Epidemiological Studies on *Vibrio parahaemolyticus* in the Mekong Delta of Vietnam

(ベトナム・メコンデルタにおける *Vibrio parahaemolyticus* に関する疫学的研究)

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The United Graduate School of Veterinary Sciences, Gifu University

(Tokyo University of Agriculture and Technology)

Tran Thi Hong To

CONTENTS

LIST OF ABBREVIATIONS

AFA	alcohol-formalin-acetic acid
AHPND	acute hepatopancreatic necrosis disease
AMP	ampicillin
APW	alkaline pepton water
bp	base pairs
CARB	carbenicillin-hydrolyzing β-lactamase
CFU	colony forming unit
CLSI	Clinical & Laboratory Standards Institute
COL	colistin
CV	CHROMagar™ Vibrio
d	day
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMS	early mortality syndrome
f237	filamentous 237 phage
g	gram
GS-PCR	group specific-PCR
h	Hour
ha	Hectare
H&E	hemotoxyline and eosin-phloxine
HP	hepatopancreas

kbp	kilobase pairs
KM	kanamycin
l	liter
LAMP	loop mediated isothermal amplification
LIM	lysin indol mobility
mg	milligram
min	minute
mm	millimeter
MR-VP	methyl red Voges Proskauer
NA	nutrient agar
NAL	nalidixic acid
NE	not examined
nm	nanometer
ORFs	open reading frames
OTT	oxytetracycline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pir^{vp}	Phortohaddus insect-related (pir) toxin like gene
ppt	parts per thousand
TCBS	thiosulfate-citrate-bile salts-sucrose
TE	Tris- EDTA
tdh	thermostable direct hemolysin gene
trh	thermostable direct hemolysin – related gene

Tris	trisaminomethane
TSI	triple sugar iron
μg	microgram
μl	microliter
μm	micrometer
rpm	revolusions per minutes
s	second
SFX	sulfisoxazole
STM	streptomycin
toxR	species-specific gene
toxRS	pandemic group specific gene
U	unite
UPGMA	unweighed pair group method using arithmetic averages
UT	untypeable
UV	ultraviolet
V/cm	volt/centimeter

PREFACE

Vibrio parahaemolyticus is a gram-negative, facultative anaerobic, halophilic, non-sporeforming and curved rod-shaped bacterium (50). It can exist freely in water or attaches to submerged, inert and animate surfaces such as suspended particulate matter, zooplankton, fish and shellfish (*35, 36*) and can be classified by 13 different O antigens and more than 71 different K antigens (33, 54). This bacterium has been considered as one of important agents of foodborne illness in human (11*)*. The foodborne illness due to *V. parahaemolyticus* was first found in Japan in 1950 (20). Since then, the infection of this pathogen in human has been reported worldwide (6, 12, 13, 19, 67) and most reported cases link to the consumption of raw or undercook seafood (12, 13). The typical symptoms of infected patients could be diarrhea with abdominal cramps, nausea, vomiting, headache, and low-grade fever (13). Several virulent factors of human pathogenic *V. parahaemolyticus* were described, in which thermostable direct hemolysin (TDH) encoded *tdh* gene and TDH-related hemolysin encoded *trh* gene are indicated as the most important virulent factors (4, 57). Most of *V. parahaemolyticus* strains from patients carried *tdh* gene and some strains lacked *tdh* gene but contained *trh* gene (67). This pathogen was reported as a common cause of foodborne illnesses in some Asian countries including Japan, China, Taiwan and Korea. It was a leading cause of foodborne illness in Japan until 1999 (28). It accounted for 86% of total bacterial foodborne outbreaks in Northern Taiwan between 1995 and 2001 (70) and 18% of foodborne outbreaks in Southern Taiwan between 2004 and 2013 (45), 24.1% of bacterial foodborne outbreaks in China between 2000 and 2014 (47) and 65.6% of bacterial foodborne outbreaks in summer months in Korea between 2007 and 2009 (25). Currently, a variety of serotypes harboring pathogenic genes have been reported (67); however, the serotype O3:K6 is identified as the predominant serotype in *V. parahaemolyticus* infection in human. In Tokyo of

Japan, the outbreak of serotype O3:K6 in total *V. parahaemolyticus* outbreaks increased approximately from 10% in 1995 to 75% in 1998 (61). This serotype dominated 46.8% and 54% of total *V. parahaemolyticus* strains isolated from human patients in Thailand between 2006 and 2010 (75) and in Southern Taiwan between 2004 and 2013 (45), respectively. In Vietnam, the infection of *V. parahaemolyticus* in human has been reported since 1983 (34). The outbreak of *V. parahaemolyticus* with the predominance of pandemic O3:K6 strain from 1997 to 1999 was reported in Nha Trang in the middle region of Vietnam (8, 77). Tai et al. (73) reported that *V. parahaemolyticus* was isolated at 8.3% from acute diarrheal patients in the South of Vietnam in 2010 and *tdh* or *trh* gene carrying strains dominated 41.7% of these *V. parahaemolyticus* infections. A few information on human pathogenic *V. parahaemolyticus* in environment has been published (73, 76) although human *V. parahaemolyticus* infection has been reported in Vietnam. In this century, a large volume of seafood and seafood products are produced in the Mekong Delta, the Southern part of Vietnam. However, the information on prevalence of *V. parahaemolyticus* in this area has not been fully understood.

Recently, *V. parahaemolyticus* was also identified as the important agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp (46). AHPND, formally known as early mortality syndrome (EMS), was reported firstly in China in 2009 and subsequently it spread to some Southeast Asian countries (53), Mexico (23, 68) and South America (65). The global economic loss of shrimp farming industry due to AHPND is estimated at more than $1 billion per year (17). This disease may occur as early as 10 days after post- larvae released in ponds and cause a mortality of up to 100% (53). Loc et al. (46) reported that the causative agent of AHPND was the specific strain of *V. parahaemolyticus*. The following studies by Lee et al. (42) found the *Photorhabdus* insect-related (pir) toxin like genes ($pirA^{vp}$ and $pirB^{vp}$) located on 70-kbp plasmid of *V. parahaemolyticus* played as virulent genes leading to this disease in shrimp. In

Vietnam, AHPND has been announced since 2010 and caused a massive loss for shrimp farming in this country, particularly in the Mekong Delta where provides approximately 95% of total shrimp production in the country (31). Shrimp disease referred to as AHPND spread on approximately 52,200 ha and 39,000 ha of shrimp farms in the Mekong Delta in 2011 and 2012, respectively (17). However, the prevalence of AHPND *V. parahaemolyticus* in environment in the Mekong Delta has not been fully understood yet.

The main objective of this research dealt with epidemiology of *V. parahaemolyticus* in seafood and water environment in the Mekong Delta.

In chapter 1, *V. parahaemolyticus* in seafood and water environment in the Mekong Delta was isolated and examined for harboring virulent genes and serotypes of virulent strains.

In chapter 2, pir^{vp} gene positive *V. parahaemolyticus* in shrimp, molluscan shellfish and water environment in the Mekong Delta was isolated and serotypes of pir^{vp} gene positive strains were investigated.

In chapter 3, several genetic and biological characteristics of human pathogenic *V. parahaemolyticus* strains and several biological characteristics of pir^{vp} gene positive *V. parahaemolyticus* strains isolated in the Mekong Delta were clarified.

CHAPTER 1

Isolation of human pathogenic *Vibrio parahaemolyticus* in seafood and water environment in the Mekong Delta of Vietnam

1.1. INTRODUCTION

V. parahaemolyticus harboring *tdh* and/or *trh* genes is reported as one of major causative agents associated with foodborne illness in human (4, 57). The infection of this pathogenic bacterium has been reported in many countries and the main source of the transmission of this illness could be via the consumption of raw or undercook seafood (1 2,13). Several studies indicated the prevalence of this pathogenic bacterium in seafood in some Southeast Asian countries (3, 5, 55). However, a few reports on prevalence of *V. parahaemolyticus* in seafood and water environment in the Mekong Delta are available.

This research aimed to isolate *V. parahaemolyticus* in seafood and water environment in the Mekong Delta to know the prevalence of this bacterium in this area and examine for harboring virulent genes and serotypes of virulent strains.

1.2. MATERIALS AND METHODS

1.2.1. Sample collection

From 2015 to 2016, a total of 449 samples including 385 seafood and 64 water samples from Can Tho city and Tra Vinh province were collected to examine for the prevalence of *V. parahaemolyticus* (Fig.1-1, Fig.1-2). Of 385 seafood samples, 330 retail samples including 32 shrimp samples [banana shrimp (*Penaeus merguiensis*) and greasyback shrimp (*Metapenaeus ensis*)] and 298 shellfish samples [white hard clam (*Meretrix lyrata*), blood cockle (*Anadara*

granosa), mud clam (*Geloina coaxans*), hakf-crenate ark (*Anadara subcrenata*) and antique ark (*Anadara antiquata*)] were purchased from wet markets in Can Tho city and Tra Vinh province in the Mekong Delta in 2015 and 2016 and 55 farming samples including 16 clam samples [white hard clam (*Meretrix lyrata*)] and 39 shrimp samples [white leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*)] were collected at 2 clam farms and 39 shrimp ponds, respectively, in Tra Vinh province in 2016. Of 64 water samples, 22 and 42 samples were collected from 2 clam farms and 42 shrimp ponds, respectively, in Tra Vinh province in 2016. All samples were kept separately in sterile plastic bags placed in polystyrene foam boxes with ice and analyzed immediately as arrival at laboratory.

1.2.2. Isolation and identification of human pathogenic *V. parahaemolyticus*

1.2.2.1. Isolation of human pathogenic *V. parahaemolyticus*

A 25 g portion of seafood sample was mixed with 225 m*l* of alkaline peptone water (APW, Nissui, Tokyo, Japan) in sterile stomacher bag to form homogenate solution. About water sample, a 100 m*l* volume of water was mixed well with 100 m*l* of 2 times high concentrate APW. The mixture was incubated at 37°C for 18 h. After that, a loopful of enrichment culture was inoculated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Nissui) and CHROMagar™ Vibrio (CV) agar (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 18 h. After incubation, green colonies on TCBS agar and violet colonies on CV agar were picked up and stored in semi-solid casiton agar for further examination.

