaemolyticus* can form biofilms on shrimp surfaces (Han *et al.*, 2016). Investigation of 35 AHPND *V. parahaemolyticus* isolates derived from shrimp farms in Mexico revealed that all of them were able to form moderate to strong biofilms (López-Leónl *et al.*, 2016). In shrimp farming, the molting process of shrimp causes an accumulation of bacterial biofilms at the bottom of the pond. Iebba *et al.*, (2014) reported that BALOs could

target their prey both in water and in biofilm. Therefore, this evidence supports the advantage of using *Bacteriovorax* as a biocontrol in shrimp aquaculture.

CHAPTER 5

CONCLUSIONS

A total of 73 isolates of *trh*$^{+}$ *V. parahaemolyticus* obtained from clinical samples were characterized. There was no significant difference in the urease production between the *tdh*$^{+}$ *trh1*$^{+}$ and *tdh*$^{+}$ *trh2*$^{+}$ and between the *tdh*$^{-}$ *trh1*$^{+}$ and *tdh*$^{-}$ *trh2*$^{+}$ isolates indicating that the *tdh* and *trh* genes were not involved in urease production in the *trh*$^{+}$ *V. parahaemolyticus* isolates. The haemolytic activity of *trh*1^{+} isolates was higher than the *trh*2^{+} isolates. Variation in biofilm production was detected in the isolates belonging to the *tdh*$^{+}$ *trh*1^{+}, *tdh*$^{+}$ *trh*2^{+}, *tdh*$^{-}$ *trh*1^{+}, and *tdh*$^{-}$ *trh*2^{+} groups. For genotyping, combination of CRISPR spacers and virulence genes provide high discriminatory power than that of CRISPR typing alone and it was able to distinguish between *trh*1^{+} and *trh*2^{+}*V. parahaemolyticus* isolates. Thus, CRISPR-virulence gene typing can be a useful method for typing *trh*$^{+}$ *V. parahaemolyticus*.

In this study, a total of 33 isolates of *V. parahaemolyticus* causing AHPND were obtained from five shrimp farms. All of them possessed the same O1 serotype but different K antigens. The most prevalent K serotype was KUT. DNA profiles of *V. parahaemolyticus* AHPND isolates were similar but different from the profiles obtained from clinical and environmental *V. parahaemolyticus*. It is postulated that the causative agent of AHPND may be originated from one clone and variant strains may be subsequently developed. In this work, the LAMP-A3 assay developed was superior to the LAMP-A2 assay and conventional PCR for detection of AHPND *V. parahaemolyticus*. The LAMP-A3 assay is easy to be performed, rapid and interpretable. It could be a practical tool for on-site investigation of AHPND *V. parahaemolyticus* in shrimp aquaculture.

The present study, the potential of BALOs to control AHPND *V. parahaemolyticus* in shrimp farms was investigated. One genus of BALOs (*Bacteriovorax*) was predominantly isolated from water and sediment samples. The optimal ratio of *Bacteriovorax* BV-A to interact with AHPND *V. parahaemolyticus* was 1:10. *In vitro*, BV-A decreased the numbers of AHPND *V. parahaemolyticus* within 2

days of the co-culture, and *in vivo*, it reduced around 50% mortality of post-larvae infected with AHPND *V. parahaemolyticus*. This indicates the preventive efficiency of BALOs to control AHPND *V. parahaemolyticus* in shrimp aquaculture.

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APPENDIX A

Media preparation

1. Alkaline peptone water (APW)

Composition of media in g/liter		
Peptone from meat	10	g
Sodium chloride	10	g
dH_2O	1,000	ml

Suspend peptone and sodium chloride in 800 ml of dH_2O. Bring to boil to dissolve the medium completely. Adjust pH to 8.6 and add dH_2O to make 1 liter. Sterilize by autoclaving at 121°C for 15 min.

2. CHROMagar Vibrio (Microbiology CHROMagar, France)

Composition of media in g/liter		
Peptone and yeast extract	8	g
Salt	51.4	g
Chromogenic mixture	0.3	g
Agar	15	g
dH_2O	1,000	ml

Suspend 74.7 g/liter in dH_2O. Bring to boil while stirring regularly until complete fusion of the agar (do not autoclave). Cool in a water bath to 45-50°C, stirring gently, mix well and pour into sterile plates (pH 9.0±0.2 at 25°C).

3. Diluted nutrient agar (DNAg)

Composition of media in g/liter		
Beef extract	0.3	g
Peptone	0.5	g
Yeast extract	0.1	g
Sodium chloride	10	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$CaCl_2 \cdot 2H_2O$	0.25	g

Casamino acid	0.3	g
Agar	15	g
dH_2O	1,000	ml

Suspend all ingredients in dH_2O, heat while stirring and adjust pH to 7.6. Sterile by autoclaving at 121°C for 15 min.

4. Diluted nutrient broth (DNB)

Composition of media in g/liter		
Beef extract	0.3	g
Peptone	0.5	g
Yeast extract	0.1	g
Sodium chloride	10	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$CaCl_2 \cdot 2H_2O$	0.25	g
Casamino acid	0.3	g
dH_2O	1,000	ml

Suspend all ingredients in dH_2O, heat while stirring and adjust pH to 7.6. Sterile by autoclaving at 121°C for 15 min.

5. Luria-Bertani agar (LB agar) (Difco, USA)

Composition of media in g/liter		
Tryptone	10	g
Yeast Extract	5	g
Sodium Chloride	10	g
Agar	15	g
dH_2O	1,000	ml

Suspend 35 g/ liter in dH_2O. Bring to boil to dissolve the medium completely and sterile by autoclaving at 121°C for 15 min (pH 7.0±0.2 at 25°C).

6. Luria-Bertani broth (LB broth) (Difco, USA)

Composition of media in g/liter		
Tryptone	10	g
Yeast extract	5	g
Sodium chloride	10	g
dH_2O	1,000	ml

Suspend 25 g/ liter in dH_2O. Bring to boil to dissolve the medium completely and sterile by autoclaving at 121°C for 15 min (pH 7.0±0.2 at 25°C).

7. Mueller Hinton agar (Difco, USA)

Composition of media in g/liter		
Beef extract powder	2	g
Acid digest of casein	17.5	g
Starch	1.5	g
Agar	17	g
dH_2O	1,000	ml

Suspend 38 g/ liter in dH_2O. Bring to boil to dissolve the medium completely and sterile by autoclaving at 121°C for 15 min (pH 7.3±0.1 at 25°C).

8. Mueller Hinton broth (Difco, USA)

Composition of media in g/liter		
Beef extract powder	2	g
Acid digest of casein	17.5	g
Starch	1.5	g
dH_2O	1,000	ml

Suspend 21 g/ liter in dH_2O. Bring to boil to dissolve the medium completely and sterile by autoclaving at 121°C for 15 min (pH 7.3±0.1 at 25°C).

9. Prawn blood agar (PBA)

Composition of media in g/liter

Basal medium

Bacto peptone	10	g
Sodium chloride	10	g
Agar	15	g
dH_2O	1,000	ml

Suspend all ingredients in dH_2O. Bring to boil to dissolve the medium completely and adjust pH to 6.8. Dispense the medium to screw-cap tubes (15 ml of medium per tube) and sterile by autoclaving at 121°C for 15 min. Cool in a water bath to 45-50°C.

Stained shrimp hemolymph

Shrimp hemolymph	1	ml
Citrate-EDTA buffer	0.2	ml
Rose bengal in citrate-EDTA buffer (3% w/v)	0.1	ml

Mix shrimp hemolymph and citrate-EDTA buffer in sterile tube and dispense 0.9 ml of hemolymph in buffer into 0.1 ml rose bengal in citrate-EDTA buffer. Mix thoroughly.

Prawn blood agar

Stained shrimp hemolymph	1	ml
Basal medium	15	ml

Add aseptically the stained shrimp hemolymph into basal medium, mix well and pour into sterile plate.

10. Sheep blood agar (SBA) (Merck, USA)

Composition of media in g/liter

Blood agar base

Heart extract	10	g
Peptone	10	g
Sodium chloride	5	g

Agar	15	g
dH_2O	1,000	ml
Defibrinated sheep blood	50	ml

Suspend 40 g/ litre in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121°C for 15 min. Cool to 45-50°C, add defibrinated sheep blood and mix well. Pour the medium into sterile plates (pH 6.8±0.2 at 25°C).

11. Soft diluted nutrient agar (soft DNAg-0.6% agar)

Composition of media in g/liter

Beef extract	0.3	g
Peptone	0.5	g
Yeast extract	0.1	g
Sodium chloride	10	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$CaCl_2 \cdot 2H_2O$	0.25	g
Casamino acid	0.3	g
Agar	6	g
dH_2O	1,000	ml

Suspend all ingredients in dH_2O. Heat and bring to boil while stirring regularly until complete fusion of the agar and adjust pH to 7.6. Sterile by autoclaving at 121°C for 15 min.

12. Trypticas soy agar (TSA) (Difco, USA)

Composition of media in g/liter

Pancreatic digest of casein	15	g
Papaic digest of soybean	5	g
Sodium chloride	5	g
Agar	15	g

Suspend 40 g/ litre in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121°C for 15 min (pH 7.3±0.2 at 25°C).

13. Urea slant agar

Composition of media in g/liter		
Bacto peptone	1	g
Dextrose	1	g
Sodium chloride	5	g
Na_2HPO_4	1.2	g
KH_2PO_4	0.8	g
Phenol red	0.012	g
Agar	15	g
dH_2O	950	ml
Urea (40% w/v)	50	ml

Suspend all ingredients in 950 ml dH_2O. Bring to boil to dissolve the medium completely and adjust pH to 6.8. Sterilize by autoclaving at 121°C for 15 min. Cool in a water bath to 45-50°C and aseptically add the sterile urea (40% w/v). Mix thoroughly and dispense into sterile tubes. Place tubes in a slanted position.

APPENDIX B

Reagent preparation

1. Cell lysis buffer for PFGE method

[50 mM Tris-HCl, 50 mM EDTA, 1% N-sodium lauroyl sarcosine]

1 M Tris-HCl pH 8.0	50	ml
0.5 M EDTA	100	ml
N-sodium lauroyl sarcosine	100	g
dH_2O	750	ml

Mix all ingredients in dH_2O and keep at 4°C.

2. Cell suspension buffer for PFGE method

[100 mM Tris-HCl, 100 mM EDTA, 20 mM NaCl]

1 M Tris-HCl pH 8.0	10	ml
0.5 M EDTA	20	ml
1 M NaCl	2	ml
dH_2O	68	ml

Mix all ingredients in dH_2O and keep at 4°C.

3. Citrate-EDTA buffer

[0.1 M Glucose, 30 mM trisodium citrate, 26 mM citric acid, 10mM EDTA, 2% (w/v) NaCl]

Glucose	1.8	g
Trisodium citrate	0.77	g
Citric acid	0.5	g
EDTA	0.29	g
Sodium chloride	2	g
dH_2O	100	ml

Mix all ingredients in dH_2O and adjust pH to 4.6. Sterile by autoclaving at 121°C for 15 min. Store at 4°C.

4. Crystal violet, 0.4%

Mix 0.4 g of crystal violet with 100 ml of dH_2O. Store at room temperature in the dark.

5. Ethanol: acetone

Mix 80 ml of ethanol and 20 ml of acetone (80:20). Store at room temperature in the dark.

6. Ethidium bromide, 10 mg/ml

Mix 1 g of ethidium bromide with 100 ml of dH_2O. Stir overnight. Store at room temperature in the dark.

7. Gel loading buffer, 6x

[0.25% (w/v) bromophenol blue and 40% (w/v) sucrose]

Weigh out 0.025 g of bromophenol blue and 4 g of sucrose. Make up volume to 10 ml with dH_2O. Store at 4°C.

8. Glutaraldehyde, 2.5% (v/v)

Mix 5 ml of 50% glutaraldehyde and 95 ml of dH_2O. Mix well and store at room temperature in the dark.

9. HEPES buffer, 50 mM pH 7.5

Suspend 2.38 g of HEPES powder in 100 ml of dH_2O to make HEPES buffer stock solution (0.1 M) and adjust pH to 7.5 with NaOH pellets. Sterile by filtration using 0.2 μm membrane filter. Mix an equal volume of 0.1 M HEPES buffer stock solution and sterile dH_2O and mix thoroughly. Store at 4°C.

10. Phosphate-buffered saline (PBS), 0.01 M pH 7.0

$NaH_2PO_4•H_2O$	27.6	g
$NaH_2PO_4•2H_2O$	31.2	g
$Na_2HPO_4•7H_2O$	53.65	g
$Na_2HPO_4•12H_2O$	71.63	g
dH_2O	1,000	ml

Mix all ingredients in dH_2O and keep at room temperature.

11. Reagent C for urease assay

Reagent A [2% (w/v) Na_2CO_3 in 0.1 N NaOH]

Na_2CO_3	20	g
0.1 N NaOH	1,000	ml

Dissolve 20 g of Na_2CO_3 in 1,000 ml of 0.1 N NaOH. Mix well and keep at 4°C.

Reagent B [0.5% (w/v) $CuSO_4•5H_2O$ in 1% (w/v) aqueous solution of sodium tartrate]

$CuSO_4•5H_2O$	0.5	g
$Na_2C_4H_4O_6•2H_2O$	1	g
dH_2O	100	ml

Dissolve 1g of $Na_2C_4H_4O_6•2H_2O$ in 100 ml of dH_2O to make 1% (w/v) aqueous solution of sodium tartrate. Dissolve 0.5 g of $CuSO_4•5H_2O$ in 100 ml of 1% (w/v) aqueous solution of sodium tartrate. Mix well and keep at 4°C.

Reagent C

Just before use, mix 50 ml of reagent A and 1 ml of reagent B. Discard after 1 day.

12. Reagent D for urease assay

Folin-Ciocalteu reagent [1 N Folin-Ciocalteu reagent]

1.8 N Folin reagent	16.7	ml
dH_2O	13.3	ml

Mix all ingredients in dH_2O and keep at 4°C in the dark.

13. Sodium acetate, 3M pH 5.2

Mix 4.08 g of sodium acetate trihydrate with 8 ml of dH_2O. Adjust pH to 5.2 with glacial acetic acid and add dH_2O to 10 ml. Sterile by autoclaving at 121°C for 15 min. Store at room temperature.

14. Solution A for urease assay

[1% Phenol, 170 mM sodium nitropusside]

Phenol	1	g
Sodium nitropusside	5	mg
dH_2O	100	ml

Mix all ingredients in dH_2O and keep at 4°C in the dark.

15. Solution B for urease assay

[125 mM NaOH, 0.05% sodium hypochlorite]

NaOH	2.5	g
8.5% NaOCl	0.6	ml
dH_2O	99.4	ml

Dissolve NaOH in 50 ml of dH_2O. Add NaOCl and add dH_2O to 100 ml. Mix well and keep at 4°C in the dark.

16. Tris-borate-EDTA (TBE) buffer, 10x

Tris base	54	g
Boric acid	27.5	g
$Na_2EDTA•2H_2O$	3.72	g
dH_2O	1,000	ml

Mix all ingredients in dH_2O. Bring to boil to dissolve completely and sterilize by autoclaving at 121°C for 15 min.

17. Tris-EDTA (TE) buffer

[10 mM Tris-HCl, 1 mM EDTA]

1 M Tris-HCl pH 8.0	10	ml
0.5 EDTA	2	ml
dH_2O	988	ml

Mix all ingredients in dH_2O. Bring to boil to dissolve completely and sterilize by autoclaving at 121°C for 15 min.

18. Urea in HEPES buffer, 25 mM

Suspend 0.6 g of urea in 100 ml of 50 mM HEPES buffer to make urea stock solution (0.1 M). Sterile by filtration using 0.2 μm membrane filter. Mix 25 ml of 0.1 M urea stock solution and 75 ml of sterile 50 mM HEPES and mix thoroughly. Store at 4°C.

APPENDIX C

1. Blood agarose plate

Kanagawa phenomenon (KP) hemolysin buffer

[0.01 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 1.5 mM NaN_3, 1.07% agarose]

1 M Tris-HCl pH 8.0	10	ml
Sodium chloride	9	g
$CaCl_2$	0.6	g
NaN_3	0.1	g
Agarose	10.7	g
dH_2O	990	ml

Suspend sodium chloride, $CaCl_2$ and agarose in 800 ml of dH_2O. Bring to boil to dissolve completely and add 10 ml of 1 M Tris-HCl (pH 8.0.) Adjust pH to 7.3 and add dH_2O to make 1 liter. Add NaN_3 and sterilize by autoclaving at 121°C for 15 min.

Human red blood cell suspension

Add 25 ml of 0.01 M PBS (pH 7.0) in 5 ml of human blood with heparin sodium. Mix gently. Centrifuge at 100 × *g* for 10 min at 4°C and remove supernatant. Repeat blood washing steps two more times and resuspend washed red blood cells in 0.01 M PBS (pH 7.0) to original volume (5 ml) of the blood.

Blood agarose plate

Mix melted based agarose agar with 50, 100 and 200 µl of washed blood to prepare blood agarose plate (in total volume of 20 ml) at low (0.25%), medium (0.5%) and high (1%) concentration of blood, respectively. Make wells using a cork borer, which was flamed (6.5 mm diameter).

2. Purification of PCR reaction for sequencing using ethanol/ sodium acetate precipitation

1.) Add 5 µl of 3 M sodium acetate to tube containing 45 µl of PCR product.
2.) Add 125 µl of 100% ethanol (stored at -20°C) to each tube and mix by inverting.
3.) Incubate at -20°C for 20 min.
4.) Centrifuge the tube at 2,500 × *g* for 30 min at 4°C.
5.) Invert the tube to remove ethanol, then centrifuge at 2,500 × *g* for 1 min to remove excess ethanol.
6.) Dry the pellet for 30 min to 1 h at room temperature (30°C) and resuspend the pellet in 20 µl of sterile MilliQ dH_2O.

3. DNA sequencing method

Preparation of cycle sequencing reaction

1.) Mix 2 µl of purified PCR product with 8 µl of BigDye terminator v3.1 ready reaction mix, 2 µl of forward primer (3.2 µM) and deionized water in a total volume of 20 µl.
2.) Seal the plate with MicroAmp™ clear adhesive film.
3.) Vortex the plate for 2- 3 s and then centrifuge briefly to collect contents to the bottom of the wells at 1,000 x *g* for 5-10 s.
4.) Place the plate in a thermal cycler to perform cycle sequencing

Purification of the sequencing product using FastGene dye terminator removal kit

1.) Swell the resin in buffer DT by vortexing for 30 min or overnight at room temperature.
2.) Apply 750 µl of hydrated resin into the column and centrifuge at 750 x *g* for 3 min.
3.) Transfer the column into a new tube and load 20 µl of sequencing product gently onto the center of the gel.

4.) Centrifuge at 750 x *g* for 3 min to obtain the purified sequencing product.

Capillary electrophoresis

1.) Mix 5 µl of purified sequencing product with 5 µl of Hi-Di formamide in the plate.
2.) Do not heat the sample and run the sample as soon as possible after mixing.
3.) Load the plate onto the ABI3130 capillary sequencer using a 36 cm capillary filled with performanceoptimized polymer 7 (POP7).

Standard calibration

1.) Add 170 µl of Hi-Di formamide to resuspend the BigDye terminator v3.1 sequencing standard (pGEM-3Zf(+) double-stranded DNA control template).
2.) Vortex thoroughly and centrifuge the mixture.
3.) Separate 50 µl of DNA standard in PCR tube to denature by heating at 95°C for 5 min and 4°C for 2 min or until used.
4.) Add 10 µl of denatured standard in the well to perform capillary electrophoresis.

4. Pulsed-field gel electrophoresis (PFGE) method

1.) Bacterial cells are grown on TSA supplemented with 1% NaCl at 30 or 37°C.
2.) Pick colonies and resuspend in 0.5 ml of cell suspension buffer (100 mM Tris-HCl, 100 mM EDTA and 20 mM NaCl, pH 8.0)
3.) Add 500 µl of 1.2% low-melting agarose (LMA) in a buffer (50°C), mix by pipetting.
4.) Add 20 µl of proteinase K (20 mg/ml) and mix by pipetting.
5.) Charge into disposable plug mold, harden the agarose at room temperature (can place it at 4°C).

6.) Push the gel and put it into 50 ml tube containing 5 ml of cell lysis buffer (50 mM Tris-HCl, 50 mM EDTA and 1% N-sodium lauroyl sarcosine) with 25 µl of proteinase K (20 mg/ml).
7.) Incubate in water bath at 55°C for 2 h until the gel become transparency.
8.) Wash each plug two times (15 min each) with 5 ml of distilled water.
9.) Wash each three times (15 min each) with 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) (Plug can be stored at 4°C for few weeks).
10.) Cut a small piece (5 x 3 mm) and equilibrate each plug with 100 µl of 1X enzyme buffer at 37°C for 1 h.
11.) Place each plug in 100 µl of 1X *Not*I buffer containing 10 U of *Not*I restriction enzyme and incubate at 37°C for 2 h.
12.) Make 100 ml of 1% agarose gel in 0.5x TBE buffer.
13.) Put a small piece of plugs and marker onto the comb, fill up two drops of 1% agarose between the top of plugs and comb and leave for 5 min to solidify.
14.) Place comb side down, then overlay with 1% agarose gel in 0.5x TBE buffer and rest for 20 min.
15.) Run PFGE power pack at 14°C, 70 rpm flow rate, plus time 3.2-54.2s linear, 6 V/cm, 120° angle in CHEF-DRIII system for 19 h.
16.) Stain the gel in ethidium bromide for 5 min and destain for 30 min.
17.) Observe under UV light with a UV transilluminator.

APPENDIX D

List of Publications

Paper I

Kongrueng, J., Yingkajorn, M., Bunpa, S., Sermwittayawong, N., Singkhamanan, K.and Vuddhakul, V. 2015. Characterization of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in southern Thailand. *Journal of Fish Diseases* 38(11):957-966.

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Characterization of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in southern Thailand

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Abstract

Vibrio parahaemolyticus was isolated from shrimp of five farms located in the Pattani and Songkhla provinces of southern Thailand. Using a PCR method targeted to the unique DNA sequences derived from the plasmid (AP2 primers) and the toxin gene (AP3 primers) of *V. parahaemolyticus* that caused acute hepatopancreatic necrosis disease (AHPND), a total of 33 of 108 isolates were positive. In contrast, all 63 and 66 isolates of clinical and environmental *V. parahaemolyticus,* respectively, obtained previously from 2008 to 2014 from the same area were negative. This implied that these strains were likely to be the cause of the outbreak of AHPND in this area. Intestinal samples proved to be a better source for the isolation of *V. parahaemolyticus* AHPND than the hepatopancreas. All isolates were investigated for haemolytic activity, virulence genes, serotypes, genotypes and antibiotic susceptibility. All the AHPND isolates had a unique O antigen, but small variations of the K antigens were detected from different farms. In addition, the DNA profiles of *V. parahaemolyticus* AHPND isolates were similar, but distinct from those clinical and environmental isolates. It is postulated that the causative agent of AHPND might have originated from one clone and then slightly different serotypes subsequently developed.

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Keywords: acute hepatopancreatic necrosis disease syndrome, serotype, shrimp, type III secretion system, type VI secretion system, *Vibrio parahaemolyticus*.

Introduction

Acute hepatopancreatic necrosis disease syndrome (AHPND), formally known as shrimp early mortality syndrome, has recently caused serious problems in the shrimp culture industry. It most frequently occurs within the first 30 days after stocking a newly prepared shrimp pond. It was first reported in China in 2009 and then spread to Vietnam, Malaysia and Thailand (Network of Aquaculture Centres in Asia-Pacific 2012). AHPND affects black tiger shrimp, *Penaeus monodon* (Fabricius) and whiteleg shrimp, *Litopenaeus vannamei* (Boone). Infected shrimp show lethargy, slow growth, an empty stomach and midgut and a pale to white atrophied hepatopancreas (Tran *et al.* 2013). The mass mortality can exceed 70%. Thailand is one of the world's leading exporters of shrimp supplying mostly to the United States, Japan and Europe. AHPND reduces shrimp production and causes a huge economic loss for the shrimp industry. It has been estimated that the quantity and financial worth of exports of Thai shrimp to Europe decreased by 38 and 34%, respectively, in the first 5 months (January–May) of 2013 due to an outbreak of AHPND (http://www.fisheries.go.th/ems/).

Journal of Fish Diseases

The aetiological agent of AHPND has been demonstrated to be *Vibrio parahaemolyticus* (Tran *et al.* 2013). Using Koch's postulates, shrimp challenged with this bacterium isolated from AHPND-positive shrimp induced shrimp mortality with typical AHPND pathology. *Vibrio parahaemolyticus* is a halophilic Gram-negative bacterium that is a normal inhabitant of marine environments. Most of the *V. parahaemolyticus* isolated from the marine environments are non-pathogenic strains. A few human pathogenic strains are detected in the environments, but they are mostly isolated from clinical samples and harbour a thermostable direct haemolysin (TDH) or the TDH-related haemolysin (TRH) encoded by the *tdh* or *trh* genes, respectively (Vuddhakul *et al.* 2000). However, any association of these genes with the *V. parahaemolyticus* isolates causing AHPND has not been detected.

Although the virulence factor(s) of *V. parahaemolyticus* that cause AHPND (*V. parahaemolyticus* AHPND) is not clearly understood, haemolysins from many *Vibrio* spp. are recognized as virulence factors, for example: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. anguillarum* and *V. mimicus* (Zhang & Austin 2005). In addition, some haemolysin genes can be detected in most pathogenic strains (Miyamoto *et al.* 1969; Honda, Ni & Miwatani 1988). The most pathogenic *V. harveyi* (VIB645) exhibited haemolytic activity to fish erythrocytes and possessed two copies of the haemolysin gene (*vhhA/vhhB*) (Zhang, Meaden & Austin 2001). A purified VHH protein was cytotoxic to flounder gill cells in tissue culture and had strong pathogenicity to flounders when injected intraperitoneally (Zhong *et al.* 2006).

The type III secretion system (T3SS) has been demonstrated in many pathogenic bacteria (Hueck 1998). T3SS is a complex needle-like structure that enables Gram-negative bacteria to secrete and inject bacterial effector proteins into the cytoplasm of eukaryotic host cells. T3SS in *Vibrio* spp. was first reported in *V. parahaemolyticus* by Park *et al.* (2004). Two sets of genes, T3SS1 and T3SS2, have been demonstrated in *V. parahaemolyticus*. T3SS1 was involved in the cytotoxicity of HeLa cells. In contrast, T3SS2 played an important role in enterotoxicity in a rabbit model. It was of interest that T3SS1 has been found in all *V. parahaemolyticus* strains and in addition has been detected in other vibrios, such as *V. alginolyticus*, *V. harveyi* and *V. tubiashii* (Park *et al.* 2004). However, T3SS2 is only found in the human pathogenic *V. parahaemolyticus* isolates that possessed the *tdh* or *trh* gene (Park *et al.* 2004; Okada *et al.* 2009).

The type VI secretion system (T6SS) is a common Gram-negative bacterial export pathway that can translocate effector proteins into many target cell types. T6SS is a complex bacteriophage-like structure. In many pathogens, T6SS is correlated to the ability of the organisms to induce host diseases. Pathogenicity processes associated with T6SS include adherence, cytotoxicity, host-cell invasion, intracellular growth within macrophages or survival and persistence within the host (Cascales 2008). The T6SS genes have been demonstrated in *V. parahaemolyticus* (Shrivastava & Mande 2008). Two types of T6SS (T6SS1 and T6SS2) have been detected in *V. parahaemolyticus* (Yu *et al.* 2012; Salomon *et al.* 2013).

Information on the virulence factors associated with *V. parahaemolyticus* that cause AHPND is still limited. Hence, investigations of any haemolytic activity and the presence of virulence genes including the bacterial secretion systems (T3SS and T6SS) would be useful to clarify its pathogenicity in shrimp. In addition, serotyping, genotyping and antibiotic susceptibility will be useful in investigations of the epidemiology and origin of this bacterium. Therefore, in this work, *V. parahaemolyticus* isolated from shrimp farms in the southern part of Thailand that had outbreaks of AHPND were characterized.