1.2.2.2. Identification of human pathogenic *V. parahaemolyticus*

1.2.2.2.1. Biochemical test

Strains stored were subcultured on nutrient agar (NA, Nissui) plates supplemented with 1% NaCl and subjected to biochemical test. These strains were inoculated into triple sugar iron (TSI) agar (Nissui), lysine indol mobility (LIM) medium (Nissui), methyl red Voges Proskauer (MR-VP) medium (Becton, Dickinson and Company, Sparks, MD, USA) and 0, 3, 8, 10% NaCl solutions and smeared on oxidase test paper (Nissui). The strain showing glucose fermentation without gas production, lysine-positive, mobility-positive, indole-positive, oxidase-positive, VP-negative, no growth without NaCl and growth from 3 to 8% NaCl was identified as *V. parahaemolyticus*.

1.2.2.2.2. Polymerase chain reaction (PCR) assay

1.2.2.2.2.1. Deoxyribonucleic acid (DNA) extraction

DNA of *V. parahaemolyticus* strains was extracted using the boiling method. A loopful of colonies on the NA plate was mixed with 1 m*l* of sterile deionized distilled water into eppendorf tubes. The mixture was mixed well using vortex mixer. Then, the mixture was boiled at 100°C for 10 min and centrifuged at 10,000 rpm for 10 min. After that, 500 μl of supernatant was moved into new eppendorf tube and kept at – 20°C.

1.2.2.2.2.2. DNA purification

DNA of bacteria was purified using isopropanol (Wako pure chemical industries, Osaka, Japan) and sodium acetate 3M (Nippon gene, Tokyo, Japan). Briefly, 500 μl and 50 μl of isopropanol and sodium acetate, respectively, were added into 500 μl of DNA aliquot. The mixture was mixed well and then centrifuged at 10,000 rpm for 10 min. After that, the supernatant was removed and the pellet was dried at room temperature. After air dry, pellet was dissolved in 1 m*l* of Tris-EDTA (TE) buffer and kept at –20°C as DNA template.

1.2.2.2.2.3. Species-specific *toxR* gene detection

V. parahaemolyticus identified was confirmed by PCR assay targeting the species-specific *toxR* gene following the protocol described by Kim et al. (38). The primer set for *toxR* genewas shown in Table 1-1. The amplification conditions were set at one cycle of 96°C for 1 min, followed by 20 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1.5 min and then followed by one cycle of 72°C for 5 min. PCR amplified products were checked in 1.5% agarose gels by electrophoresis. After that, the gel was stained with ethidium bromide (0.5 μg/m*l*), then, washed with distilled water and photographed under a ultraviolet (UV) transilluminator.

1.2.3. Pathogenic gene detection

1.2.3.1. *tdh* and *trh* gene detection by PCR assay

The PCR assay was used to detect *tdh* and *trh* genes positive *V. parahaemolyticus* strains following the instruction described in the previous study (3). The primer set for the *tdh* and *trh* gene detection was shown in Table 1-1. The conditions of *tdh* and *trh* gene amplification was set at one cycle of 96°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and then followed by one cycle of 72°C for 7 min. Gel electrophoresis and stained were done following 1.2.2.2.2.3.

1.2.3.2. *tdh* gene detection by loop mediated isothermal amplification (LAMP) assay

The LAMP assay was used to detect *V. parahaemolyticus* strains harboring *tdh* gene using a Loopamp DNA amplification kit (Eiken Chemical, Tokyo, Japan) by following the protocol described by Yamazaki et al (81). The primer set for the *tdh* gene detection by LAMP assay was described in Table 1-2. The incubation was carried out in a Loop realtime tubidimeter (Realoop-30, Eiken Chemical) at 65°C for 60 min, following by 80°C for 2 min. A reaction was considered positive as the turbidity reached 0.1 within 60 min.

1.2.4. Serotyping

V. parahaemolyticus strains harboring *tdh* and/or *trh* genes isolated in this study were

serotyped using commercial antisera test kit (Denka Seiken, Tokyo, Japan) by following manufacturer's instructions. Bacteria were subcultured on NA supplemented with 1% NaCl. After that, a loopful of colonies was collected and mixed well in 4 m*l* of 3% NaCl solution supplemented with 5% glycerol. The mixture was autoclaved at 121°C for 60 min and then centrifuged at 30,000 rpm for 20 min. Next, the supernatant was removed and the pellet was dissolved in 500 μl of 3% NaCl solution. This mixture was used to examine for O antigen. The remaining colonies on NA plate was used directly for K antigen examination.

1.3. RESULTS

1.3.1. Prevalence of *V. parahaemolyticus* in seafood and water samples in the Mekong Delta

1.3.1.1. Seafood samples

The prevalence of *V. parahaemolyticus* in seafood samples obtained from wet markets and clam and shrimp farms was summarized in Table 1-3. *V. parahaemolyticus* strains were isolated in 288 of 330 (87.3%) samples from retail shops and in 44 of 55 (80.0%) samples from farms. Of 330 retail seafood samples, *tdh* and/or *trh* gene positive *V. parahaemolyticus* strains were detected in 24 (7.3%) samples. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (7.4%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 4 (1.3%) samples. Of 24 pathogenic *V. parahaemolyticus* strains, 2 strains harbored both *tdh* and *trh* genes and the other 22 strains carried either *tdh* or *trh* gene. Regarding to farming seafood samples, none of *V. parahaemolyticus* strains isolated from these samples was positive for *tdh* gene; meanwhile, *V. parahaemolyticus* harboring *trh* gene was found in 1 (1.8%) sample. Of farming seafood samples examined, the *trh* gene positive strain was detected only in the clam sample, dominating 6.3% clam samples collected. No pathogenic *V. parahaemolyticus* strains were found in shrimp samples including in retail shops and in shrimp ponds. It is reported that the *toxR* gene presents in almost *V. parahaemolyticus* and it was used to detected *V. parahaemolyticus* by PCR assay (38). In this study, all pathogenic strains isolated from seafood samples were *toxR* gene positive.

1.3.1.2. Water samples

In 64 water samples collected from clam farms and shrimp ponds, 50 (78.1%) samples were *V. parahaemolyticus* positive (Table 1-4). No *tdh* gene positive *V. parahaemolyticus* was isolated from all samples, although only 1 (1.6%) sample harbored *trh* gene positive *V. parahaemolyticus* (Table 1-4). This sample also collected from clam farming environment, accounting for 4.5% samples collected at this area. The pathogenic strain isolated from water sample was also *toxR* gene positive.

1.3.2. Serotyping

The serotypes of human pathogenic *V. parahaemolyticus* strains obtained in this study were shown in Table 1-5. All strains reacted to O antisera, forming 6 different O serogoups including O1, O2, O3, O4, O5 and O8. Of O serogroups identified, *tdh* gene positive strains belonged to O1, O2, O3 and O4, dominating for 4, 3, 7 and 8 strains, respectively; meanwhile, *trh* gene positive strains was involved in 4 O serogroups consisting of O1, O3, O5 and O8, accounting for 3, 1, 1 and 1 strains, respectively. Regarding to K antigens, 14 strains was typed into several K antisera; meanwhile, 12 strains did not react to any K antisera. Notably, the serotype O3:K6 was recognized in 4 *tdh* gene positive strains.

1.4. DISCUSSION

The pathogenicity of *V. parahaemolyticus* causing foodborne illness in human is usually associated with *tdh* and *trh* genes (56). The contamination of this pathogen has been reported in seafood samples in some Southeast Asian countries such as Thailand (5, 55), Malaysia (3, 55, 58) and Indonesia (55). However, few information on the prevalence of this pathogen in environment in the Mekong Delta has been reported. In this study, *tdh* and/or *trh* gene positive *V. parahaemolyticus* was isolated from retail shellfish and shellfish farms. This is the first report on the detection of human pathogenic *V. parahaemolyticus* in food in the Mekong Delta, Vietnam. In this study, the *tdh* gene positive *V. parahaemolyticus* was detected relatively at a high rate in retail molluscan shellfish samples; however, this pathogen was not found in samples from farms. This pathogenic bacterium was also detected at high rates in retail molluscan shellfish in other Southeast Asian countries such as 12% in Thailand (5), 11.1% in Malaysia and 9.1% in Indonesia (55). Many of retail shops which I brought the samples in this study were located near the coast in the Mekong Delta. Shellfish from those shops were usually sold immediately after they were harvested at the coast. Therefore, the prevalence of pathogenic *V. parahaemolyticus* in retail shellfish seems to reflect that in the environment in this coastal area although *tdh* gene positive *V. parahaemolyticus* was not detected from farming shellfish and environment samples. Moreover, the prevalence rate of pathogenic *V. parahaemolyticus* in shellfish samples seems to be higher than that in shrimp in this study. In agreement to our study, Vuddhakul et al. (79) examined seafood in Thailand and indicated the pathogenic bacteria could be found in molluscan shellfish but not in shrimp and fish. Malcolm et al. (48) examined seafood in Malaysia and indicated no detection of this pathogenic bacterium in shrimp although high

prevalence of this pathogenic bacterium was found in molluscan shellfish. It is known that molluscan shellfish are filter feeders and can accumulate pathogenic *V. parahaemolyticus* in their guts, providing high prevalence of this pathogenic bacterium compared to other species such as shrimp and fish (63).

Human pathogenic *V. parahaemolyticus* harboring *tdh* and/or *trh* genes were involved in a variety of serotypes in which serotype O3:K6 is the most important because it was found as the predominant serotype in *V. parahaemolyticus* infection in human (54, 79). The pathogenic *V. parahaemolyticus* strains isolated from seafood in the Mekong Delta, the South of Vietnam in this study were classified into several serotypes and serotype O3:K6 was detected. Therefore, we should pay more attention on human *V. parahaemolyticus* infection in this region.