Material and methods

Isolation and identification of *V. parahaemolyticus* from shrimp farms experiencing an outbreak of AHPND

This study was conducted on shrimp farms in Songkhla and Pattani provinces that were around 100 km apart, in southern Thailand. Shrimp samples were collected from 11 ponds of five different farms that exhibited the AHPND phenomenon by massive death of shrimp 1 month after the release of post-larva to the earth pond. In each pond, 5–7 diseased shrimp that showed specific signs of AHPND including a pale to white hepatopancreas with empty stomach and intestine (Figure S1) were collected, and their intestines and hepatopancreases were dissected. Each organ was treated separately. *V. parahaemolyticus* was isolated using

CHROAMagar Vibrio (CHROMagar Microbiology). Briefly, a loopful of sample was streaked onto the agar, and the plate was incubated overnight at 37 °C. Around five mauve colonies (except isolates from the Songkhla farm) that were most likely to be *V. parahaemolyticus* were selected and confirmed to be *V. parahaemolyticus* using PCR targeted to the *toxR* gene (Kim *et al.* 1999). PCR identification of *V. parahaemolyticus* isolated from the shrimp with AHPND was performed using specific AP2 primers, unique to the DNA sequences derived from the plasmid of this bacterium, details of which were released from the Network of Aquaculture Centres in Asia-Pacific, (http://www.enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf; Joshi *et al.* 2014). In addition, confirmation was obtained using AP3 primers specific to a toxin gene of *V. parahaemolyticus* AHPND, which encoded for toxin that was homologous to bacterial toxins active against insects (Sirikharin *et al.* 2014). Briefly, the tested isolates were grown in Luria-Bertani (LB) broth containing 1% NaCl with shaking (160 rpm) at 37 °C overnight. One millilitre of the broth culture was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water and used as the template for PCR amplification. The reaction was carried out with a mixture consisting of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 0.025 U of *Taq* DNA polymerase and 2.0 μL DNA template in a 20-μL volume. Amplification of the AP2 primers was performed with a single cycle at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, an extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. All the positive isolates were investigated for haemolytic activity and virulence genes. For the AP3 primers, the thermocycle was started with a cycle at 95 °C for 5 min. This was followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 5 min, the thermocycle finished with one cycle at 72 °C for 5 min. The amplification products of AP2 (700 bp) and AP3 (336 bp) were analysed by electrophoresis in a 1% agarose gel.

Bacterial strains

The 63 isolates of clinical *V. parahaemolyticus* obtained from stool cultures during 2008 to 2014 at Hat Yai hospital, Songkhla (10 isolates/year except in 2009), and 66 environmental isolates obtained from shrimp and mollusks collected in Hat Yai city, Songkhla, between 2008 and 2014 (10 isolates/year except in 2011) were included in this study. All of them have been deposited in the culture collection of the Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand (Thongchan *et al.* 2013a; Thongjun *et al.* 2013b).

Determination of haemolytic activity

To investigate any haemolytic activity, the tested isolates were inoculated onto 5% sheep red blood cell agar. After incubation for 18 h, clear zones around the bacterial colonies were evaluated for complete haemolysis. In addition, haemolytic activity was also performed using prawn blood agar (PBA) (Chang, Liu & Shyu 2000). Briefly, 1 mL of citrate-EDTA prawn haemolymph was mixed with Rose Bengal (at a final concentration of 0.3% w/v) and was added to 15 mL of basal medium. Haemolytic activity was detected by the presence of a clear zone around the *V. parahaemolyticus* colonies on prawn blood agar within 7 days of incubation at 30 °C.

Detection of virulence genes

Using the PCR technique, all tested isolates were screened for the *V. parahaemolyticus* haemolysin genes *tdh* and *trh* (Tada *et al.* 1992), T3SS1 *vcr*D1 (Yu *et al.* 2013), T3SS2 *vcr*D2 (Okada *et al.* 2009), T6SS1 *vip*A1 and T6SS2 *vip*A2 (Salomon *et al.* 2013) using the bacterial DNA template that was described above. The PCR mixture (final volume, 20 μL) consisted of 1.5 μL of DNA template, 2 μL of 10× reaction buffer, 1.6 μL of 25 mM $MgCl_2$, 1.6 μL of 2.5 mM dNTPs, 5 μL of 2 μM of each primer mix, 0.1 μL of *Taq* DNA polymerase (5 U μL^{-1}) and 8.2 μL of sterile MilliQ water. The reactions were performed with a GeneAmp PCR System 2400 as follows: 5 min for a hot start at 96 °C, followed by 35 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 55 °C 1 min for *tdh* and *trh* genes, 55 °C 30 s for *vcr*D2, 58 °C 1 min for *vcr*D1 and *vip*A2, 60 °C 1 min for *vip*A1, and extension at 72 °C for 1.5 min and final extension at 72 °C for 7 min.

Electrophoresis was performed on a 1.5% agarose gel, and the amplicons were detected using a UV transilluminator.

Serotype investigation

To determine the somatic (O) and capsular (K) serotypes of *V. parahaemolyticus*, the slide agglutination technique using anti-O and anti-K antibodies (Denka Seiken) was performed following the manufacturer's instructions with a slight modification. Briefly, the tested bacteria were grown in trypticase soy agar containing 3% NaCl at 37 °C for 18 h. For determination of the O serotypes, bacterial cells were washed with a solution containing 3% NaCl and 5% glycerol and the suspension was autoclaved at 121 °C for 1 h. After centrifugation, the cells were resuspended in 3% NaCl. A heavy suspension was subjected to the agglutination test with specific anti-O antibodies. For the K antigen, bacterial cells were washed with a 3% NaCl solution and made into a smooth heavy suspension of cells. The suspension was tested first with pooled K antisera (I-IX). Any positive isolate using the pooled K antisera was then subjected to testing for the monovalent K antisera.

Genotype investigation using pulse field gel electrophoresis (PFGE)

DNA profiles of all *V. parahaemolyticus* AHPND isolates including some environmental and clinical *V. parahaemolyticus* isolates were investigated using the PFGE technique. *Vibrio parahaemolyticus* was grown in LB broth supplemented with 1% NaCl at 37 °C overnight. An agarose plug was prepared by mixing equal volumes of a bacterial suspension with melted agarose. After lysis of the bacteria, DNA was cleaved by the *Not* I restriction enzyme (NEB), and the digested DNA fragments were separated on 1% Pulse-Field Certified agarose gel (Bio-Rad Laboratories) using a 0.5× TBE buffer on a CHEF-DRIII system (Bio-Rad). Electrophoresis was performed at 6 V cm^{-1} with a field angle of 120° at 14 °C. The pulse times were 1–18 s for 36 h. After electrophoresis, the gel was stained with ethidium bromide and DNA was visualized with a UV transilluminator. A dendrogram was constructed using a Bioprofile image analysis system (Viber Lourmat).

Antibiotic sensitivity

Susceptibility to seven antibiotics, ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulphamethoxazole/trimethoprim (25 μg), gentamycin (10 μg), erythromycin (15 μg) and norfloxacin (10 μg), that historically and currently are often used in aquaculture was examined using the disc diffusion method. In brief, the tested isolate was cultured on Luria-Bertani agar supplemented with 1.5% NaCl. Three to four colonies were selected and cultured in Muller Hinton broth containing 1.5% NaCl for 3 h, then, and the turbidity of bacterial suspension was adjusted to be 1×10^8 cfu mL^{-1} using the 0.5 McFarland Standard (CLSI 2010). A bacterial lawn was produced by spreading the suspension on Muller Hinton agar supplemented with 1.5% NaCl, and the antibiotic-loaded paper discs (Oxoid) were dispensed on it. After incubation at 28 °C for 24–28 h, the diameter of the inhibition zone was recorded and interpreted according to the interpretive chart provided by the Performance Standards for Antimicrobial Disk Susceptibility Tests. *Escherichia coli* ATCC 25922 was used as a standard strain. All tests were done in duplicate.

Results and discussion

In this study, four shrimp farms located in Pattani and one in Songkhla province were investigated. All 108 suspected *V. parahaemolyticus* isolates obtained on CHROMagar Vibrio that were positive for the *tox*R gene were subjected to PCR using the specific primer pairs AP2 and AP3. These 2 primer pairs have been demonstrated to target specifically *V. parahaemolyticus* AHPND isolates (Joshi *et al.* 2014; Sirikharin *et al.* 2014). All 63 and 66 clinical and environmental *V. parahaemolyticus* isolates, respectively, obtained during 2008 to 2014 from the same area were also included for comparison. A total of 33 of the 108 isolates were PCR positive for the *V. parahaemolyticus* AHPND (data not shown). Both primers provided identical detections, even though the specificity of AP2 primers for AHPND isolates has been demonstrated to be lower (Sirikharin *et al.* 2014). The highest numbers of these bacteria detected were obtained from the intestine (Table 1). It was of interest that all clinical and environmental *V. parahaemolyticus* obtained from the present year and 6 years previously were

Table 1 Isolation of *Vibrio parahaemolyticus* AHPND from shrimp

No. of farms	Locations	Pond code	No. of isolates from		Serotypes (no. of isolate)	Isolate number	DNA profiles
			hepatopancrease	intestine			
1	Pattani	1	0/3[a]	0/4	–[b]	–	–
		2	0/4	0/4	–	–	–
2	Pattani	3	1/4	0/4	O1:KUT(1)[c]	1	A2
		4	0/4	2/5	O1:KUT(2)	2,3	A2
		5	2/5	1/3	O1:KUT(3)	4,5,6	A1, A2
		6	3/3	3/3	O1:KUT(6)	7,8,9,10,11,12	A2
		7	3/3	2/3	O1:KUT(5)	13,14,15, 16,17	A2
3	Pattani	8	1/5	3/4	O1:K33(4)	18,19,20,21	B
4	Pattani	9	1/5	4/5	O1:KUT (1),O1:K68 (4)	22,23,24,25,26	A2, C
5	Songkhla	10	7/19	0	O1KUT(7)	27,28,29,30,31,32,33	A3
		11	0/18	0	–	–	–
Total (%)			18/73 (24.7)	15/35 (42.9)	–	–	–

[a]no. of selected colonies
[b]not determined
[c]no. of isolate (s)

negative for both the AP2 and AP3 primers (data not shown). This indicated that the *V. parahaemolyticus* AHPND may be an emerging strain in this area. Previously, there has been no report of any pathology similar to AHPND in this area. The disease was first reported in China and has subsequently spread to many countries in Asia. Thus, the spreading of these strains to Thailand via sea water or some other vehicle needs to be resolved.

In this work, *V. parahaemolyticus* AHPND was isolated from 8 of 11 ponds (Table 1). Colonies selected from shrimp samples of the remaining three ponds were negative for *V. parahaemolyticus* AHPND and indicated that those shrimp might not be infected with *V. parahaemolyticus* AHPND or a mild infection might have occurred; therefore, more bacterial isolates would be needed for confirmation.

Acute hepatopancreatic necrosis disease is limited to the hepatopancreas and is described as a severe atrophy of the shrimp hepatopancreas that displays an acute unique histopathology. It is characterized by a massive sloughing of the epithelial cells of the hepatopancreas (Tran *et al.* 2013). To determine the virulence factors involved in the pathogenesis of AHPND, all 33 *V. parahaemolyticus* AHPND isolates were subjected to tests for haemolytic activity and detection of virulence genes. All the AHPND isolates exhibited haemolytic activity on PBA but not on SRBC agar (Table 2). These results correlated with the work of Chang *et al.* (2000) who demonstrated that the highest percentage of bacteria isolated from the hepatopancreas of prawn (90%) caused haemolysis on PBA. In addition, haemolytic activity on PBA was correlated to the mortality rate of the shrimp (Chang *et al.* 2000; Rattanama *et al.* 2009). Therefore, this haemolytic activity may be one factor involved in shrimp pathogenesis, although five environmental isolates of *V. parahaemolyticus* in the control set also displayed a similar activity.

Table 2 Investigation of haemolytic activity and virulence genes of *Vibrio parahaemolyticus* AHPND isolates

Test	AHPND Vp[a]positive/ total	Env. Vp positive/total	Clin. Vp positive/total
Haemolysis on SRBC[b]	0/33	0/5	ND[d]
Haemolysis on PBA[c]	33/33	5/5	ND
tdh	0/33	0/66	50/63
trh	0/33	0/66	5/63
vcrD1(T3SS1)	33/33	66/66	63/63
vcrD2(T3SS2)	0/33	0/66	51/63
vipA1(T6SS1)	33/33	49/66	53/63
vipA2(T6SS2)	33/33	42/66	58/63

[a]*V. parahaemolyticus.*
[b]sheep red blood cell.
[c]prawn blood agar.
[d]not determined.

In this study, all *V. parahaemolyticus* AHPND isolates were negative for the *tdh* and *trh* genes that are the important virulence genes detected in human pathogenic *V. parahaemolyticus* (Table 2). All of them were positive for *vcr*D1 (T3SS1) but were negative for *vcr*D2 (T3SS2). This is not

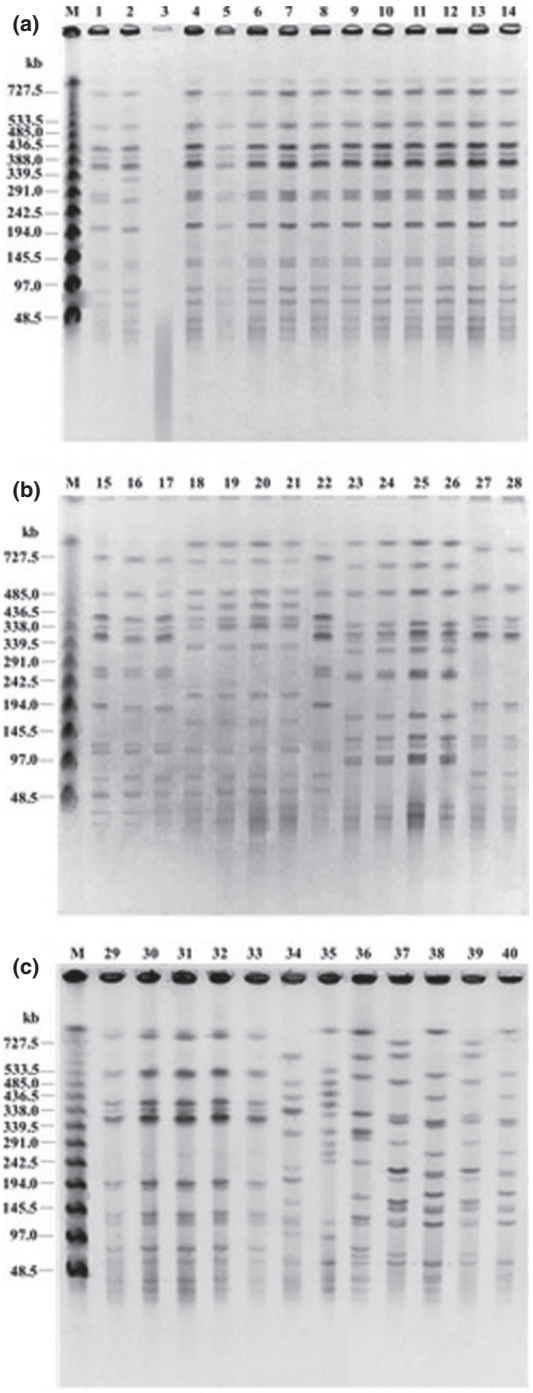

Figure 1 DNA profiles of the isolates from *Vibrio parahaemolyticus* AHPND, the environmental and clinical samples. M = lambda ladder PFG marker (NEB), (a) lanes 1–14, *V. parahaemolyicus* AHPND isolates number 1–14 (b) lanes 15–28, *V. parahaemolyicus* AHPND isolates number 15–28 (c) lanes 29–40, *V. parahaemolyicus* AHPND isolates number 29–33, environmental isolates number 34–35 and clinical isolates number 36–40, each lane is correlated to the isolate number listed in Table 1 NB. DNA of an isolate in pond 4 (lane 3) was degraded.

surprising because T3SS1 has been demonstrated in all *V. parahaemolyticus* isolates, whereas T3SS2 has been identified only in human pathogenic *V. parahaemolyticus* that harbour the *tdh* and/or *trh* genes (Park *et al.* 2004; Okada *et al.* 2009). Detection of those genes in the control sets of *V. parahaemolyticus* from clinical and environment isolates supported this view because all the isolates were positive for T3SS1, but only the isolates that harboured *tdh* or *trh* or both genes were positive for T3SS2 (Table 2). Thus, this confirmed that the clone of *V. parahaemolyticus* AHPND isolates was different from the human pathogenic *V. parahaemolyticus* clone.