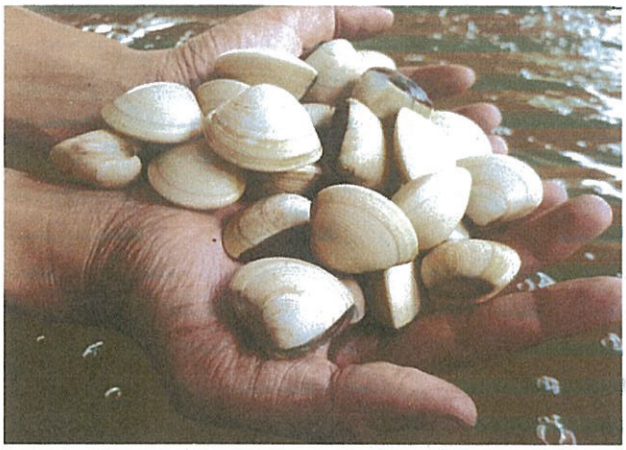
The *tdh* gene can be examined by the presence of beta-haemolysis on blood agar called kanagawa phenomenon (66, 78) and *trh* gene can be examined by urease production (72). Recently, the examination of these genes via molecular techniques such as PCR and LAMP assay has been developed for more successful detection. Several reports indicated LAMP assay was more sensitive than PCR assay in *tdh* gene detection (59). Therefore, aside from PCR assay, LAMP assay was also used to examine *tdh* gene in this study.

In conclusion, human pathogenic *V. parahaemolyticus* presented in seafood relatively at a high rate in the Mekong Delta and the serotype O3:K6 existed in this area. These results can be used for understanding microbiological risks of seafood in the Mekong Delta.

1.5. SUMMARY

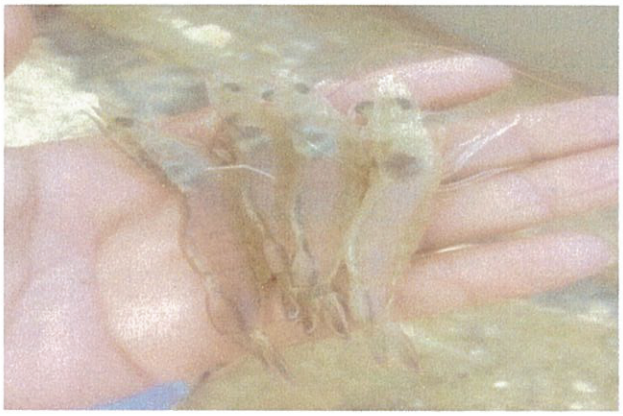
A total of 449 samples including 385 seafood and 64 water samples collected in the Mekong Delta in the year 2015 and 2016 were examined in order to determine the prevalence of *V. parahaemolyticus* in this area. Of 385 seafood samples, 332 (86.2%) samples were contaminated with *V. parahaemolyticus* and 25 (6.5%) samples were pathogenic *V. parahaemolyticus* carrying *tdh* and/or *trh* gene. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (5.7%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 5 (1.3%) samples. Of 25 pathogenic *V. parahaemolyticus* strains, 2 strains harbored both *tdh* and *trh* genes and the other 23 strains carried either *tdh* or *trh* gene. Of 64 water samples from aquaculture farms, 50 (78.1%) samples were contaminated with *V. parahaemolyticus*. No *V. parahaemolyticus* harboring *tdh* gene were found; meanwhile, *V. parahaemolyticus* harboring *trh* gene was detected in 1 (1.6%) sample. Twenty-six pathogenic *V. parahaemolyticus* strains isolated were classified into 6 types of O antigen, in which the serotype O3:K6 was detected in 4 strains. These findings can be used for understanding microbiological risk of seafood in the Mekong Delta.

Fig.1-1. Seafood samples collected at retail shops



White hard clam

Clam farm



White leg shrimp

Shrimp pond

Fig.1-2. Seafood samples collected at farms

Table 1-1. Primer sets used in this study for *tdh*, *trh* and *toxR* gene detection by PCR

Target genes	Primer sequence (5'- 3')	Amplicon sizes (bp)	References
tdh	5'-CCACTACCACTCTCATATGC-3' 5'-GGTACTAAATGGCTGACATC-3'	251	Bilung et al. (3)
trh	5'-GGCTCAAAATGGTTAAGCG-3' 5'-CATTTCCGCTCTCATATGC-3'	250	
toxR	5'-GTCTTCTGACGCAATCGTTG-3' 5'-ATACGAGTGGTTGCTGTCATG-3'	368	Kim et al. (38)

Table 1-2. Primer set used in this study for *tdh* gene detection by LAMP assay

Primer	Sequence (5' - 3')	Reference
Tdh-FIP	GTACCTGACGTTGTGAATACTGATTGTCTCTGACTTTTGGACAAAC	Yamazaki et al. (81)
Tdh-BIP	TGACATCCTACATGACTGTGAACACTTATAGCCAGACACCGC	
Tdh-F3	AGATATTGTTTGTTGTTCGAGAT	
Tdh-B3	AACACAGCAGAATGACCG	
Tdh-LF	GTACGGTTTTCTTTTTACATTACG	
Tdh-LB	AAGACTATACAATGGCAGCG	

Table 1-3. Prevalence of *V. parahaemolyticus* in seafood samples in the Mekong Delta, Vietnam

Origins	Samples	No. of samples	No. of *V. parahaemolyticus* positive samples (%)	No. of human pathogenic *V. parahaemolyticus* positive samples (%)		
				tdh gene	*trh* gene	Total
Retail shops	Molluscan shellfish					
	White hard clam	87	79 (90.8)	10 (11.5)	2 (2.3)	11 (12.6)[a]
	Blood cockle	85	80 (94.1)	5 (5.9)	1 (1.2)	5 (5.9)[a]
	Mud clam	60	51 (85.0)	4 (6.7)	1 (1.7)	5 (8.3)
	Antique ark	40	32 (80.0)	1 (2.5)	0 (0.0)	1 (2.5)
	Hakf-crenate ark	26	18 (69.2)	2 (7.7)	0 (0.0)	2 (7.7)
	Subtotal	298	260 (87.2)	22 (7.4)	4 (1.3)	24 (8.0)
	Shrimp					
	Banana shrimp	28	25 (89.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Greasyback shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	32	28 (87.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	330	288 (87.3)	22 (6.7)	4 (1.2)	24 (7.3)
Farms	Molluscan shellfish					
	White hard clam	16	16 (100.0)	0 (0.0)	1 (6.3)	1 (6.3)
	Shrimp					
	White leg shrimp	35	25 (71.4)	0 (0.0)	0 (0.0)	0 (0.0)
	Black tiger shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	39	28 (71.8)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	55	44 (80.0)	0 (0.0)	1 (1.8)	1 (1.8)
Total		385	332 (86.2)	22 (5.7)	5 (1.3)	25 (6.5)

[a]: One *V. parahaemolyticus* strain harbored both *tdh* and *trh* genes.

Table 1-4. Prevalence of *V. parahaemolyticus* in water samples in the Mekong Delta, Vietnam

Water samples	No. of samples	No. of *V. parahaemolyticus* positive samples (%)	No. of human pathogenic *V. parahaemolyticus* positive samples (%)	
			tdh gene	*trh* gene
Molluscan shellfish farms				
White hard clam	22	17 (77.3)	0 (0.0)	1 (4.5)
Shrimp ponds				
White leg shrimp	38	30 (78.9)	0 (0.0)	0 (0.0)
Black tiger shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)
Subtotal	42	33 (78.6)	0 (0.0)	0 (0.0)
Total	64	50 (78.1)	0 (0.0)	1 (1.6)

Table 1-5. Serotypes of *tdh* and/or *trh* gene positive *V. parahaemolyticus* strains isolated from seafood and water samples in the Mekong Delta, Vietnam

Strain names	Serotypes	Origins	*tdh* gene	*trh* gene	*toxR* gene
VP-HP1	O1:K1	Retail shellfish	-	+	+
VP-HP2	O1:K32	Retail shellfish	+	-	+
VP-HP3	O1:K_{UT}[a]	Retail shellfish	+	+	+
VP-HP4	O1:K_{UT}	Retail shellfish	+	+	+
VP-HP5	O1:K_{UT}	Retail shellfish	+	-	+
VP-HP6	O2:K_{UT}	Retail shellfish	+	-	+
VP-HP7	O2:K_{UT}	Retail shellfish	+	-	+
VP-HP8	O2:K_{UT}	Retail shellfish	+	-	+
VP-HP9	O3:K6	Retail shellfish	+	-	+
VP-HP10	O3:K6	Retail shellfish	+	-	+
VP-HP11	O3:K6	Retail shellfish	+	-	+
VP-HP12	O3:K6	Retail shellfish	+	-	+
VP-HP13	O3:K7	Retail shellfish	+	-	+
VP-HP14	O3:K7	Retail shellfish	+	-	+
VP-HP15	O3:K_{UT}	Retail shellfish	+	-	+
VP-HP16	O3:K_{UT}	Clam at clam farm	-	+	+
VP-HP17	O4:K29	Retail shellfish	+	-	+
VP-HP18	O4:K34	Retail shellfish	+	-	+
VP-HP19	O4:K42	Retail shellfish	+	-	+
VP-HP20	O4:K42	Retail shellfish	+	-	+
VP-HP21	O4:K42	Retail shellfish	+	-	+
VP-HP22	O4:K_{UT}	Retail shellfish	+	-	+
VP-HP23	O4:K_{UT}	Retail shellfish	+	-	+
VP-HP24	O4:K_{UT}	Retail shellfish	+	-	+
VP-HP25	O5:K47	Retail shellfish	-	+	+
VP-HP26	O8:K_{UT}	Water at clam farm	-	+	+

[a]: Untypeable

CHAPTER 2

Isolation of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) of shrimp in shrimp, molluscan shellfish and water environment in the Mekong Delta, Vietnam

2.1. INTRODUCTION

V. parahaemolyticus naturally distributes in estuarine and marine environment. The high prevalence of this bacterium is usually observed in shellfish species (5, 48). Recently, *V. parahaemolyticus* harboring pir^{vp} genes has been indicated as the causative agent of AHPND in shrimp (42, 46). It is postulated that pir^{vp} genes were recently acquired by *V. parahaemolyticus* and caused shrimp disease (27). The outbreak of AHPND has been reported in several commercial shrimp species such as white leg shrimp (*P. vannamei*), black tiger shrimp (*P. monodon*) and fleshy shrimp (*P. chinensis*) (18). In the Mekong Delta, shrimp farming mainly relays on white leg and black tiger shrimp. AHPND has appeared and led to a big economic loss for shrimp farming in this area since 2010. However, a few reports on the prevalence of AHPND pathogen in environment in the Mekong Delta have been published.