The T6SS model is composed of heterodimers exterior sheath of VipA and VipB and interior tube of Hcp hexamers which is capped with a VgrG trimer (Basler *et al.* 2012). To determine whether the *V. parahaemolyticus* AHPND isolates harboured the T6SS1 and T6SS2, detection of the genes that encode for VipA, *vip*A1 (T6SS1) and *vip*A2 (T6SS2) was investigated. All *V. parahaemolyticus* AHPND isolates were positive for both the *vip*A1 and *vip*A2 genes, whereas 49 of the 66 isolates (74.2%) and 53 of the 63 isolates (84.1%) of the environmental and clinical isolates, respectively, were positive for *vip* A1 and 42 of the 66 isolates (63.6%) and 58 of the 63 isolates (92.1%) of the environmental and clinical isolates, respectively, were positive for *vip*A2 (Table 2). Although both T6SS1 and T6SS2 were demonstrated to be involved in adhesion to cell cultures, T6SS1 of *V. parahaemolyticus* contributed to interbacterial competition and was shown to kill several Gram-negative bacteria such as *Escherichia coli*, *V. cholerae*, *Yersinia pseudotuberculosis* and *Vibrio natriegens* (Salomon *et al.* 2013). In this work, the presence of T6SS1 and T3SS2 in all the *V. parahaemolyticus* AHPND isolates indicated their abilities to adhere to host cells as well as to inhibit various other bacteria in the environment. Although T6SS1 has been detected predominantly in clinical isolates, T6SS2 has been detected in all tested strains of *V. parahaemolyticus* (Yu *et al.* 2012); however, in this work, not all *V. parahaemolyticus* isolates in both environmental and clinical samples were positive for T6SS2 (Table 2).

An investigation of the serotypes of all 33 *V. parahaemolyticus* AHPND isolates revealed that

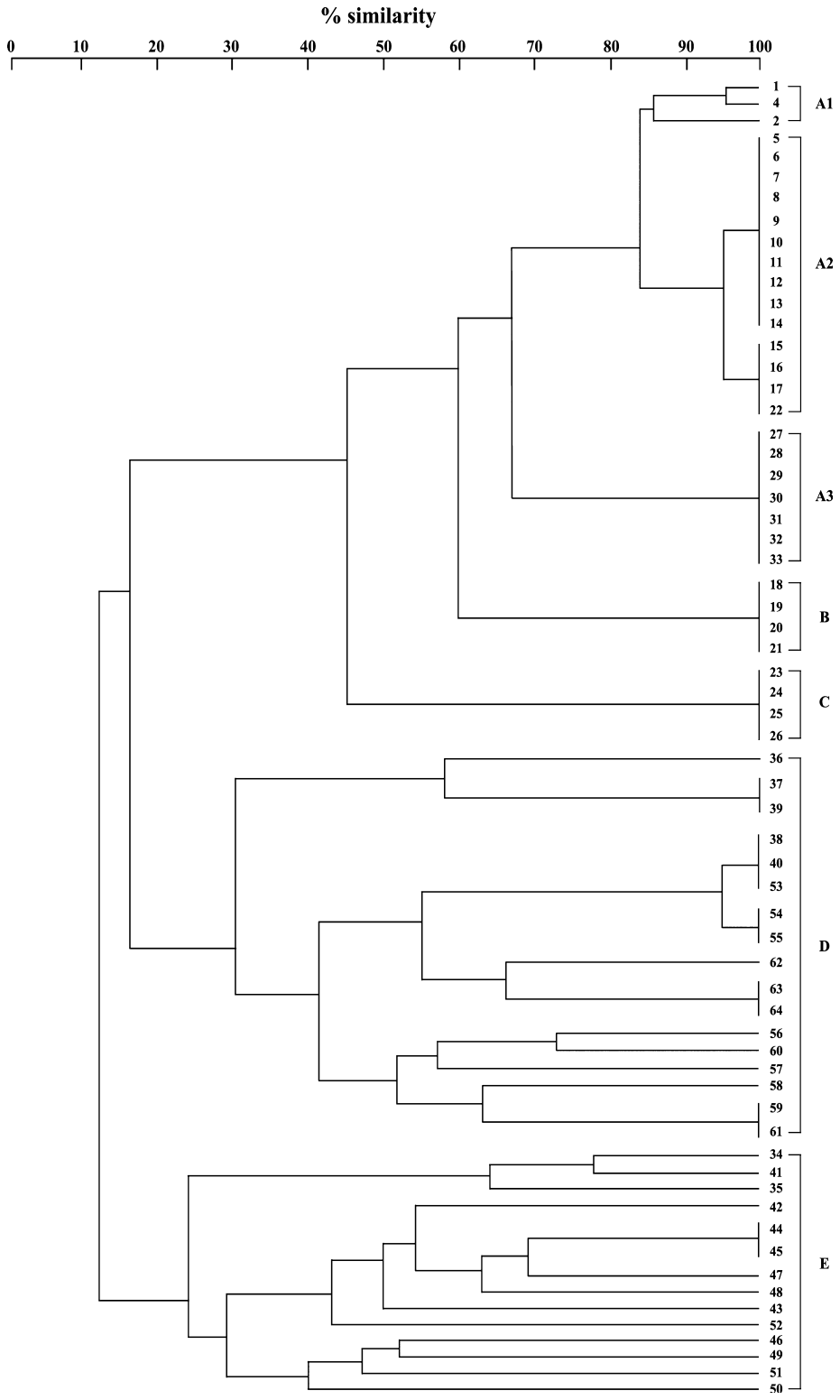

Figure 2 A dendrogram constructed from PFGE. Numbers 1 to 33 are *V. parahaemolyicus* AHPND isolates, numbers 34–35 and 41–52 are environmental isolates, and numbers of 36–40 and 53–64 are clinical isolates, each number is correlated to the isolate number listed in Table 1, Fig.1 & Figure S2. A1 to E indicate the groups of *V. parahaemolyicus*.

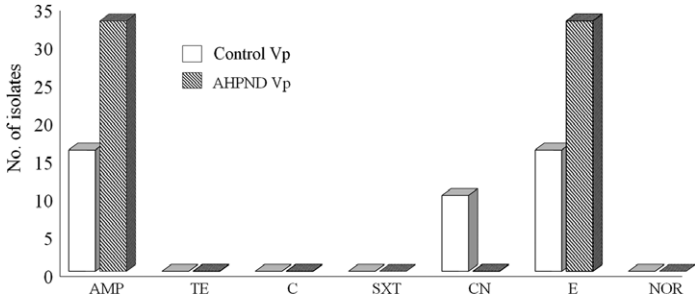

Figure 3 Antibiotic resistances of 33 *Vibrio parahaemolyticus* AHPND isolates (AHPND Vp) and 16 environmental isolates (Control Vp). Amp (ampicillin), TE (tetracycline), C (chloramphenicol), SXT (sulphamethoxazole/trimethoprim), CN (gentamycin), E (erythromycin) and NOR (norfloxacin).

all of them had the O1 serotype but some possessed different K antigens that included KUT (K untypeable), K33 and K68 (Table 1). Most of the isolates were KUT, and identical serotypes were detected with the isolates obtained from the same ponds except pond no. 9 from which both O1:KUT and O1:K68 were detected. It was previously demonstrated that most of the isolates of *V. parahaemolyticus* derived from the environment are KUT (Thongchan *et al.* 2013a). This may be due to the schematic serotyping of *V. parahaemolyticus* that has been established from clinical isolates. In this work, it is postulated that the AHPND isolates might have originated from one clone of *V. parahaemolyticus,* and subsequently, new serovarients developed gradually. In the pandemic of *V. parahaemolyticus* in which O3:K6 was first reported to cause a worldwide disease, serovarients including O4:K68, O1:K25, O1:KUT, O4:K12, etc. isolates were subsequently demonstrated (Nair *et al.* 2007).

DNA profiles of all 33 *V. parahaemolyticus* AHPND isolates including the 17 clinical and 14 environmental *V. parahaemolyticus* isolates were investigated using PFGE technique (Fig. 1). All the DNA profiles obtained from *V. parahaemolyticus* AHPND isolates were organized into groups A1, A2, A3, B and C, whereas the DNA profiles of *V. parahaemolyticus* isolates from the clinical and environmental samples were classified into group D and E, respectively (Fig. 2). All the isolates in group A1 to A3 were O1:KUT serotype, and isolates in group B and C were O1:K33 and O1:K68, respectively (Table 1). Most of the isolates that originated from the same pond and possessed the same O1:KUT serotype exhibited similar DNA profiles (Fig. 1a–c). The DNA profile of isolate no.3 obtained from pond 4 was not included because it was degraded (Fig. 1a, lane 3), although its DNA profile was repeated, suggesting that autolysis might occur in this isolate.

Dendrogram analysis indicated that the similarity of the DNA profile of isolates in the group A2 was 95%, and those isolates were slightly different from the isolates in group A1 (Figs 1 a, b and 2). The DNA profiles of the isolates in both groups (A1 and A2) were 16% different from the profiles obtained from the isolates in group A3 (from Songkhla farm) (Figs 1 b, c and 2). The DNA profiles of isolates in group B that possessed O1:K33 were identical. In addition, the DNA profiles of O1:K68 belonging to the isolates in group C were indistinguishable (Fig. 1b). However, the DNA profiles of the isolates in group B and C were, respectively, 8% and 23% different from the DNA profile of the isolates in group A (A1 to A3) (Fig. 2).

All *V. parahaemolyticus* AHPND isolates seemed to share a similar DNA profile, whereas those from the clinical and environmental isolates appeared to be more diverse (Fig. 1c & Figure S2). The similarity of the DNA profiles of the clinical isolates (group D) was 30%, whereas that from the environmental isolates (group E) was 24% (Fig. 2). However, the DNA profiles of the clinical and environmental isolates were between 84% and 88% different from those of the *V. parahaemolyticus* AHPND isolates (Fig. 2). Thus, the causative agent of AHPND might have originated from the same clone, and variant strains may have subsequently developed. It would be of interest to see whether there is any difference between the strain diversity and pathogenicity. Recently, a few *V. parahaemolyticus* AHPND isolates were obtained from a single Thai shrimp and caused a slightly different histopathology of the hepatopancreas (Joshi *et al.* 2014). Future work is needed to clarify this.

Antibiotics are frequently used in aquaculture, and antibiogram patterns can be used to evaluate different populations of organisms. In this study, the antibiotic susceptibility of the 33 *V. parahaemolyticus* AHPND isolates and 16 environmental

V. parahaemolyticus isolates was evaluated. All *V. parahaemolyticus* AHPND isolates were resistant to ampicillin (Amp) and erythromycin (E), whereas they were susceptible to tetracycline (T), chloramphenicol (C), sulphamethoxazole/trimethoprim (SXT), gentamycin (CN) and norfloxacin (NOR) (Fig. 3). It was of interest that the antibiogram pattern of the *V. parahaemolyticus* AHPND to gentamycin was different from the control isolates; thus, it implies that *V. parahaemolyticus* AHPND isolates are different from the *V. parahaemolyticus* that exist in this environment. Biocontrol should be investigated to limit these AHPND strains.

In conclusion, a total of 33 isolates of *V. parahaemolyticus* causing AHPND were obtained from five shrimp farms. There was no significant difference in the detection of haemolytic activity and virulence genes between those isolates and the *V. parahaemolyticus* control groups, except that all the *V. parahaemolyticus* AHPND isolates were positive for T6SS1 and T6SS2. It was of interest that all of them had the O1 serotype but some possessed different K antigens. The most prevalent K serotype detected was KUT. Most of the DNA profiles of *V. parahaemolyticus* AHPND isolates seemed to have similar characteristics. It is postulated that the causative agent of AHPND might have originated from one clone and variant strains may have subsequently developed. The inability to detect *V. parahaemolyticus* AHPND from clinical and environmental samples from the present and the past 6 years implied that these strains were likely to be the cause of AHPND. To limit their ability to contaminate shrimp cultures, the sources of these isolates need to be determined.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gross signs of AHPND-infected shrimps showing pale hepatopancreas with empty stomach and intestine compared to non-infected shrimps.

Figure S2. DNA profiles of the environmental and clinical isolates of *V. parahaemolyticus*. M = lambda ladder PFG marker (NEB); (d) lanes 42 to 52 are environmental *V. parahaemolyicus* isolates; (e) lanes 53 to 64 are clinical *V. parahaemolyicus* isolates.

Paper II

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LAMP assay to detect *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in shrimp

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Abstract Acute hepatopancreatic necrosis disease (AHPND) is a serious disease in shrimp and results in considerable losses for the shrimp aquaculture industry. The etiologic agent of AHPND has recently been identified as a unique strain of *Vibrio parahaemolyticus*: One that is different from human pathogenic *V. parahaemolyticus* strains. In this study, two sets of primers (LAMP-A2 and LAMP-A3) were developed and validated for use in a LAMP assay to specifically identify *V. parahaemolyticus* causing AHPND (*V. parahaemolyticus* AHPND). LAMP-A2 and LAMP-A3 detected all 33 *V. parahaemolyticus* AHPND isolates except the non-*V. parahaemolyticus* AHPND isolates and 19 other closely related bacterial species. In pure culture and in spiked shrimp experiments, the LAMP assay was superior to PCR for the detection of *V. parahaemolyticus* AHPND. In pure cultures, the detection limit of LAMP-A3 was 53 CFU/ml or 0.1 CFU per reaction (10× lower than LAMP-A2), whereas in spiked shrimp experiments, the detection limit was 4.4×10^5 CFU/ml or 8.8×10^2 CFU per reaction. Further testing of 24 post-larvae, shrimp, sediment and water samples collected from a shrimp farm revealed that *V. parahaemolyticus* AHPND was detected mostly in the sediment samples. Taken together, the results suggested that the LAMP-A3-based LAMP assay, but not LAMP-A2 or PCR, was suitable for the identification of *V. parahaemolyticus* AHPND in shrimp aquaculture.

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Keywords *Vibrio parahaemolyticus* · AHPND · Shrimp · LAMP · Vibriosis

Introduction

Acute hepatopancreatic necrosis disease (AHPND) is an early mortality syndrome that occurs in shrimp approximately 1 month after the release of post-larvae into an earthen pond culture system. While first documented in China, AHPND has now also been reported in Vietnam, Malaysia and Thailand. It has been demonstrated that *Vibrio parahaemolyticus*, a marine bacterium that can be found worldwide, is the causative agent of this disease (Tran et al. 2013). The identification of this bacterium to the species level can be performed by PCR targeting the *tlh* or *toxR* genes (Bej et al. 1999; Kim et al. 1999). While most *V. parahaemolyticus* strains are nonpathogenic, some possess hemolysins such as the thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) and can cause gastroenteritis in humans after consumption of *V. parahaemolyticus*—contaminated seafood (Nishibuci and Kaper 1995; Yeung and Boor 2004). However, both TDH and TRH have not been found in *V. parahaemolyticus* strains that cause AHPND (Tran et al. 2013). Furthermore, *V. parahaemolyticus* causing AHPND (*V. parahaemolyticus* AHPND) strains have not been reported to cause any diseases in humans.

When infected, shrimp with AHPND present a massive shedding of hepatopancreatic tubule epithelial cells, a pale hepatopancreas and empty intestinal tract (Tran et al. 2013). Despite their severity, the *V. parahaemolyticus* AHPND virulence factors that are responsible for these marked disease phenotypes are not known. However, certain *V. parahaemolyticus* AHPND proteins show homology to bacterial toxins that are known to effect insects (http://www.enaca.org/modules/news/article.php?article_id=2030).

As *V. parahaemolyticus* are natural and mostly nonpathogenic inhabitants of the marine environment, it is common to find *V. parahaemolyticus* contamination in shrimp farming facilities. However, as *V. parahaemolyticus* AHPND has a clearly detrimental effect on shrimp yield and shrimp aquaculture economics, the ability to rapidly identify the specific strain(s) of this bacterium that cause AHPND from broodstock, larvae, post-larvae, adult shrimp, surrounding water and sediment is necessary to monitor the presence and spread of such shrimp aquaculture contamination events. One molecular detection method that has the potential to rapidly identify targeted bacteria and is amenable to use on-site is loop-mediated isothermal amplification (LAMP). LAMP was developed to synthesize large amounts of nucleic acid using four primers that recognize six-specific sites on target DNA. The autocycling reaction is carried out using DNA polymerase, and the amplified DNA molecules incorporate magnesium pyrophosphate which results in a solution turbidity that can be detected by a turbiditometer or the naked eye. A LAMP assay has been developed to detect *V. parahaemolyticus* using the *tlh* gene as a target, and this technique identified 143 *V. parahaemolyticus* strains, but did not detect 33 other *Vibrio* spp. and 56 non-*Vibrio* strains (Yamazaki et al. 2008). Chen and Ge (2010) developed another LAMP assay targeting the *toxR* gene that was able to correctly detect 36 *V. parahaemolyticus* strains and did not generate false positive responses with 39 other bacteria. LAMP assays have also been developed to detect human pathogenic *V. parahaemolyticus* strains that harbored the *tdh*, *trh*1 and *trh*2 genes (Yamazaki et al. 2010), *Vibrio cholerae, Vibrio vulnificus*, *Campylobacter* spp., and *Salmonella* spp. (Hara-Kudo et al. 2005; Han and Ge 2008; Yamazaki et al. 2009; Srisuk et al. 2010). Perhaps most importantly for on-site use, unlike

PCR, LAMP assays do not require thermal cycling. Thus, in the shrimp farming environment, an appropriately designed and validated LAMP assay could be used for the rapid identification of *V. parahaemolyticus* AHPND simply using a water bath or thermal block set to 60–65 °C.

Materials and methods

Bacterial strains

V. parahaemolyticus, *V. furnissii*, *V. fluvialis*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus*, *V. harveyi*, *V. metschnikovii*, *V. cholerae*, *V. campbellii*, *V. cincinatiensis*, *V. splendidus*, *V. gazogenes*, *V. mytili*, *Grimontia hollisae*, *Photobacterium damselae subsp. damselae*, *Aeromonas* sp., *Pseudomonas* sp., *Escherichia coli*, *Plesiomonas* sp. and *Listeria* sp. were used in this study. Thirty-three *V. parahaemolyticus* AHPND isolates were obtained from the hepatopancreas and intestines of diseased shrimp (stock culture from the Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand).

LAMP assay primers

Six primers of each of two sets were designed using PrimerExplorer V4 software (Fujitsu System Solution Ltd., Tokyo, Japan) (Table 1). LAMP-A2 primers were designed from the 700 bp AP2 DNA sequence (http://www.enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf; Joshi et al. 2014), and LAMP-A3 primers were designed from the 336 bp AP3 DNA sequence (http://www.shrimpnews.com/FreeReportsFolder/NewsReportsFolder/ThailandNewPCRDetectionMethodFlegel.html).

Table 1 LAMP primers used in this study

Primer	Sequence (5′–3′)	Length (bp)
LAMP-A2		
FIB	GGTTTTGGCTCTGGGTACACTCGTCCAACATTTTAACGACT	41
BIP	TGCTTCCTACGTTGAAAGACCGACAGTCATTCAGCGATCC	40
F3	GCTTAATCGATTATTCAAATGCG	23
B3	CTGAGATTGATCAATCTTTATTGTC	25
LF	TCTTGCCGTTTCGCCCA	17
LB	GCGTTATCAGCTTCATATCCCC	22
LAMP-A3		
FIB	GGTATTGAATGGTAAGCTCCCCGGAAGTCGGTCGTAGTGT	40
BIP	AATGGGGTGCGCCATTTATGAAGTTTCATCACGTTGTACC	40
F3	GCAAACATACACCTATCATCC	21
B3	GCATTATCAGGGCGTTGT	18
LF	ACGTCCCGTATTCTCAATGTCT	22
LB	GCTGGCGGCTGGAAAGT	17

LAMP assay

The LAMP assay was performed in 25 µl reaction volumes containing 2 µl of extracted DNA, 1.6 µM of primers FIP and BIP, 0.8 µM of primers F3 and B3, 0.2 µM of primers LF and LB, 1 µl of *Bst* DNA polymerase (NEB) in 2 µl of buffer [0.25 M Tris–HCl (pH 8.8), 0.125 M KCl, 0.125 M $(NH_4)_2SO_4$, 2.5 % Tween 20], 16 mM $MgSO_4$, 1.6 M Betaine (Sigma-Aldrich) and 2.8 mM dNTP. The reaction mixtures were incubated in a Loopamp realtime turbidimeter (Eiken) for 60 min at 65 °C and were inactivated for 2 min at 80 °C. The reactions were considered positive when the turbidity reached 0.1 within 60 min. The presence of *V. parahaemolyticus* in all positive tubes was confirmed using a LAMP assay targeting the *tlh* gene as previously described (Yamazaki et al. 2008).

Specificity and sensitivity of the LAMP assays

LAMP-A2 and LAMP-A3 primer specificity and sensitivity were first evaluated using bacterial pure cultures. Briefly, the tested bacteria included 14 *Vibrio* spp., 6 non-*Vibrio* spp. and 33 *V. parahaemolyticus* AHPND isolates that were cultured in Luria-Bertani (LB) broth supplemented with 1 % NaCl at 37 °C overnight. One milliliter of each culture was centrifuged at $900\times g$ for 1 min, and the resulting supernatants were centrifuged at $10{,}000\times g$ for 5 min. Each cell pellet was resuspended in 500 µl of sterile distilled water and boiled for 10 min. The cell lysates were immediately cooled on ice and then centrifuged at $20{,}000\times g$ at 4 °C for 5 min, and the resulting supernatant was used as the DNA template source for specificity testing. For sensitivity testing, the LB broth *V. parahaemolyticus* AHPND cultures were adjusted to a 0.5 McFarland standard (10^8 CFU/ml), and tenfold dilutions were performed to obtained a range of template concentrations (10–10^5 CFU/ml). Bacterial CFU were confirmed by plating the same cultures on Tryptic Soy Agar supplemented with 1 % NaCl and incubated overnight at 37 °C. All experiments were done in triplicate, and the detection limit was defined as the highest dilution of sample in all three experiments that was considered positive.

LAMP assay sensitivity in spiked shrimp

Normal shrimp were purchased from the local market and first tested to confirm the absence of *V. parahaemolyticus* AHPND by PCR (described below). Samples free of *V. parahaemolyticus* AHPND were homogenized in alkaline peptone water (APW) and inoculated with 100 µl volumes containing *V. parahaemolyticus* AHPND at final concentrations of 10–10^5 CFU. After mixing, the APW supernatant was harvested, and the DNA templates for use in the LAMP assays were prepared as described above.

Shrimp farm environmental samples

A total of 24 samples including post-larvae, shrimp, sediment and water were collected from shrimp farm as previously described (Yingkajorn et al. 2014). Each sample was enriched in APW at the ratio of 1:10 and incubated for 6 h. Then, 1 ml of the APW supernatant was subjected to DNA extraction as described above. LAMP assays were performed using both LAMP-A2 and LAMP-A3 as specified above. In addition, all extracted DNA was tested by PCR for comparison. Specificity was defined as the (number of samples negative by both techniques)/(number of samples negative by both techniques + number of samples positive by LAMP but negative by PCR), and sensitivity was

defined as the (number of samples positive by both techniques)/(number of samples positive by both techniques + number of samples positive by PCR but negative by LAMP) (Lau et al. 2010).

Confirmatory DNA sequencing

To confirm the specificity of the LAMP assays, PCR was performed with the outer primers (B3 and F3 of each primer pair). The PCR assays were performed in 20 µl reaction volumes containing 2 µl of extracted DNA, 4 µl of 5× buffer supplemented with 7.5 mM $MgCl_2$, 8.3 µl of milliQ water, 1.6 µl of 2.5 mM dNTP mixture, 2 µl of 2 µM of each primer of F3 and B3 and 0.1 µl of 5 U/µl *Go Taq* DNA polymerase (Promega). For the cycling conditions, the initial denaturation was done at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30, 30 s at 55 °C for LAMP-A2 and 60 °C for LAMP-A3, 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were purified using a QIAGEN kit (QIAGEN) and sequenced.

Detection of *V. parahaemolyticus* AHPND by PCR

Samples were enriched in APW at a ratio of 1:10. After incubation at 37 °C for 6 h, serial dilutions were performed, and each dilution was plated on CHROMagar *Vibrio* (CHROMagar Microbiology, Paris). After incubation overnight at 37 °C, five to ten mauve-colored colonies were selected and tested by PCR using AP3 primers which target a gene purported to encode a *V. parahaemolyticus* AHPND-specific protein (http://www.enaca.org/modules/news/article.php?article_id=2030). DNA templates were prepared by boiling for 10 min, and the supernatant obtained after centrifugation was diluted 10× in distilled water. PCR was performed in 20 µl reaction volumes consisting of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.025 U of *Taq* DNA polymerase and 2.0 µl DNA template. For the cycling conditions, the initial denaturation was done at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 5 min and a final extension at 72 °C for 5 min. The 336 bp amplicons were analyzed by electrophoresis in a 1 % agarose gel.

Results and discussion

In this study, two sets of LAMP assay primers (LAMP-A2 and LAMP-A3) were designed based on the *V. parahaemolyticus* AHPND AP2 primers (targeting a unique plasmid DNA sequence) and AP3 primers (targeting a homolog encoding a bacterial toxin effecting insects) (Table 1). Their specificities were evaluated using 14 *Vibrio* spp., 6 non-*Vibrio* spp. and 33 *V. parahaemolyticus* AHPND isolates. With the exception of the 33 *V. parahaemolyticus* AHPND isolates, every other isolate tested, including ten environmental *V. parahaemolyticus* isolates, generated a true negative outcome by LAMP assay (Table 2). There was no difference in the specificity of the LAMP-A2 and LAMP-A3 assays for the detection of pure culture *V. parahaemolyticus* AHPND. However, the sensitivity of the LAMP-A3 assay in pure culture was better than that of the LAMP-A2 assay [the detection limit of LAMP-A3 assay was 0.1 CFU per reaction or 53 CFU/ml (Table 3)]. When comparing the sensitivity of the LAMP assays with PCR, both LAMP-A2 and LAMP-A3 were able to detect 1.1 CFU of *V. parahaemolyticus* AHPND per reaction in contrast to the 10.6 CFU per reaction detection limit using PCR in which only

Table 2 Primer specificity for the detection of *V. parahaemolyticus* AHPND via LAMP assay

Bacteria	No. of tested isolates	No. of positive identifications	
		LAMP-A2	LAMP-A3
V. parahaemolyticus	10	0	0
V. furnissii	1	0	0
V. fluvialis	1	0	0
V. vulnificus	1	0	0
V. mimicus	1	0	0
V. alginolyticus	2	0	0
V. harveyi	2	0	0
V. metschnikovii	1	0	0
V. cholerae	2	0	0
V. campbellii	1	0	0
V. cincinatiensis	1	0	0
V. splendidus	1	0	0
V. gazogenes	1	0	0
V. mytili	1	0	0
V. parahaemolyticus AHPND	33	33	33
Grimontia hollisae	1	0	0
Photobacterium damselae	1	0	0
Pseudomonas sp.	1	0	0
Escherichia coli	1	0	0
Plesiomonas sp.	1	0	0
Listeria sp.	1	0	0

two of three experiments were positive. These findings corroborate to the findings of Yamazaki et al. (2008) which demonstrated that the detection limit of a LAMP assay targeting the *tlh* gene for the detection *V. parahaemolyticus* in pure culture was 2 CFU per reaction—a sensitivity that was ten times greater than that obtained by conventional PCR.