This research aimed to isolate pir^{vp} gene positive *V. parahaemolyticus* strains in the Mekong Delta of Vietnam and examine for serotypes of these strains.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection

A total of 481 samples including 449 samples described in chapter 1 (330 retail shrimp and molluscan shellfish samples, 55 farming shrimp and molluscan shellfish samples and 64 water samples collected in the Mekong Delta in 2015 and 2016) and 32 white leg and black tiger shrimp samples collected from 32 intensive shrimp ponds in Tra Vinh province in 2017 were examined in this study.

2.2.2. Isolation and identification of *pir*^{vp} genes positive *V. parahaemolyticus*

The isolation and identification of *pir*^{vp} genes positive *V. parahaemolyticus* were followed the protocol described in 1.2.2.

2.2.3. Pathogenic gene detection by PCR assay

Pathogenic genes (*pirAB*^{vp}) were examined using duplex PCR following the protocol described by Han et al. (26). Primer sets used to examine for *pir*^{vp} genes were displayed in Table 2-1. The conditions of duplex PCR for *pirAB*^{vp} gene amplification were set at one cycle of 94°C for 3 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s and then followed by one cycle of 72°C for 7 min. PCR amplified products were checked in 1.5% agarose gels by electrophoresis. After that, the gel was stained with ethidium bromide (0.5 μg/m*l*), then, washed with distilled water and photographed under a UV transilluminator.

2.2.4. Serotyping

The *pir*^{vp} gene positive *V. parahaemolyticus* strains were serotyped following the method described in 1.2.4.

2.3. RESULTS

2.3.1. Isolation of AHPND *V. parahaemolyticus* in shrimp, molluscan shellfish and water samples in the Mekong Delta

The isolation of pir^{vp} gene positive *V. parahaemolyticus* in shrimp, molluscan shellfish and water samples obtained in this study was described in Table 2-2. The pir^{vp} gene positive *V. parahaemolyticus* strains were isolated in 2 of 298 (0.7%) retail molluscan shellfish samples, 7 of 71 (9.9%) farming shrimp samples and 2 of 42 (4.8%) water samples from shrimp ponds. All except 2 pathogenic strains detected in this study carried both $pirA^{vp}$ and $pirB^{vp}$ genes. Only 2 strains isolated from two shrimp samples harbored $pirB^{vp}$ gene without $pirA^{vp}$ gene. All pir^{vp} gene positive strains were *toxR* gene positive.

2.3.2. Serotyping

Sixteen pir^{vp} gene positive *V. parahaemolyticus* strains were serotyped. They were classified into 2 types of O antigen including O1 and O3, in which O1 was predominant. Regarding to K antigen, 9 strains reacted to several types of K antigen including K25, K31, K64, K68, whereas, the other 7 strains did not react to any K antigen (Table 2-3).

2.4. DISCUSSION

In this study, *pir*^{vp} gene positive *V. parahaemolyticus* was isolated not only in shrimp and water samples from shrimp ponds but also in molluscan shellfish from retail shops. Molluscan shellfish samples in this study were the same those described in the previous chapter which were collected from retail shops located near the coast in the Mekong Delta. Therefore, the prevalence of *pir*^{vp} gene positive *V. parahaemolyticus* in retail shellfish may also reflect those in environment in the Mekong Delta although this pathogen was not isolated from environment samples. These results suggest that *pir*^{vp} gene positive *V. parahaemolyticus* seems to be prevalent widely in environment in the Mekong Delta. Further studies should clarify factors related to the outbreak of AHPND.

It is known that some pathogens were found to be correlated to the specific serotype. For example, the serogroup O1 and O139 of *V. chorelae* was able to produce cholera enterotoxins which cause gastrointestinal illness in human; meanwhile the non O1 and non O139 serogroups rarely encored these toxins (32). However, in several situations, the diversity of pathogenic serotypes following the specific pathogenic serotype found previously which could be because of the evolution of pathogenic bacteria has been reported. For instant, the new clone of the serotype O3:K6 identified as pandemic serotype causing foodborne illness in human was reported firstly in 1996 (62). Subsequently, several reports indicated other serovars such as O4:K68; O1:K25, O1:K41 and O3:K46 also carried pandemic traits (67, 75, 79). Regarding to AHPND *V. parahaemolyticus*, the previous study by Kongrueng et al. (40) indicated virulent *V. parahaemolyticus* isolated from shrimp ponds in Thailand were involved in the unique O antigen (O1) with some types of K antigen (K33, K68 and K untypeable). However, the present study detected 2 types of O antigen and 5 types of K antigen of *V. parahaemolyticus* harboring virulent

genes. Similar to our study, the recent study by Chonsin et al. (9) in Thailand demonstrated several types of O antigen (O1, O3 and O8) and K antigen (K25, K68 and K untypeable) of virulent strains. Although several O antigens were detected, O1 antigen was the predominant serogroup. This phenomenon indicated that the evolution may occur with this pathogen. This is the first report on the serotype associated with AHPND *V. parahaemolyticus* in Vietnam. It is reported that the virulent genes of AHPND are encoded in plasmid of *V. parahaemolyticus* (42). Han et al. (26) characterized AHPND plasmid and described pir^{vp} genes are flanked by a transposase coding sequence which acts as a mobile genetic element and can promote horizontal gene transfer. A variation of serotypes of *V. parahaemolyticus* harboring pir^{vp} genes suggests that the virulent genes could be transferred among *V. parahaemolyticus* strains. The existence of pir^{vp} genes was also reported in non-*V. parahaemolyticus* strains such as *V. harveyi*-like strains (39) and *V. campbellii* (15). Factors affecting transfer of the virulent genes should be investigated.

In conclusion, pir^{vp} gene positive *V. parahaemolyticus* presented widely in environment in the Mekong Delta and the serotype of pir^{vp} gene positive strains was diversity. These findings can be used for understanding the risk of AHPND infection in the Mekong Delta.

2.5. SUMMARY

A total of 481 samples including 330 retail shrimp and molluscan shellfish, 87 farming shrimp and molluscan shellfish and 64 water samples collected in 2015, 2016 and 2017 were examined for the prevalence of pathogenic *V. parahaemolyticus* related to AHPND in shrimp. The pir^{vp} gene positive strains were found in 2 of 298 (0.7%) retail molluscan shellfish samples, 7 of 71 (9.9%) farming shrimp samples and 2 of 42 (4.8%) water samples from shrimp ponds. These strains belonged to 2 types of O serogroup including O1 and O3 in which O1 was predominant. These findings can be used for understanding the risk of AHPND infection in the Mekong Delta.

Table 2-1. Primer sets used in this study for *pir*^{vp} and *toxR* gene detection by PCR

Target genes	Primer sequence (5' - 3')	Amplicon sizes (bp)	Reference
pirA^{vp}	5'-TGACTATTCTCACGATTGGACTG-3' 5'- CACGACTAGCGCCATTGTTA-3'	284	Han et al. (26)
pirB^{vp}	5'- TGATGAAGTGATGGGTGCTC-3' 5'- TGTAAGCGCCGTTTAACTCA-3'	392	
toxR	5'-GTCTTCTGACGCAATCGTTG-3' 5'-ATACGAGTGGTTGCTGTCATG-3'	368	Kim et al. (38)

Table 2-2. Prevalence of *pir*^{vp} gene positive *V. parahaemolyticus* in shrimp, molluscan shellfish and water samples in the Mekong Delta, Vietnam

Origins	Sample types	No. of samples	No. of AHPND *V. parahaemolyticus* positive samples (%)
Seafood samples			
Retail shops	Molluscan shellfish	298	2 (0.7)
	Shrimp	32	0 (0.0)
	Subtotal	330	2 (0.6)
Farms	Molluscan shellfish	16	0 (0.0)
	Shrimp	71	7 (9.9)
	Subtotal	87	7 (8.0)
Total		417	9 (2.2)
Water samples			
Farms	White hard clam farms	22	0 (0.0)
	Shrimp ponds	42	2 (4.8)
Total		64	2 (3.1)

Table 2-3. Serotypes of *pir*^{vp} gene positive *V. parahaemolyticus* strains isolated in the Mekong Delta, Vietnam

No. of samples	Strain names	Serotypes	Pathogenic genes		Species-specific gene (*toxR*)	Origins
			pirA^{vp}	*pirB*^{vp}		
1	VP-AHPND-1	O1:K64	+	+	+	Shellfish in shop
2	VP-AHPND-2	O1:K68	+	+	+	Shellfish in shop
3	VP-AHPND-3	O1:K$_{UT}$[a]	+	+	+	Water at shrimp pond
4	VP-AHPND-4	O1:K$_{UT}$	+	+	+	Water at shrimp pond
5	VP-AHPND-5	O1:K68	+	+	+	Shrimp at shrimp pond
6	VP-AHPND-6	O1:K$_{UT}$	+	+	+	Shrimp at shrimp pond
	VP-AHPND-7	O1:K$_{UT}$	-	+	+	Shrimp at shrimp pond
7	VP-AHPND-8	O1:K25	+	+	+	Shrimp at shrimp pond
8	VP-AHPND-9	O1:K25	+	+	+	Shrimp at shrimp pond
	VP-AHPND-10	O1:K$_{UT}$	+	+	+	Shrimp at shrimp pond
9	VP-AHPND-11	O1:K$_{UT}$	+	+	+	Shrimp at shrimp pond
	VP-AHPND-12	O1:K25	+	+	+	Shrimp at shrimp pond
10	VP-AHPND-13	O1:K25	+	+	+	Shrimp at shrimp pond
	VP-AHPND-14	O1:K$_{UT}$	+	+	+	Shrimp at shrimp pond
11	VP-AHPND-15	O3:K31	+	+	+	Shrimp at shrimp pond
	VP-AHPND-16	O3:K31	-	+	+	Shrimp at shrimp pond

[a]: Untypeable

CHAPTER 3

Genetic and biological characteristics of human pathogenic and AHPND *Vibrio parahaemolyticus* strains originated in the Mekong Delta, Vietnam

3.1. INTRODUCTION

V. parahaemolyticus is one of leading causative agents of foodborne illness in human (57). The infection of *V. parahaemolyticus* was associated with diverse serovars in which serotype O3:K6 was associated with many outbreaks of foodborne illness worldwide (54, 67). The new clone of O3:K6 with specific genetic markers was found in an outbreak in India in 1996 and after that, it spread to other parts of the world (62). Nasu et al. (56) identified ORF8 gene, one of the open reading frames (ORFs) of the f237 phage genome of O3:K6 strains, as a genetic marker of pandemic strains. Matsumoto et al. (52) developed group-specific PCR (GS-PCR) targeting the unique *toxRS* sequence harbored by the new pandemic clone to distinguish pandemic and non-pandemic strains. A recent research indicated several pandemic strains were ORF8 negative but GS-PCR positive (79). In Vietnam, the infection of *V. parahaemolyticus* in humans has been reported since 1983 (34). The outbreak of O3:K6 *V. parahaemolyticus* from 1997 to1999 was reported in Khanh Hoa province in the Middle of Vietnam (8, 77) and the isolation of *tdh* or *trh* gene positive *V. parahaemolyticus* from acute patients in 2010 was reported in the South of Vietnam. Moreover, *V. parahaemolyticus* harboring pir^{vp} genes has been recently identified as the causative agent of AHPND in shrimp in the Mekong Delta and caused a big economic loss of shrimp farming in this area (17, 42, 46). However, characteristics of human and shrimp pathogenic *V. parahaemolyticus* in environment in the Mekong Delta have been not fully understood.

This chapter aimed to clarify some characteristics of human and shrimp pathogenic *V. parahaemolyticus* strains isolated in the Mekong Delta including several genetic characteristics of human pathogenic strains such as harboring pandemic trait of *tdh* and/or *trh* gene positive strains and pulsed-field gel electrophoresis (PFGE) patterns of O3:K6 strains and some biological

characteristics of human and shrimp pathogenic strains such as antimicrobial susceptibility and pathogenicity to shrimp via challenge experiment.

3.2. MATERIALS AND METHODS

3.2.1. Determination of some characteristics of human pathogenic *V. parahaemolyticus* strains isolated in the Mekong Delta, Vietnam

3.2.1.1. Antimicrobial susceptibility

The antimicrobial susceptibility test was done using the disk diffusion method following the guideline of Clinical and Laboratory Standard Institute (CLSI) (10). Nine antimicrobial agents were used in this experiment including ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), ofloxacin (10 μg), oxytetracycline (30 μg), streptomycin (10 μg) and sulfisoxazole (250 μg). The density of bacteria was adjusted based on 0.5McFarland standard. After that, bacteria were inoculated on Muller Hilton agar (Becton, Dickinson and Company) plates supplemented with 3 % NaCl. Antimicrobial disks (Becton, Dickinson and Company) were then placed on agar plates and these agar plates were incubated at 37°C for 18 h. Clearance zones (mm) were measured and compared to criteria as described by CLSI (10) to determine the resistance of bacteria to these antimicrobial agents.

3.2.1.2. Pandemic trait detection by GS-PCR

The pandemic trait of pathogenic *V. parahaemolyticus* strains was examined basing on GS-

PCR by following the protocol described by Matsumoto et al. (52). The primer set used for GS-PCR were displayed in Table 3-1. The amplification conditions were set at one cycle of 96°C for 5 min, followed by 25 cycles of amplification consisting of denaturation at 96°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 3 min, and then followed by one cycle of 72°C for 7 min. PCR amplified products were checked in 1.5% agarose gels by electrophoresis. After that, the gel was stained in ethidium bromide (0.5 μg/m*l*), then, washed in distilled water and photographed under a UV transilluminator.

3.2.1.3. PFGE pattern determination of O3:K6 strains

3.2.1.3.1. O3:K6 strains used in this study

O3:K6 strains used in this study included 4 strains originated from the Mekong Delta and 5 pandemic strains originated from Japan and China (Table 3-2).

3.2.1.3.2. PFGE analysis

PFGE was performed by following the protocol described by PulseNet (64) and Hara-Kudo et al. (29) in order to compare characteristics of O3:K6 strains. Bacterium on NA was collected and mixed well in 2 m*l* of cell suspension (100 mM Tris:100 mM EDTA, pH 8.0). Bacteria density was adjusted using spectrophotometer at 610 nm wavelength to read optical turbidity of 0.8 to 1.0. After that, 400 μl of cell suspension was mixed with 20 μl of protein K stock solution (20 mg/m*l*) and 400 μl melted 1% NA agarose (GE Healthcare, Uppsala, Sweden) prepared in 1X TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) and 10% sodium dodecyl sulfate solution. The

mixture was mixed gently up and down for several times and dispended into plug mold. The plugs were allowed to solid at room temperature for 10 to 15 min. Then, each plug was put into a 10 m*l* tube containing a mixture prepared by adding 25 μl of protein K stock solution into 5 m*l* of cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl). The tube was incubated in 55°C water bath with sharking (150 rpm) for 2 h. Next, solution in each tube was replaced with 5 m*l* of sterile deionized water and poured off after incubated in 55°C water bath with sharking for 10 min. This washing was repeated 4 times. Then, the plugs were washed with 1X TE buffer. The procedure of washing with 1X TE buffer was similar to that with water. After that, the plugs were kept in 5 m*l* of 1X TE buffer and stored at 4°C before transferred into the process of DNA digestion. In the process of restriction digestion of DNA in agarose plugs, each plug was removed from 1X TE buffer and cut to obtain 2.0 to 2.5 mm wide slice. This slice was placed into 200 μl of 1X restriction buffer and incubated at 37°C for 15 min. After incubation, buffer from the plug slice was removed and the DNA was digested with 40U *Not*I (Takara, Kyoto, Japan) in restriction enzyme master mix in accordance with the manufacturer's instruction by incubation at 37°C for 3 h. After incubation, the mixture was removed and the slice was placed in 1% agarose NA gel for electrophoresis. Lambda ladders (Bio-Rad Laboratories, CA, USA) were used as size markers. The gel was covered with 0.5X Tris-borate-EDTA buffer (50mM Tris base, 50mM boric acid, 2nM EDTA) on CHEF-DR II pulsed field electrophoresis system (Bio-Rad Laboratories). The running condition was 6 V/cm at 14°C for 24 h and switch times were 1 to 18 s for 12 h and 3 to 80 s for 12 h. After that, the gel was stained with ethidium bromide for 30 min and then washed in distilled water for 30 min. The gel was photographed under a UV transilluminator. The dendrogram was designed using bionumeric software, version 12 (Applied Maths) applying clustering analysis of unweighted pair-group method with arithmetic average (UPGMA).

3.2.2. Determination of some characteristics of *pir*^{vp} gene positive *V. parahaemolyticus* strains isolated in the Mekong Delta, Vietnam

3.2.2.1. Antimicrobial susceptibility

The antimicrobial susceptibility test of *pir*^{vp} gene positive strains was followed protocol described in 3.2.1.1. Ten antimicrobial agents were used in this experiment including ampicillin (10 μg), chloramphenicol (30 μg), colistin (10 μg), gentamicin (10 μg), nalidixic acid (30 μg), ofloxacin (10 μg), oxytetracycline (30 μg), sulfisoxazole (250 μg) and tebipenem (10 μg). Clearance zones (mm) were measured and compared to criteria as described by CLSI (10) to determine the resistance of bacteria to these antimicrobial agents except for colistin and tebipenem. Criteria for colistin and tebipenem were based on the instruction of Galani et al. (22) and Fujisaki et al. (21), respectively.

3.2.2.2. AHPND pathogenicity confirmation

3.2.2.2.1. Challenge experiment

Pathogenicity of *pir*^{vp} gene positive *V. parahaemolyticus* strains was confirmed using challenge test by immersion method. Five strains harboring *pirAB*^{vp} genes were selected and pathogenicity of each strain was examined. White leg shrimp (*L. vannamei*) at about 1 g was immersed in 5 *l* aerated seawater (20 ppt) containing jars. Five shrimps per 1 *pir*^{vp} gene positive strain were contained in each jar. The concentration of *pir*^{vp} gene positive *V. parahaemolyticus* in each jar was adjusted at 10^6 CFU/m*l*. The experiment was observed for at least 7 d. Shrimp was examined for mortality rates and AHPND clinical signs. Moribund shrimp was picked up for re-

isolation of AHPND strains and several moribund shrimps were also used for histopathological examination of hepatopancreas. In this experiment, two human pathogenic *V. parahaemolyticus* strains were also selected and each was examined for its pathogenicity to experimental shrimp.

3.2.2.2.2. Histopathology examination

Histopathological examination was done by the protocol described by Lightner (44). Briefly, hepatopancreas of shrimp samples wase removed and fixed in Davidson's AFA (alcohol-formalin-acetic acid) fixative. The samples were processed using automatic tissue processing system, Tissue-Tek@ VIP™ 5 Jr vacuum infiltration processor (Sakura Finetek, CA, USA). Then, the sample was embedded, cut and sectioned. The sectioned samples were stained with hemotoxyline and eosin-phloxine (H&E) using automatic slice stainer, Varistain V24-4 (ThermoFisher Scientific, MA, USA) and observed by light microscopy to detect AHPND lesions in the tissue.

3.3. RESULTS

3.3.1. Characteristics of human pathogenic *V. parahaemolyticus* strains isolated in the Mekong Delta, Vietnam

3.3.1.1. Antimicrobial susceptibility

Many of human pathogenic *V. parahaemolyticus* strains isolated in this study showed resistance against streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%) (Table 3-3). Of 26 human pathogenic strains, 20 (76.9%) strains showed multi-resistance against two or more antimicrobial agents, while only 1 strain did not show resistance to antimicrobial agents (Table 3-4).

3.3.1.2. Pandemic trait of human pathogenic *V. parahaemolyticus* strains

In this study, 4 O3:K6 strains were GS-PCR positive. However, other all human pathogenic *V. parahaemolyticus* strains isolated in the Mekong Delta were GS-PCR negative (Table 3-5).