In spiked shrimp experiments, the sensitivity of *V. parahaemolyticus* AHPND detection was lower as both LAMP-A2 and LAMP-A3 assays could detect 8.8×10^2 CFU per reaction in all triplicate experiments and 8.8 CFU per reaction in one out of three experiments using the LAMP-A3 assay (Table 3). This is not particularly surprising for spiked shrimp experiments as a variety of chemicals and particles in complex matrices (such as shrimp tissue) may interfere with the amplification reaction. However, better LAMP assay sensitivities (i.e., between 0.8 and 21.3 CFU per reaction) have been observed in a previous study that detected human pathogenic *V. parahaemolyticus* harboring the *tdh*, *trh*1 and *trh*2 genes in spiked shrimp samples (Yamazaki et al. 2010). In this study, PCR was also found to be ten times less sensitive for the detection of AHPND *V. parahaemolyticus* in spiked shrimp samples, although detection limits of 8.8×10^2 CFU per reaction were observed in two out of three experiments using this method (Table 3). Nevertheless, the LAMP assay does not require expensive instruments or sophisticated techniques. In general, it is easier to perform, and the results can be obtained more rapidly than conventional PCR. The testing of pure culture isolates and shrimp spike in experiments in this study using the LAMP-A3 assay resulted in assay time of 15–35 min and

Table 3 Sensitivity of the LAMP-A2 and LAMP-A3 primers for the detection of *V. parahaemolyticus* AHPND

Samples	Assays	No. of isolates	Dilutions				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Pure culture		CFU/ml	5.3×10^4	5.3×10^3	5.3×10^2	53	0.5
		CFU/ reaction	106	10.6	1.1	0.1	0.01
	LAMP-A2		+	+	+[a]	−[b]	−
	LAMP-A3		+	+	+	+	−
	PCR		+	±[c](2/3)	−	−	−
Spiked shrimp experiment		CFU/ml	4.4×10^7	4.4×10^6	4.4×10^5	4.4×10^4	4.4×10^3
		CFU/ reaction	8.8×10^4	8.8×10^3	8.8×10^2	88	8.8
	LAMP-A2		+	+	+	−	−
	LAMP-A3		+	+	+	± (1/3)	−
	PCR		+	+	± (2/3)	−	−

[a] Positive for all triplicate experiments

[b] Negative for all triplicate experiments

[c] Variable detection in triplicate experiments (positive identifications/number tested)

22–40 min, respectively. Both assays times are considerably less than 90 min of conventional PCR. In addition, the LAMP results could be interpreted by eye and did not require subsequent gel electrophoresis for the detection of DNA amplicons.

We also compared the efficacy of the LAMP-A2, LAMP-A3 and PCR assays using shrimp aquaculture environmental samples, such as post-larvae ($n = 3$), shrimp ($n = 7$), sediment ($n = 7$) and water ($n = 7$). All of the LAMP assay positive identifications were confirmed using a LAMP assay targeting the *tlh* gene; in addition, the LAMP amplification product was further amplified using primers B3 and F3 of each primer set of LAMP-A2 and LAMP-A3 and sequenced. All post-larvae samples were found to be negative for *V. parahaemolyticus* AHPND with both LAMP assays and PCR. In contrast, two out of seven water samples were positive for *V. parahaemolyticus* AHPND by all of the methods tested (Table 4). The LAMP-A3 assay is better than the LAMP-A2 assay and PCR in detection of this organism in shrimp samples because no false positive was detected. However, using LAMP-A2, one shrimp sample collected from pond number 10/5 was *V. parahaemolyticus* AHPND positive, but its product could not be amplified with the B3 and F3 primers of LAMP-A2 (although the LAMP assay targeting the *tlh* gene was positive) (Table 4). It has previously been demonstrated that in shrimp farming, the numbers of total and human pathogenic *V. parahaemolyticus* are higher in sediment than those collected from overlying water (Yingkajorn et al. 2014). Using the LAMP-A3 assay, we found that all of the sediments collected from every shrimp pond tested were positive for *V. parahaemolyticus* AHPND. All of the LAMP amplification products were confirmed by DNA sequencing. Thus, the presence of *V. parahaemolyticus* AHPND in all sediment samples indicated that the organisms already exist in the shrimp farm environment. Therefore, an examination of post-larvae, prior to their release into the earthen pond, does not guarantee that shrimp in the pond will not be infected by *V. parahaemolyticus* AHPND. In this case, the

Table 4 Comparison of LAMP and PCR for the detection of *V. parahaemolyticus* AHPND in shrimp culture

Pond number	Post-larvae			Shrimp			Sediment			Water		
	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR	LAMP-A2	LAMP-A3	PCR
2/2	−	−	−	−	−	−	−	+	−	−	−	−
3/1	−	−	−	−	−	−	+	+	−	−	−	−
3/2	ND	ND	ND	+	+	−	+	+	−	−	−	−
4/1	ND	ND	ND	+	+	+	+	+	−	−	−	−
7/5	ND	ND	ND	+	+	+	+	+	−	+	+	+
10/5	−	−	−	−[a]	−	−	+	+	−	−	−	−
11/4	ND	ND	ND	−	+	−	−	+	−	+	+	+
total	0	0	0	3	4	2	5	7	0	2	2	2

[a] False positive detected but the product could not be amplified with F3 and B3 primers of LAMP-A2

Table 5 Specificity and sensitivity of LAMP compared to PCR for the detection of *V. parahaemolyticus* AHPND in shrimp culture

Test		PCR		Total	Specificity (%)	Sensitivity (%)
		Positive	Negative			
LAMP-A2	Positive	4	6	10	70	100
	Negative	0	14	14		
	Total	4	20	24		
LAMP-A3	Positive	4	9	13	55	100
	Negative	0	11	11		
	Total	4	20	24		

decontamination of *V. parahaemolyticus* AHPND in shrimp pond sediment and subsequent confirmation of this decontamination by LAMP assay may be one course of action to take to reduce the risk of infection.

The sensitivity and specificity of the LAMP assays for the detection of *V. parahaemolyticus* AHPND were compared to PCR. A total of 24 samples were analyzed, and where 10 and 13 samples were found to be positive by LAMP-A2 and LAMP-A3, respectively, only four samples were deemed positive by PCR (Table 5). Therefore, sensitivity of both LAMP assays was 100 %. Conventional PCR indicated 20 negative results, while LAMP-A2 and LAMP-A3 gave six and nine positive results, respectively. Thus, LAMP-A2 and PCR shared up to 14 matched negative results indicating higher specificity (70 %). On the other hand, LAMP-A3 and PCR shared only 11 matched negative results thus indicating lower specificity (55 %). However, the ability of LAMP-A3 to detect more positive samples than LAMP-A2 without any false positive results is more critical to its usefulness for the investigation of *V. parahaemolyticus* AHPND in shrimp aquaculture. In addition, LAMP-A2 was designed from AP2 PCR primers which have a positive predictive value of 97.4 %, whereas the AP3 PCR primers had a 100 % positive predictive value (http://www.enaca.org/modules/news/article.php?article_id=2030).

In conclusion, the LAMP-A3 assay developed in this study is superior to the LAMP-A2 assay and conventional PCR for the detection of *V. parahaemolyticus* AHPND. Overall, the LAMP-A3 assay is easy to perform, generates rapid and easily interpretable results and could be a practical tool for on-site monitoring of *V. parahaemolyticus* AHPND in shrimp aquaculture environments.

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Paper III

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Isolation of *Bdellovibrio* and like organisms and potential to reduce acute hepatopancreatic necrosis disease caused by *Vibrio parahaemolyticus*

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ABSTRACT: Acute hepatopancreatic necrosis disease, a severe disease of shrimp, is caused by *Vibrio parahaemolyticus* (AHPND Vp), a halophilic bacterium harboring a plasmid that contains toxin genes homologous to *Photorhabdus* insect-related toxins. We obtained 9 isolates of *Bdellovibrio* and like organisms (BALOs) from water and sediment samples in Thailand. Using 16S rRNA sequencing, all of the organisms were identified as *Bacteriovorax* spp. and were able to attack all tested AHPND Vp isolates. In addition, their various susceptible hosts, including Gram-positive and Gram-negative bacteria, were observed. The optimal ratio for interaction between the *Bacteriovorax* isolate BV-A and AHPND Vp was determined to be 1:10. The suitable conditions applied for co-culture between BV-A and AHPND Vp were 30°C, 2% NaCl, and pH 7.6. The capability of BV-A to reduce numbers of AHPND Vp *in vitro* was observed in co-culture after incubation for 2 d and continued until the end of the incubation period. *In vivo*, BV-A was able to reduce mortality of shrimp post-larvae infected with AHPND Vp. In addition, BV-A significantly decreased the formation of biofilm by AHPND Vp. These findings provide evidence for using *Bacteriovorax* as a biocontrol of AHPND Vp in shrimp aquaculture.

KEY WORDS: *Bdellovibrio* and like organisms · BALOs · *Bacteriovorax* · *Vibrio parahaemolyticus* · Acute hepatopancreatic necrosis disease · AHPND · Shrimp

INTRODUCTION

Shrimp aquaculture is an enterprise that has generated substantial export income for many countries. Previously, Thailand was the world's leading producer of cultured shrimp, followed by China, India, Ecuador, Vietnam, and Indonesia. However, in 2009, a new shrimp disease, named acute hepatopancreatic necrosis disease (AHPND), emerged in China and subsequently spread to Vietnam, Malaysia, Thailand, and Mexico (Tran et al. 2013, Joshi et al. 2014, Nunan et al. 2014). The causative agent of AHPND is *Vibrio parahaemolyticus*, a halophilic Gram-negative bacterium found in marine and estuarine environments (Tran et al. 2013). AHPND *V. parahaemolyticus* (AHPND Vp) harbors a unique large plasmid containing genes encoded for toxin homologs to the *Photorhabdus* insect-related toxins PirA and PirB (Yang et al. 2014). The bacterium affects post-larvae approximately 30 to 35 d after stocking the shrimp pond, causing massive rounding and sloughing of hepatopancreatic tubule epithelial cells in the early to mid stages of the disease (Hong et al. 2016). PirA is only present at the later stage, whereas PirB is detected in the hepatopancreas at the early stage of infection but is sufficient to cause cellular damage (Lai et al. 2015). This bacterium has been disastrous for the shrimp industry and has reduced shrimp production worldwide by up to 20%

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(Hong et al. 2016). Strenuous efforts to solve this problem have included cessation of shrimp cultivation and drying out of shrimp ponds for a period of time. However, the disease reoccurs after cultivation recommences. Therefore, pond management including biocontrol should be incorporated.

Bdellovibrio and like organisms (BALOs) are a group of small Gram-negative predatory bacteria that are ubiquitous in aquatic and terrestrial environments (Williams & Pineiro 2006). BALOs can invade the periplasm and multiply inside many prey bacteria, lysing them and further attacking other bacteria. This makes BALOs potentially powerful inhibitors of environmentally and clinically undesirable bacteria (Fratamico & Cooke 1996, Sockett & Lambert 2004). BALOs have been investigated as alternative organisms for the prevention and control of bacterial disease in aquaculture. *Bdellovibrio* F16, isolated from sturgeon gut, displayed bacteriolytic activity against the fish pathogen *Aeromonas hydrophila* (Cao et al. 2012). Fish challenged by immersion in water containing *A. hydrophila* and *Bdellovibrio* C-1 suffered lower mortality than fish in water containing *A. hydrophila* alone, suggesting the possibility of using *Bdellovibrio* to control this bacterium in fish (Chu & Zhu 2010). Two marine BALOs isolated from the sediment of a bay in China reduced total vibrios and *V. parahaemolyticus* in water and oyster intestine (Li et al. 2011). BALO strain BDHSH06 reduced the numbers of total bacteria and vibrios in pond water and shrimp intestine (Li et al. 2014). To solve the problem of drug resistance, which causes complications in bacterial infection, 2 genera of BALOs (*Bdellovibrio* and *Micavibrio*) have been demonstrated to be capable of attacking many human pathogenic bacteria such as *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Shigella*, and *Yersinia* (Dashiff et al. 2011). Although BALOs attack bacteria nonspecifically, they preferentially select prey to different degrees (Williams & Pineiro 2006). Therefore, it is of interest to investigate the potential of BALOs to eliminate AHPND Vp. The aims of this work were to isolate BALOs from various samples collected from AHPND shrimp ponds and to evaluate their potential to control the numbers of AHPND Vp *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

AHPND Vp designated as PSU 5429, PSU 5499, PSU 5562, and PSU 5579 were isolated from AHPND-afflicted shrimp collected from various farms in southern Thailand (Kongrueng et al. 2015) and were used as mixed prey bacteria to isolate BALOs. AHPND Vp PSU 5429 was used as a host prey for other assays throughout this study. Each bacterium was grown in Luria-Bertani (LB) broth supplemented with 1% NaCl at 30°C with shaking at 150 rpm for 3 h and adjusted to 10^8 CFU ml^{-1} using a densitometer (Densimat, bioMérieux). The bacterial cells were then spun down, and LB broth was replaced with diluted nutrient broth (DNB). Each 1 ml of bacterial strain was mixed and used as a prey cocktail to isolate BALOs.

Isolation and purification of BALOs

Water and sediment field samples were collected off an island and from AHPND Vp-infected shrimp farms located in central and southern Thailand. Isolation of BALOs was performed using the double-layered plaque assay (Medina et al. 2008). Briefly, in the pre-enrichment step, a test sample of either 10 g or 10 ml of sediment or water was mixed with 100 ml of DNB (Starr 1975) and 1 ml of prey cocktail (10^8 CFU ml^{-1}). The mixture was incubated with shaking (200 rpm) at 30°C for 7 d; it was then centrifuged and the supernatant was passed through a 0.45 µm membrane filter (Millipore). The filtrate was concentrated by centrifugation at 22 000 × *g* for 1 h, and the pellet was re-suspended in DNB and mixed with 300 µl of prey cocktail (10^8 CFU ml^{-1}); it was then overlaid on diluted nutrient agar (DNAg). The plate was incubated at 30°C, and the development of plaque formation within 3 to 7 d indicated the presence of BALOs. Individual plaques were purified by 3 passages, and the BALOs were cultivated in DNB with AHPND Vp (PSU 5429) to enhance the titer and kept at –80°C with 10% glycerol.

For any assay, each BALO was recovered from the stock and its titer was enhanced as described above. To enumerate the BALOs, a 10-fold dilution of BALO suspension was performed, and 100 µl of each dilution were mixed with 4 ml of soft DNAg (0.6% agar) containing 300 µl of AHPND Vp PSU 5429 at a concentration of 10^8 CFU ml^{-1}. The mixture was then overlaid on a DNAg plate and incubated at 30°C. The numbers of plaques were counted after at least 3 d of incubation.

Molecular identification

To identify the genus of the BALOs, genomic DNA of BALOs was extracted by boiling. The 16S rRNA

gene was amplified by PCR using primers specific to the *Bdellovibrio* 16S rRNA gene (63F primer: 5′-GAG GCC TAA CAC ATG CAA GTC-3′; 842R primer: 5′-CGW CAC TGA AGG GGT CAA-3′) (Jurkevitch et al. 2000) and the *Bacteriovorax* 16S rRNA gene (Bac676F primer: 5′-ATT TCG CAT GTA GGG GTA-3′; Bac1442R primer: 5′-GCC ACG GTT CAG GTA AG-3′) (Davidov et al. 2006). The PCR product was electrophoresed on 1% agarose gel and was detected using a UV transilluminator. For sequencing, the PCR product was purified using a Qiagen kit and sequenced.