3.3.1.3. PFGE patterns of O3:K6 strains

The dendrogram of PFGE analysis indicated 4 Vietnamese strains formed 4 PFGE patterns and were grouped into 2 different clusters (Fig.3-1). Two Mekong Delta strains (VP-HP11 and and VP-HP12) belonged to 1 cluster and shared a similarity at larger than 75%. The other 2

Mekong Delta strains were comprised in another cluster together with Japanese strains and the Chinese strain. However, in this cluster, the strains examined were divided into 2 subclusters. The 2 Mekong Delta strains (VP-HP9 and VP-HP10) were comprised in the same subcluster to the Chinese strain, although only 1 Mekong Delta strain had a highly similar PFGE pattern to this strain (Similarity > 95%). All Japanese strains were included in 1 subcluster and generally shared a similarity to the Mekong Delta strains at larger than 70%.

3.3.2. Characteristics of *pir*^{vp} gene positive strains isolated in the Mekong Delta, Vietnam

3.3.2.1. Antimicrobial susceptibility

Table 3-6 indicated that *pir*^{vp} gene positive *V. parahaemolyticus* strains showed high resistance rates to colistin (100%), ampicillin (93.8%) and streptomycin (87.5%).

Of *pir*^{vp} gene positive strains examined, 1 strain showed resistance to five antimicrobial agents, 3 to four, 9 to three and 3 to two antimicrobial agents (Table 3-7). All (100.0%) strains showed resistance against more than one antimicrobial agent.

3.3.2.2. AHPND pathogenicity

The 5 *pir*^{vp} gene positive *V. parahaemolyticus* strains examined showed pathogenicity to experimental shrimp (Table 3-7). The mortality of challenged shrimp varied from 40 to 100%. No mortality was observed in shrimp without challenged with *pir*^{vp} gene positive strains. Shrimp infected with AHPND strains showed clinical signs of AHPND such as atrophied and pale HP, empty stomach and midgut (Fig.3-2). Histopathology of HP of shrimp infected with AHPND

strains indicated sloughing and necrosis of HP tubule epithelial cells and hemocytic infiltration surrounding HP tubules (Fig.3-3). Regarding to human pathogenic strains used in experimental infection, they showed no pathogenicity to experimental shrimp (Table 3-8). No mortality was observed in shrimp challenged with these strains.

3.4. DISCUSSION

3.4.1. Characteristics of human pathogenic *V. parahaemolyticus* isolated in the Mekong Delta, Vietnam

The high resistance rates of human pathogenic *V. parahaemolyticus* to several antimicrobial agents have been reported in some Southeast Asian countries. Al-Othrubi et al. (2) reported 63.1% of pathogenic *V. parahaemolyticus* isolated from retail seafood in Selangor, Malaysia showed resistance to ampicillin. Elmahdi et al. (16) indicated 72% of pathogenic *V. parahaemolyticus* isolated in seafood samples in Thailand showed resistance to ampicillin. Marlina et al. (51) reported human pathogenic strains isolated in shellfish from Malaysia showed high resistant rates to ampicillin (41.2%), cefuroxime (88.3%), ciprofloxacin (59%), gentamicin (59%), rifampicin (70.6%), sulfamethosazone (59%), teicoplanin (94.1%) and tetracycline (88%). In this study, pathogenic *V. parahaemolyticus* strains showed high resistance rates to streptomycin, ampicillin and sulfisoxazole. However, our isolates showed no resistance to tetracycline and gentamicin although strains from Malaysia showed high resistance rates to those antimicrobial agents. Almost all pathogenic *V. parahaemolyticus* strains examined in this study demonstrated multiple antimicrobial resistances. Similarly, Marlina et al. (51) and Al-Othrubi et al. (2) indicated Malaysian strains resisted to minimum of 4 antimicrobial agents. Elmadhi et al. (16) showed Thai strains resisted to 2 antimicrobial agents. In fact, several antimicrobial agents such as streptomycin, ampicillin and sulfisoxazone have been frequently used in agriculture and aquaculture for the last some decades; therefore, the resistance of bacteria to those antimicrobial agents could be facilitated by selective pressure (14, 30). Recently, the intrinsic ampicillin

resistance of *V. parahaemolyticus* has been mentioned because *V. parahaemolyticus* is commonly resistant to ampicillin. Chiou et al. (7) clarified this phenomenon and indicated that a novel class A carbenicillin-hydrolyzing β-lactamase (CARB) family of β-lactamases, $bla_{CARB-17}$, was identified in *V. parahaemolyticus* and this gene might respond for nature penicillin resistance of this bacterium.

Serotype O3:K6 is known to be the predominant serotype isolated from *V. parahaemolyticus* infection in human (54). The GS-PCR positive O3:K6 strain is indicated as the pandemic strain (52). In this study, all 4 O3:K6 strains detected in retail shellfish samples showed GS-PCR positive. These results indicate that the pandemic *V. parahaemolyticus* strain is prevalent in environment in the Mekong Delta. This may post a risk for an outbreak of foodborne illness caused by *V. parahaemolyticus* in this area. O3:K6 strains isolated after 1996 tended to closely relate each other despite geographical location. Alam et al. (1) reported that the similarity at larger than 75% of PFGE patterns of O3:K6 strains were isolated after 1996 among various countries including Thailand, Hong Kong, the United State and Chile. Wong et al. (80) examined PFGE patterns of O3:K6 strains among four countries including Japan, Korea, Taiwan and India and found a similarity at larger than 90% of PFGE patterns among strains isolated after 1996. Hara-Kudo et al. (29) examined PFGE patterns of O3:K6 strains from Japan environment and compared to those isolated from clinical in some countries and indicated that O3:K6 strains producing *tdh* after 1996 showed diversity in PFGE patterns and shared a similarity at larger than 70%. In present study, O3:K6 strains in the Mekong Delta also showed a diversity of PFGE patterns. They had some strains having close genetic relationship to the Chinese strain although they were genetically far from those originated from Japan. However, the similarity of PFGE patterns among the Mekong Delta, Japanese and Chinese strains was relatively at a high rate (> 70%). Therefore, they possibly diverged from one ancestral clone.

3.4.2. Characteristics of AHPND *V. parahaemolyticus* strains isolated in the Mekong Delta, Vietnam

In this study, most of AHPND *V. parahaemolyticus* strains were resistant to colistin, ampicillin and streptomycin and all AHPND strains were multidrug resistance. In agreement to our finding, Obaidat et al. (60) reported 100% of *V. parahaemolyticus* strains isolated from seafood samples in some developing countries showed resistance to colistin. The high resistance rates to ampicillin and other antimicrobials were also recorded on AHPND *V. parahaemolyticus* strains in other countries. Kongrueng et al. (40) reported 100% of AHPND strains isolated from Thailand were resistant to ampicillin and erythromycin. Han et al. (27) indicated 100% of AHPND strains isolated from Mexico were resistant to ampicillin and oxytetracycline. The explanation for antimicrobial resistance of AHPND *V. parahaemolyticus* could be similar to that used for human pathogenic *V. parahaemolyticus* such as selective pressure and bacteria's innate ability. Particularly, colistin was limited in use since 1980s due to its side effect such as renal and neurological toxicity (24, 69). It is expected that bacteria isolated in this study showed high susceptible to this antimicrobial agents. However, we observed a convert result. Notably, the intrinsic resistance to colistin has been reported in *V. cholerae* and *V. vulnificus* (69). Therefore, this phenomenon could also have in *V. parahaemolyticus* strains in this study.

The typical symptom of shrimps infected with AHPND was empty stomach and midgut, pale to white HP and atrophy of HP with dysfunction of HP tubule epithelial cells (18). Histopathology of AHPND shrimp HP indicated several lesions such as enlargement and necrosis and sloughing of tubule epithelial cells, less diversity of tubule epithelial cells and infiltration of hemocytes surrounding HP tubules (46). Of these lesions, the sloughing of HP tubule epithelial cells is indicated as the principal diagnostic characteristic of AHPND (17). In this study,

challenged shrimp showed similar symptoms described for shrimp infected with AHPND such as high mortality rate, atrophied and pale HP, empty stomach and midgut, sloughing and necrosis of HP tubule epithelial cells and hemocytic infiltration surrounding HP tubules. These results confirmed the previous finding demonstrated *V. parahaemolyticus* harboring pir^{vp} genes related to AHPND (42, 46) and $pirAB^{vp}$ gene positive *V.paraheamolyticus* isolated from the environment samples in this study cause AHPND in shrimp although all $pirAB^{vp}$ gene positive *V. paraheamolyticus* isolates could not be challenged in shrimp. Kongrueng et al. (40) reported that DNA profiles of AHPND *V. parahaemolyticus* strains were similar to but different from clinical and environmental *V. parahaemolyticus* strains. Therefore, they estimated that the causative agent of AHPND might have originated from one clone.

In conclusion, most of human pathogenic *V. parahaemolyticus* strains showed resistance to some antimicrobial agents and multidrug resistance. The pandemic O3:K6 strains existed in environment in the Mekong Delta and had a diversity of PFGE pattern. The presence of pandemic *V. parahaemolyticus* serotype O3:K6 may post a risk of an outbreak of *V. parahaemolyticus* in this area. Therefore, we should pay much attention to developing solutions to prevent outbreaks by this pathogenic bacterium in this region. Regarding to AHPND strains, all strains showed resistance to one or more antimicrobial agent. The strains examined in experimental infection showed AHPND pathogenicity. The antimicrobial alternative methods should be developed to prevent the outbreak of AHPND in shrimp culture in the Mekong Delta.

3.5. SUMMARY

The result of antimicrobial susceptibility test showed that most of human pathogenic strains were resistant to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%). Twenty-five of 26 strains showed resistance to one or more antimicrobial agents. All 4 O3:K6 strains showed GS-PCR positive and they performed a diversity of PFGE patterns. They had some strains having close genetic relationship to the Chinese strain although they were genetically far from those originated from Japan. The present of pandemic *V. parahaemolyticus* serotype O3:K6 in the Mekong Delta may post a risk of an outbreak of *V. parahaemolyticus* in this area. Regarding to AHPND *V. parahaemolyticus* isolated in this study, most of AHPND strains were resistant to colistin (100.0%), ampicillin (93.8%) and streptomycin (87.5%). All (100.0%) pir^{vp} gene positive strains showed multidrug resistance. The strains examined in challenge experiment showed AHPND pathogenicity to experimental shrimp. The prevention methods without antimicrobial application should be developed to prevent AHPND infection in shrimp in the Mekong Delta.