Phylogenetic tree analysis

The 16S rRNA sequences of BALOs obtained in this study were analyzed against the database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program. The sequences of BALO isolates were aligned by ClustalW with reference strains. The aligned sequences were analyzed using MEGA 6.0 software (Tamura et al. 2013). The phylogenetic tree was constructed by neighbor joining using the TN93 model (Tamura & Nei 1993). Statistical significance levels of interior nodes were determined by bootstrap analysis with 1000 replications.

Susceptibility of Gram-positive and Gram-negative bacteria to BALOs

The ability of BALOs to attack both Gram-positive and Gram-negative bacteria was investigated with *Staphylococcus aureus*, *Escherichia coli*, *Vibrio vulnificus, V. cholerae, V. alginolyticus,* AHPND Vp, and clinical and environmental *V. parahaemolyticus* isolates. A bacterial lawn was produced by shaking a loop full of each bacterium in LB broth for 3 h before mixing with melted DNAg and spreading on DNAg. To increase the titer of BALOs, each pure culture of BALO strain in DNB was mixed with AHPND Vp PSU 5429 and incubated with shaking at 30°C for 3 d. The mixture was then filtered to remove the host bacterium, and 10 µl of the filtrate were dropped on the plate containing the bacterial lawn. A clear zone was observed after incubation at 30°C for 3 to 7 d. All experiments were performed in triplicate independently, and at least 1 positive out of the 3 experiments was defined as susceptible, whereas 3 negative experiments indicated non-susceptibility.

Optimal conditions of interaction between a BALO strain and AHPND Vp

To assess factors that affect the ability of a BALO to inhibit AHPND Vp, we evaluated the multiplicity of infection of a selected BALO isolate and an AHPND Vp isolate including temperature, salinity, and pH. Each 1 ml of a BALO designated as BV-A at concentrations of 10^3 to 10^6 plaque-forming units (PFU) ml^{-1} was incubated with 1 ml AHPND Vp PSU 5429 suspension (10^7 CFU ml^{-1}) in 100 ml DNB at a ratio between 1:10 and 1:10 000. This co-culture was incubated with shaking at 30°C for 7 d, and the numbers of AHPND Vp were enumerated daily by the plate count technique. To investigate the effect of temperature, the co-culture was incubated with shaking at 25, 30, and 37°C, and for salinity and pH evaluations, the co-culture was inoculated in DNB supplemented with 1 to 6% NaCl at pH between 7 and 9. For controls, general shrimp pond conditions were applied, AHPND Vp was inoculated in DNB supplemented with 2% NaCl, pH 7.6, and incubated with shaking at 30°C. The experiment was performed in triplicate. The reduction of *V. parahaemolyticus* was calculated based on the percent difference between the initial numbers of the bacterium in the control before and after treatment.

Potential of a BALO strain to suppress AHPND Vp *in vitro*

Co-culture of a BALO strain and an AHPND Vp isolate was performed in the optimal conditions described above for 7 d. A control for each organism was included. The numbers of BALO and AHPND Vp were enumerated daily using double-layer agar and spread plate techniques, respectively.

Efficacy of a BALO strain to inhibit AHPND Vp in shrimp larvae

To assess the ability of a BALO strain to reduce mortality in shrimp larvae infected with AHPND Vp, whiteleg shrimp *Litopenaeus vannamei* post-larvae (PL24) were obtained from a shrimp farm in Songkhla Province, Thailand. Larvae were acclimatized in a tank containing 5 l of 2% artificial sea water (ASW) with aeration at room temperature (30°C) and fed with a commercial diet twice a day. The experiment was divided into 6 groups: ASW (PL24) control, AHPND Vp, BALO control, and AHPND Vp interactions with BALO at final concen-

trations of 10^2, 10^4 and 10^6 PFU ml^{-1}. Briefly, PL24 were introduced into the tanks containing AHPND Vp at the final concentration of 10^7 CFU ml^{-1}, and various concentrations of BALO were added to the tanks after 15 min. In each group, 20 shrimp larvae were investigated and 2 replicates were performed. The mortality of larvae was recorded daily for 7 d.

Biofilm assay

To evaluate the ability of BALOs to remove biofilm formed by *V. parahaemolyticus*, the formation of biofilm by AHPND Vp and clinical and environmental *V. parahaemolyticus* isolates was assessed by quantitative determination (Nesper et al. 2001, Chanyi & Koval 2014). Briefly, 200 µl of an overnight culture of *V. parahaemolyticus* grown in LB broth supplemented with 2% NaCl were inoculated into 96-well microtiter plates and incubated at room temperature (30°C) for 24 h. The wells were washed 3 times with water to remove planktonic cells, and 200 µl of a BALO suspension (10^6 PFU ml^{-1}) in DNB were inoculated into the preformed *V. parahaemolyticus* biofilms and incubated at room temperature for 24 h. The wells were then washed 3 times and fixed with glutaraldehyde. The cells were stained with 200 µl of crystal violet (0.4% w/v) for 15 min, washed with water and dried, then destained with ethanol-acetone (80:20). The biofilm production was analyzed by color development using a microplate reader at a wavelength of 570 nm. Biofilm formation of either *V. parahaemolyticus* alone or a BALO strain alone including LB broth supplemented with 2% NaCl was used as a control. In all experiments, the results obtained were an average of 12 wells.

Statistical analysis

Statistical analysis was carried out using SPSS 11.5 software to observe differences in each experiment. All values were subjected to 1-way analysis of variance (ANOVA). The data are presented as the mean and standard deviation (SD) for the indicated numbers of each experiment. Means were separated using Duncan's test at $p < 0.05$. The differences observed were considered statistically significant at $p < 0.05$.

RESULTS

Isolation and identification of BALOs

In total, 25 samples were collected including 13 water and 12 sediment samples from Yor Island and shrimp farms. Three BALO strains were isolated from the water and 6 from the sediment samples (Table 1). Confirmation by 16S rRNA sequencing revealed that all of them were *Bacteriovorax* spp. with 89 to 100% homology to the reference strains in GenBank. A comparison of the phylogenetic tree analysis with 11 *Bacteriovorax* spp. reference strains in GenBank (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d124p223_supp.pdf) indicated that *Bacteriovorax* NBV 2-5 were 89 to 99% similar in sequence to the NE1, DA5, DD1, and NB2 reference strains. *Bacterivorax* MBV 5-6 showed 100% homology to the RM2T4-S, RM3S1-S, RM2B2-S, RL1T4-S, RM3T4-S, and RM3S3-S reference strains, whereas *Bacterivorax* BV-A was 91% homologous to this group. *Bacterivorax* MBV 4 and NBV1 displayed around 94 to 100% similarity to BV-A and the rest of the *Bacteriovorax* obtained in this study (Table 1 and Fig. S1).

Table 1. Isolation of *Bdellovibrio* and like organisms (BALOs) from environmental samples and shrimp farms

Source	Sample type	No. of positive isolates	Isolate code	NCBI description	Homology (%)
Yor island	Water	3	MBV4	*Bacteriovorax* sp. B3S2-S	94
			MBV5	*Bacteriovorax* sp. RM3T4-S	100
			MBV6	*Bacteriovorax* sp. RM3T4-S	100
	Sediment	2	NBV1	*Bacteriovorax* sp. NE1	100
			NBV2	*Bacteriovorax* sp. NE1	89
Trang farm	Sediment	1	BV-A	*Bacteriovorax* sp. DA5	91
Samutsongkram farm	Sediment	2	NBV3	*Bacteriovorax* sp. NE1	99
			NBV4	*Bacteriovorax* sp. NE1	99
Songkhla farm	Sediment	1	NBV5	*Bacteriovorax* sp. NE1	98

Susceptibility of Gram-positive and Gram-negative bacteria to BALOs

The ability of 9 isolates of *Bacteriovorax* spp. to lyse Gram-positive and Gram-negative bacteria, including *Vibrio* spp., was evaluated. Two isolates of *Bacteriovorax* spp. could attack *Staphylococcus aureus*, and 4 could predate *E. coli*, whereas 5 to 6 isolates possessed the ability to lyse *Vibrio vulnificus*, *V. cholerae*, and *V. alginolyti-*

Table 2. Susceptibility of other bacteria (*Staphylococcus aureus*, *Escherichia coli*) and *Vibrio* spp. to *Bacteriovorax* spp. The experiment was performed independently 3 times; numbers shown are the numbers of positive experiments out of 3; dashes indicate that all 3 experiments were negative. Vv: *Vibrio vulnificus*; Vc: *V. cholerae*; Va: *V. alginolyticus*; Vp: *V. parahaemolyticus*; AHPND Vp: acute hepatopancreatic necrosis disease-causing *V. parahaemolyticus*

Bacteriovorax isolates	Hosts													
	Other bacteria		*Vibrio* spp.			AHPND Vp					Vp			
											Clinical		Environmental	
	S. aureus	*E. coli*	Vv	Vc	Va	EMS_1S_2	VP12	7.2L3	PeP_{16}	6.1L3	PSU5666	PSU5668	PSU5147	PSU5150
BV-A	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	–	–	+3
MBV4	–	–	+2	–	–	+3	+3	+3	+3	+3	+3	–	+3	+3
MBV5	+3	–	–	+2	+2	+3	+3	+3	+3	+3	–	–	–	–
MBV6	–	–	–	–	–	+3	+3	+3	+3	+3	–	–	–	–
NBV1	–	–	+2	–	+3	+3	+3	+3	+3	+3	–	–	–	–
NBV2	–	+2	+3	+3	+3	+3	+3	+3	+3	+3	+3	–	–	–
NBV3	–	+3	+3	+3	+2	+3	+3	+3	+3	+3	+3	+3	+3	+1
NBV4	–	+3	–	+2	+3	+3	+3	+3	+3	+3	+3	–	–	–
NBV5	–	–	–	+3	–	+3	+3	+3	+3	+3	–	+1	–	–
Total	2	4	5	6	6	9	9	9	9	9	5	2	2	3

cus (Table 2). All of them could lyse all 5 AHPND Vp tested isolates; however, only 2 to 5 isolates of *Bacteriovorax* spp. could attack clinical and environmental *V. parahaemolyticus*.

Factors involved in interaction between a BALO strain and AHPND Vp

To investigate the optimal ratio for the interaction of BALOs with AHPND Vp, *Bacteriovorax* BV-A was selected because it was isolated from a shrimp farm that was less severely affected by AHPND. In addition, it was able to attack many bacterial strains (Table 2). BV-A was incubated with AHPND Vp PSU 5429 at a ratio between 1:10 and 1:10 000. Although the numbers of AHPND Vp continuously decreased in every interaction ratio after 1 d of incubation, the highest reduction in numbers of AHPND Vp (91.1%) was detected at a ratio of 1:10 (Fig. 1). Thus, this ratio was used for interaction evaluation between these organisms throughout this study.

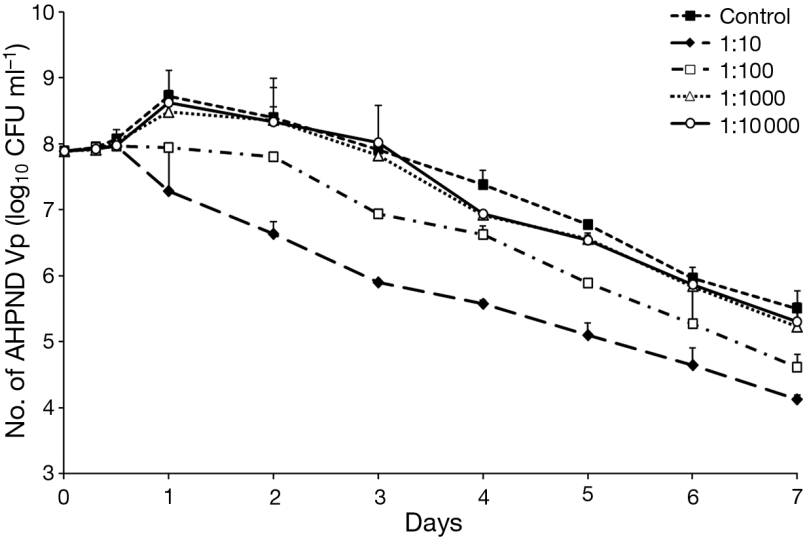

Fig. 1. Determination of the optimal ratio for interaction between *Bacteriovorax* BV-A and acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp). BV-A was incubated with AHPND Vp at ratios between 1:10 and 1:10 000 for 7 d, and the numbers of AHPND Vp were enumerated daily. The control contained only AHPND Vp. Values are mean ± SD of 2 experiments; each experiment was performed in triplicate. The highest reduction in numbers of AHPND Vp (91.1%) was detected at a ratio of 1:10

Reduction in numbers of AHPND Vp in co-culture between BV-A and AHPND Vp in the whole period of time was greater at 30°C than at 25 or 37°C (Fig. S2A in the Supplement). In addition, 90.8 to 95.3% reduction in numbers of AHPND Vp was detected in the co-culture at NaCl concentrations between 1 and 3% (Fig. S2B). No significant difference in the reduction of AHPND Vp isolate was observed after co-cultivation with BV-A at pH between 7 and 8 (Fig. S2C). In Thailand, the level of salinity in shrimp farms is between 1 and 3% (Flaherty et al. 2000), and the ambient temperature is 30°C. Therefore, 2% NaCl and 30°C were applied for subsequent co-culture of *Bacteriovorax* and AHPND Vp in DNB at pH 7.6. This pH was selected because the typical pH of seawater lies between 7.5 and 8.4, and the appropriate pH of

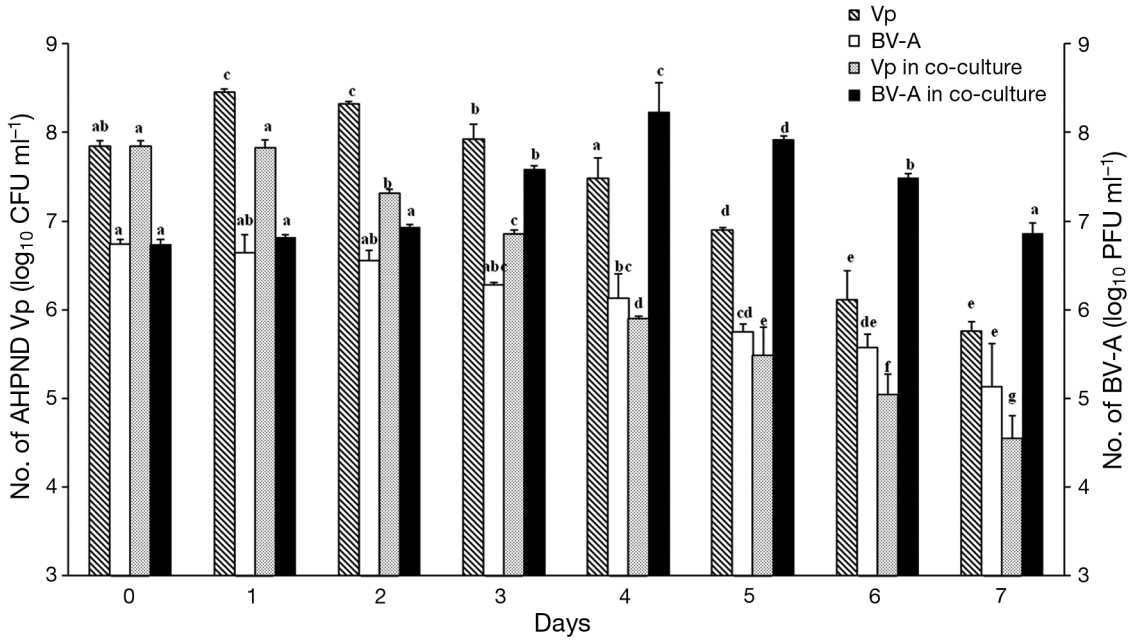

Fig. 2. Potential of *Bacteriovorax* BV-A to inhibit acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp) *in vitro*. BV-A was co-cultured with AHPND Vp for 7 d, and each organism was enumerated daily and compared to the BV-A and AHPND Vp controls. Values are mean ± SD of 2 experiments. Different letters above bars show values that are significantly different ($p < 0.05$). Each value is compared within each category

water in shrimp ponds should be maintained between 7.5 and 8.5 (Anh et al. 2010).

In vitro interaction between a BALO strain and AHPND Vp

One milliliter each of BV-A (10^6 PFU ml^{-1}) and AHPND Vp PSU 5429 (10^7 CFU ml^{-1}) were mixed in 100 ml of DNB containing 2% NaCl, pH 7.6, and incubated at 30°C. After 2 d of incubation, the numbers of AHPND Vp were significantly lower than in the AHPND Vp control and continuously decreased over the following days (Fig. 2). However, the numbers of BV-A significantly increased after 3 d of incubation and continued to do so until Day 5 of incubation, before decreasing until Day 7. In the AHPND Vp control group, the bacterial numbers significantly increased during Day 1 and 3 of incubation and subsequently decreased from Days 5 to 7, whereas a slight decrease in numbers of the BV-A control was detected on Day 1 and carried on to Day 7.

In vivo reduction of shrimp larval mortality by BV-A

Shrimp post-larvae (PL24) were inoculated with AHPND Vp PSU 5429 at a final concentration of 10^7 CFU ml^{-1}, and various final concentrations of BV-A between 10^2 and 10^6 PFU ml^{-1} were added into the shrimp tanks. The mortality of the post-larvae was determined daily for 7 d. On Day 1, approximately 7.5, 2.5, 2.5, and 0% of PL24 were dead in the AHPND Vp control and in the infected post-larval groups treated with 10^2, 10^4, and 10^6 PFU ml^{-1} BV-A, respectively; dead post-larvae in those groups increased to 50.0, 30.0, 20.0, and 17.5% on Day 3 (Fig. 3). At the end of treatment, more than 90% of post-larvae were dead in the AHPND Vp control, whereas in the infected groups containing BV-A at final concentrations of 10^2, 10^4, and 10^6 PFU ml^{-1}, mortality was 72.5, 62.5, and 47.5%, respectively. No post-larvae died in the post-larvae and BV-A control groups.

Reducing *V. parahaemolyticus* biofilm formation by BV-A

AHPND Vp and clinical and environmental *V. parahaemolyticus* isolate biofilms were pre-formed in 96-well microtiter plates for 24 h, the planktonic cells were removed, and the remaining biofilms were evaluated after adding BV-A and further incubated for 24 h. Biofilm formation of the 3 categories of *V. parahaemolyticus* was significantly reduced by BV-A (Fig. 4).

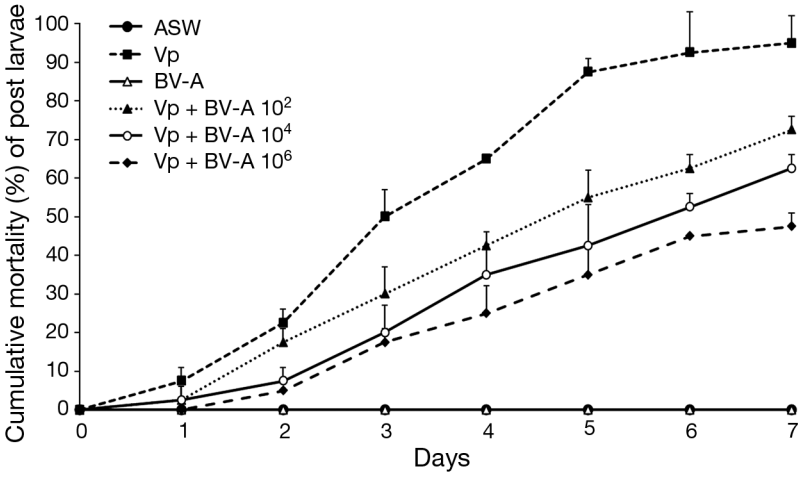

Fig. 3. Efficacy of *Bacteriovorax* BV-A to decrease shrimp post-larval mortality after treatment with acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp). *Litopenaeus vannamei* post-larvae (PL24) were exposed to AHPND Vp and subsequently treated with *Bacteriovorax* BV-A at concentrations of 10^2, 10^4, and 10^6 plaque-forming units (PFU) ml^{-1}. Dead post-larvae were counted daily. PL24 treated with artificial seawater (ASW), AHPND Vp (10^7 CFU ml^{-1}), or BV-A (10^6 PFU ml^{-1}) alone were used as controls

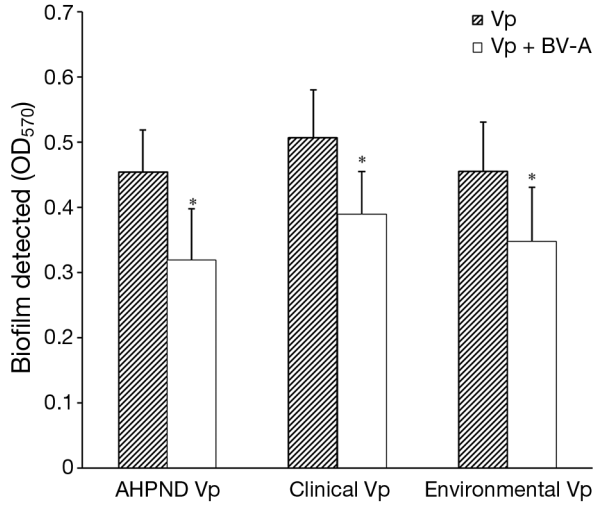

Fig. 4. Capability of *Bacteriovorax* BV-A to decrease biofilm formation of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp) and clinical and environmental isolates of *V. parahaemolyticus*. *V. parahaemolyticus* biofilms were pre-formed in 96-well microtiter plates for 24 h, and BV-A suspension was added after removal of planktonic cells. Biofilm was quantitated using crystal violet staining. * indicates significant differences ($p < 0.05$)

DISCUSSION

AHPND has caused an economic crisis in shrimp aquaculture. BALOs are ubiquitously distributed in the environment and target many prey bacteria. This makes them an attractive candidate for control of *Vibrio parahaemolyticus*, the causative agent of AHPND. In this work, various samples were obtained from Yor Island and infected shrimp farms. This island was selected because it is close to many shrimp farms located on the peninsular coast, and BALOs prefer to attack bacteria from the same environment (Pineiro et al. 2004). The organisms were more predominant in sediment than in water; 6 out of 12 sediments were positive (50%), but they were only detected in 3 out of 13 water samples (23%; Table 1). 16S rRNA sequencing was performed for identification of all BALOs obtained in this study without any further identification; therefore, the isolates were specified as the genus of their closest BLAST homology. Although all of the isolates collected from Yor Island were identified as *Bacteriovorax* spp., phylogenetic analysis differentiated 2 of them (MBV4 and NBV1) with 100% confidence because they formed an independent branch (Fig. S1). Previously, there had been no evidence that Gram-positive bacteria were susceptible to BALOs; recently, however, *Bdellovibrio bacteriovorus* HD 100 has been demonstrated to decrease numbers of *Staphylococcus aureus* in broth culture (Iebba et al. 2014). In the present study, only 2 out of 9 *Bacteriovorax* spp. (BV-A and MBV-5) were able to attack *S. aureus* (Table 2). However, all of them could lyse all AHPND Vp tested isolates, whereas their capabilities to attack clinical and environmental *V. parahaemolyticus* were lower. This may be because most of the *Bacteriovorax* spp. were isolated from AHPND-affected shrimp ponds, and AHPND Vp PSU 5429 was used as a host for propagation. In this work, although MBV-5 and MBV-6 showed 100% homology to *Bacteriovorax* RM3S3-S including the other 6 *Bacteriovorax* reference strains (Fig. S1), their bacteriolytic activities toward prey bacteria were slightly different (Table 2). The information of 16S sequencing might not be enough to differentiate some BALO strains. The gene encoding the β-subunit of RNA polymerase (*rpoB*) is more discriminating than the 16S rRNA gene for differentiation of saltwater members of the genus *Bacteriovorax* (Pineiro et al. 2008). Additionally, the mechanism of BALOs to select their prey is not clearly understood, and variability in prey range between 2 closely related BALO strains has been documented (Chanyi et al. 2013).

The optimal ratio for interaction between *Bacteriovorax* BV-A and AHPND Vp was evaluated. In this work, AHPND Vp PSU 5429 at a concentration of 10^7 CFU ml^{-1} was selected for the assay because investigation of AHPND in a shrimp farm in Mexico revealed that different virulence of *V. parahaemolyticus* depended on bacterial density, and the minimum infective density was 10^4 CFU ml^{-1} (Soto-Rodriguez et al. 2015). In addition, no shrimp mortality was observed at densities below this value, and less virulent strains did not induce 100% mortality. In our study, the reduction of AHPND Vp isolates was highest at a ratio of 1:10 between BV-A and AHPND Vp. Thus, this ratio was used for the consecutive assays.

To evaluate the capability of *Bacteriovorax* BV-A to decrease numbers of AHPND Vp *in vitro*, co-culture of those organisms was conducted. Each of AHPND Vp and BV-A was included for comparison. We found that at the end of incubation (Day 7), the numbers of both control organisms had decreased (Fig. 2). This might be due to the depletion of nutrients in DNB as the growth of AHPND Vp approached the decline phase, whereas reduction of BV-A was due to the lack of prey. In co-culture, the numbers of AHPND Vp decreased significantly more than that of the control group, indicating the potential of BV-A to eliminate this bacterium. This was clearly apparent on Day 2 of incubation, and the numbers of BV-A started increasing in the subsequent days. On Day 5, the numbers of BV-A in co-culture started decreasing, and this continued until the end of the incubation period, suggesting that this decline might be due to the decrease in numbers of prey.

To evaluate the effectiveness of BALOs in the prevention of AHPND Vp infection in shrimp, a different concentration of BV-A was applied to AHPND Vp-infected PL24. Post-larval mortality was decreased after treatment with the high concentration of BV-A (Fig. 3). This indicates the protective efficacy of BALO for post-larval shrimp. However, in this study, the mortality of post-larvae was reduced by approximately 50% with a 1:10 ratio of BV-A to AHPND Vp. Therefore, to improve the effectiveness of the treatment, a higher concentration of BALO is suggested.

V. parahaemolyticus can form biofilms on shrimp surfaces (Han et al. 2016). Investigation of 35 AHPND Vp isolates derived from shrimp farms in Mexico revealed that all of them were able to form moderate to strong biofilms (López-Leónl et al. 2016). In shrimp farming, the molting process of shrimp causes an accumulation of bacterial biofilms at the bottom of the pond. Iebba et al. (2014) reported that BALOs could target their prey both in water and in biofilm. In the current work, we demonstrated the potential of BV-A to significantly diminish biofilm formed by AHPND Vp and by clinical and environmental *V. parahaemolyticus* isolates (Fig. 4). Therefore, this evidence supports the advantage of using *Bacteriovorax* as a biocontrol in shrimp aquaculture.

BALOs have a wide prey range, but the way they select their prey is not well understood (Rogosky et al. 2006, Chen et al. 2011). *Bdellovibrio* directs itself towards its prey by flagellar motility and chemotactic responses (Straley & Conti 1977, Lambert et al. 2003). Pilus fibers may also be involved in the early stage of predation. Three *pilA* mutants of *Bdellovibrio bacteriovorus* were unable to attack their prey compared with the wild type (Evans et al. 2007). In addition, Bd0112 (homologue to *pilQ*) and Bd3852 (homologue to 1 of 2 *pilT*) are implicated in *B. bacteriovorus* predation (Medina et al. 2008). BALOs generally possess a periplasmic life cycle; however, recently, an epibiotic life cycle has been demonstrated in the Gram-negative bacteria *Acinetobacter*, *Aeromonas*, *Caulobacter*, and *Delftia* (Chanyi et al. 2013). In the epibiotic life cycle, predators attach to the prey surface and perform binary fission on the outer surface of the prey cell while prey cytoplasmic contents decrease. Iebba et al. (2014) demonstrated that *B. bacteriovorus* HD 100 attacked *S. aureus* in the epibiotic style. In addition, Monnappa et al. (2014) reported extracellular protease released from *B. bacteriovorus* HD 100 to degrade *S. aureus* biofilm and reduce its virulence. We do not know which life cycle(s) *Bacteriovorax* BV-A obtained in this work uses for attacking AHPND Vp. It would be of interest to determine the attack mechanism of this predator in the future.

In conclusion, this work demonstrates the potential of BALOs to control AHPND Vp in shrimp farms. One genus of BALOs (*Bacteriovorax*) was predominantly isolated from water and sediment samples. The optimal ratio of *Bacteriovorax* BV-A to interact with AHPND Vp was 1:10. *In vitro*, BV-A decreased the numbers of AHPND Vp within 2 d of the co-culture, and *in vivo*, it reduced mortality of post-larvae infected with AHPND Vp by around 50%. This indicates the preventive efficiency of BALOs in the control of AHPND Vp in shrimp aquaculture.

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List of Proceedings and Publications

Kongrueng, J., Yingkajorn, M., Nishibuchi, M., and Vuddhakul, V. Effects of time and temperature on numbers of pathogenic *Vibrio parahaemolyticus* in retail cockles. 16th International conference on emerging infectious diseases (EID) in the Pacific Rim: antimicrobial drug resistance in bacterial and Pacific diseases. 9-11 February 2014, Dhaka, Bangladesh.

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