Table 3-1. Primer set used in this study for GS-PCR

Target gene	Primer sequence (5'- 3')	Amplicon size (bp)	Reference
toxRS	5'-TAATGAGGTAGAAACA-3' 5'-ACGTAACGGGCCTACA-3'	651	Matsumoto et al. (52)

Table 3-2. O3:K6 strains originated from other countries used in this study

Strain names	Origins	GS-PCR
VP80	Shellfish in Japan	+
VP526	Shellfish in Japan	+
VP540	Patient in Japan	+
VP1123	Patient in Japan	+
VP1239	Shellfish in China	+

Table 3-3. Antimicrobial resistance of human pathogenic *V. parahaemolyticus* (n = 26)

Antimicrobial agents	No. of resistant strains (%)
Streptomycin	22 (84.6)
Ampicillin	15 (57.7)
Sulfisoxazole	15 (57.7)
Kanamycin	2 (7.7)
Chloramphenicol	0 (0.0)
Gentamicin	0 (0.0)
Nalidixic acid	0 (0.0)
Oxfloxacin	0 (0.0)
Oxytetracycline	0 (0.0)

Table 3-4. Resistant patterns of human pathogenic *V. parahaemolyticus* strains against 9 antimicrobial agents

Antimicrobial agents	Resistant patterns
KM-AMP-SFX-STM[a]	1
KM-AMP-STM	1
AMP-SFX-STM	6
SFX-STM	6
AMP-STM	4
AMP-SFX	2
STM	4
AMP	1
No resistance	1
Total	26

[a]: AMP: Ampicillin; KM: Kanamycin; STM: Streptomycin; SFX: Sulfisoxazole

Table 3-5. Pandemic trait of human pathogenic *V. parahaemolyticus* strains isolated from seafood and water samples in the Mekong Delta, Vietnam

Strain names	Serotypes	GS-PCR
VP-HP1	O1:K1	-
VP-HP2	O1:K32	-
VP-HP3	O1:K_{UT}[a]	-
VP-HP4	O1:K_{UT}	-
VP-HP5	O1:K_{UT}	-
VP-HP6	O2:K_{UT}	-
VP-HP7	O2:K_{UT}	-
VP-HP8	O2:K_{UT}	-
VP-HP9	O3:K6	+
VP-HP10	O3:K6	+
VP-HP11	O3:K6	+
VP-HP12	O3:K6	+
VP-HP13	O3:K7	-
VP-HP14	O3:K7	-
VP-HP15	O3:K_{UT}	-
VP-HP16	O3:K_{UT}	-
VP-HP17	O4:K29	-
VP-HP18	O4:K34	-
VP-HP19	O4:K42	-
VP-HP20	O4:K42	-
VP-HP21	O4:K42	-
VP-HP22	O4:K_{UT}	-
VP-HP23	O4:K_{UT}	-
VP-HP24	O4:K_{UT}	-
VP-HP25	O5:K47	-
VP-HP26	O8:K_{UT}	-

[a]: Untypeable

Table 3-6. Antimicrobial resistances of *pir*^{vp} gene positive *V. parahaemolyticus* strains (n = 16)

Antimicrobial agents	No. of resistant strains (%)
Colistin	16 (100.0)
Ampicillin	15 (93.8)
Streptomycin	14 (87.5)
Oxytetracycline	4 (25.0)
Nalidixic acid	1 (6.3)
Chloramphenicol	0 (0.0)
Gentamicin	0 (0.0)
Kanamycin	0 (0.0)
Ofloxacin	0 (0.0)
Tebipenem	0 (0.0)

Table 3-7. Some characteristics of *pir*^{vp} gene positive *V. parahaemolyticus* strains isolated in shrimp, molluscan shellfish and water samples in the Mekong Delta, Vietnam

No. of samples	Strain names	Origins	Pathogenicity to shrimp	Antimicrobial resistance patterns
1	VP-AHPND1	Shellfish in shop	+	AMP-COL[b]
2	VP-AHPND2	Shellfish in shop	+	COL-STM
3	VP-AHPND3	Water at shrimp pond	+	AMP- COL- STM
4	VP-AHPND4	Water at shrimp pond	+	AMP-COL-OTT-STM
5	VP-AHPND5	Shrimp at shrimp pond	+	AMP-COL- NAL-OTT-STM
6	VP-AHPND6	Shrimp at shrimp pond	NE[a]	AMP-COL-OTT-STM
	VP-AHPND7	Shrimp at shrimp pond	NE	AMP-COL-OTT-STM
7	VP-AHPND8	Shrimp at shrimp pond	NE	AMP-COL
8	VP-AHPND9	Shrimp at shrimp pond	NE	AMP-COL-STM
	VP-AHPND10	Shrimp at shrimp pond	NE	AMP-COL-STM
9	VP-AHPND11	Shrimp at shrimp pond	NE	AMP-COL-STM
	VP-AHPND12	Shrimp at shrimp pond	NE	AMP-COL-STM
10	VP-AHPND13	Shrimp at shrimp pond	NE	AMP-COL-STM
	VP-AHPND14	Shrimp at shrimp pond	NE	AMP-COL-STM
11	VP-AHPND15	Shrimp at shrimp pond	NE	AMP-COL-STM
	VP-AHPND16	Shrimp at shrimp pond	NE	AMP-COL-STM

[a]: Not examined in challenge experiment

[b]: AMP: Ampicillin; COL: Colistin; NAL: Nalidixic axid; OTT: Oxytetracycline; STM: Streptomycin

Table 3-8. Pathogenicity of human pathogenic *V. parahaemolyticus* to experimental shrimp

Strain names	Serotypes	Origins	Pathogenic genes		Pathogenicity to shrimp
			tdh	*trh*	
VP-HP3	O1:K$_{UT}$[a]	Shellfish in shop	+	+	-
VP-HP10	O3:K6	Shellfish in shop	+	-	-

[a]: Untypeable

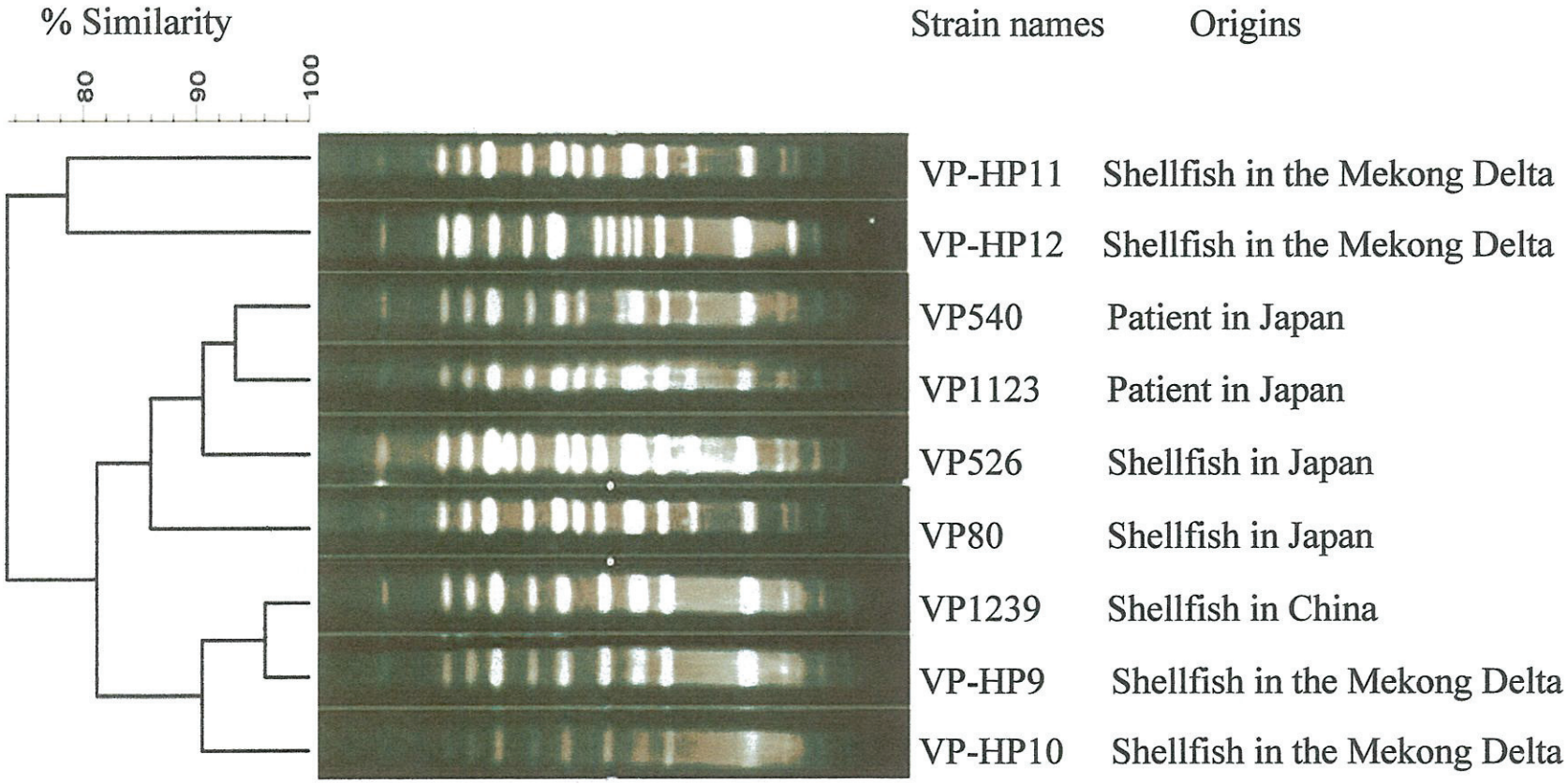


Fig.3-1. The comparison by PFGE patterns of O3:K6 strains originated from Vietnam, Japan and China

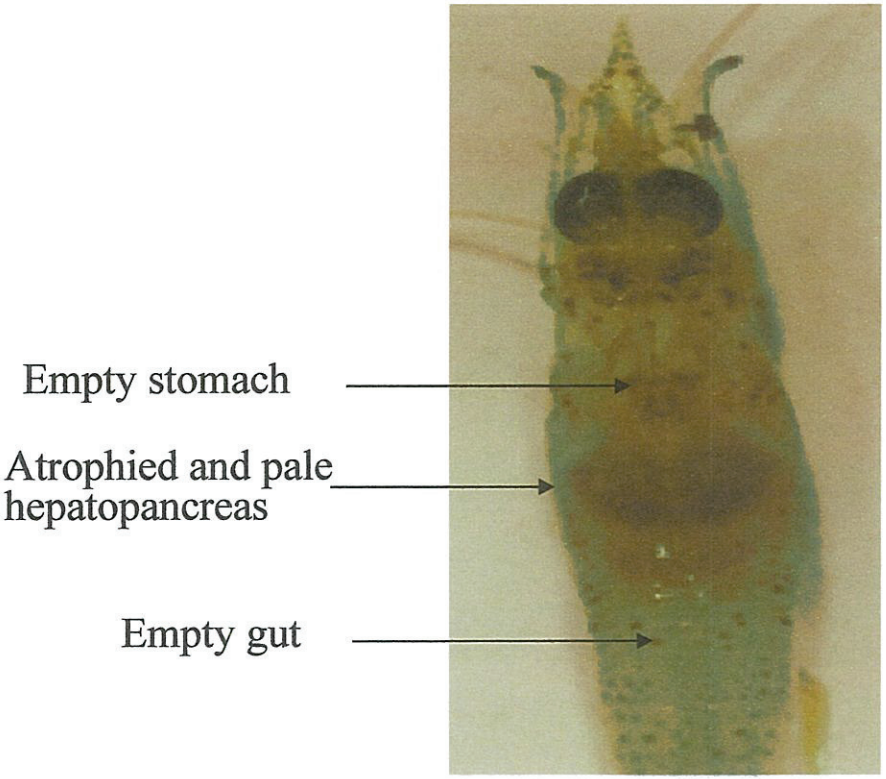


Fig 3.2. Clinical signs of shrimp challenged with AHPND *V. parahaemolyticus*

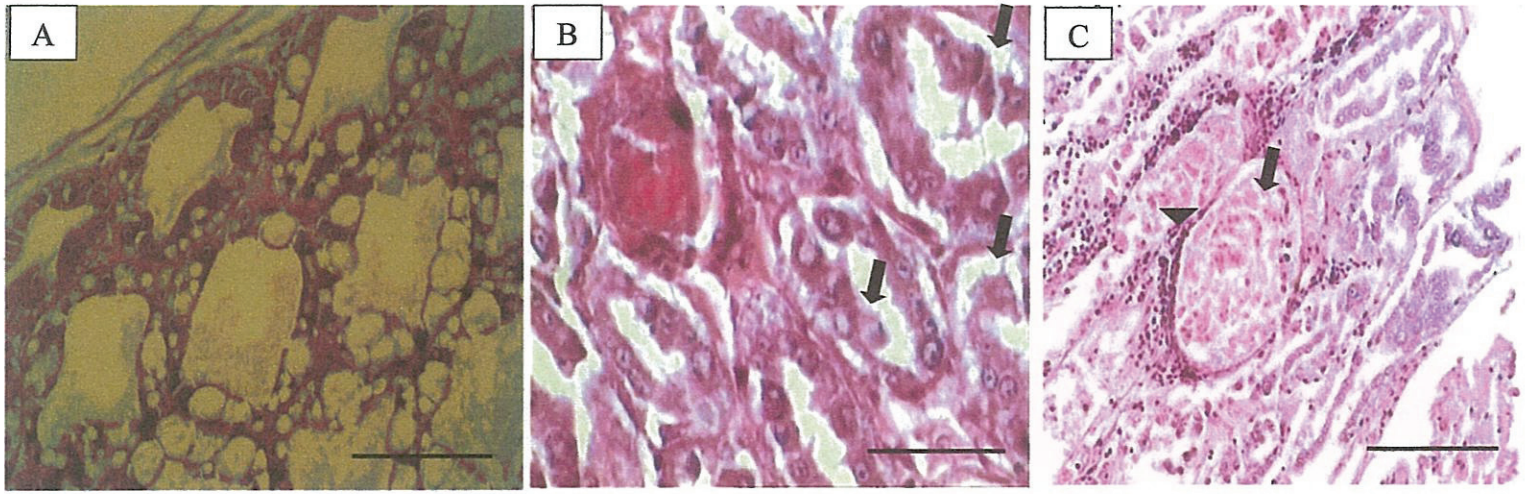


Fig.3-3. Histopathology of shrimp hepatopancreas (HP). Unchallenged shrimp showed normal structure of shrimp HP (A). Challenged shrimp showed sloughing of HP tubule epithelial cells (B, arrows), sloughing and necrosis of tubule epithelial cells (C, arrow) and hemocytic infiltration surrounding HP tubules (C, arrowhead). H&E stain. Magnification 400X. Scale bars = 50 μm

CONCLUSION

The purpose of the present study is to know epidemiological aspects of *V. parahaemolyticus* in the Mekong Delta, Vietnam. The main findings of the present study are summarized as follows:

1. Of 385 seafood samples, 332 (86.2%) samples were contaminated with *V. parahaemolyticus* and 25 (6.5%) samples were pathogenic *V. parahaemolyticus* carrying *tdh* and/or *trh* gene. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (5.7%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 5 (1.3%) samples. Of 25 pathogenic *V. parahaemolyticus* strains, two strains harbored both *tdh* and *trh* genes and the other 23 strains carried either *tdh* or *trh* gene. Of 64 water samples from aquaculture farms, 50 (78.1%) samples were contaminated with *V. parahaemolyticus*. No *V. parahaemolyticus* strains harboring *tdh* gene were found; meanwhile, *V. parahaemolyticus* strains harboring *trh* gene was detected in 1 (1.6%) sample. Twenty-six pathogenic *V. parahaemolyticus* strains isolated were classified into 6 types of O antigen, in which the serotype O3:K6 was detected in 4 strains. Those results indicated that human pathogenic *V. parahaemolyticus* seems to be prevalent widely in seafood and water environment in the Mekong Delta. Therefore, more attention should be paid on human foodborne illness caused by *V. parahaemolyticus* in the Mekong Delta.

2. Of 481 samples including 32 shrimp samples and 298 molluscan shellfish samples from retail shops, 16 molluscan shellfish samples and 71 shrimp samples from farms and 64 water samples from molluscan shellfish and shrimp farms, the pir^{vp} gene positive *V. parahaemolyticus* strains were isolated in 2 of 298 (0.7%) retail molluscan shellfish samples, 7 of 71 (9.9%) farming shrimp samples and 2 of 42 (4.8%) water samples from shrimp ponds. The pir^{vp} gene

positive strains belonged to O1 and O3 serogroups in which O1 was predominant. These results indicated that AHPND *V. parahaemolyticus* presents widely in seafood and water environment in the Mekong Delta.

3. Most of human pathogenic strains were resistant to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%). Twenty-five of 26 strains showed resistance to one or more antimicrobial agents. All 4 O3:K6 strains showed GS-PCR positive and they performed a diversity of PFGE patterns. They had some strains having close genetic relationship to the Chinese strain although they were genetically far from those originated from Japan. The present of pandemic *V. parahaemolyticus* serotype O3:K6 in the Mekong Delta may post a risk of an outbreak of *V. parahaemolyticus* in this area. Regarding to AHPND *V. parahaemolyticus* isolated in this study, most of AHPND strains were resistant to colistin (100.0%), ampicillin (93.8%) and streptomycin (87.5%). All (100.0%) pir^{vp} gene positive strains showed multidrug resistance. The 5 strains examined in challenge experiment showed AHPND pathogenicity to experimental shrimp.

These findings can be useful for understanding microbiological risk of seafood and can be used for understanding the risk of AHPND infection in the Mekong Delta, Vietnam. Moreover, the prevention methods without antimicrobial application should be developed to prevent AHPND infection in shrimp in the Mekong Delta of Vietnam.

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ABSTRACT

Vibrio parahaemolyticus has been considered as one of vital agents of foodborne illness in human and the infection of this bacterium frequently links to seafood consumption. The most important factors which associated with this illness are thermostable direct haemolysin (TDH), encoded *tdh* gene and thermostable related haemolysin (TRH), encoded *trh* gene. In Vietnam, human pathogenic *V. parahaemolyticus* has been reported since 1983. The outbreak of *V. parahaemolyticus* with the predominance of pandemic O3:K6 strain from 1997 to 1999 was reported in Nha Trang in the middle region of Vietnam. *V. parahaemolyticus* was isolated at 8.3% from acute diarrheal patients in the South of Vietnam in 2010 and *tdh* or *trh* gene carrying strains dominated 41.7% of these *V. parahaemolyticus* infections. A few information on human pathogenic *V. parahaemolyticus* in environment has been published although human *V. parahaemolyticus* infection has been reported in Vietnam. In this century, a large volume of seafood and seafood products are produced in the Mekong Delta, the South of Vietnam. However, the information on prevalence of *V. parahaemolyticus* in this area has been not fully understood.

Moreover, recently, *V. parahaemolyticus* has been identified as an important agent of acute hepatopancreatic necrosis disease (AHPND) in shrimp. This disease caused the big economic losses of more than $ 1 billion per year for shrimp farming industry in the world. In Vietnam, AHPND has appeared since 2010 and spread on approximately 52,200 ha and 39,000 ha of shrimp farms in the Mekong Delta of Vietnam in 2011 and 2012, respectively. Some researches indicated *Photorhabdus* insect-related (pir) toxin like genes (pir^{vp}) located on plasmid of *V. parahaemolyticus* could relate to AHPND in shrimp. However, the information of *V.*

parahaemolyticus causing AHPND in the Mekong Delta has been not well documented.

In this study, seafood and water samples in the Mekong Delta were examined for the prevalence of *V. parahaemolyticus* associated with foodborne illness in human and AHPND in shrimp. Some genetic and biological characteristics of pathogenic *V. parahaemolyticus* strains including several genetic characteristics of human pathogenic strains such as harboring pandemic trait of *tdh* and/or *trh* gene positive strains and PFGE patterns of O3:K6 strains and some biological characteristics of human and shrimp pathogenic strains such as antimicrobial susceptibility and pathogenicity to shrimp via challenge experiment were examined to know the characteristic of *V. parahaemolyticus* strains originated from the Mekong Delta.

Chapter 1: Isolation of human pathogenic *V. parahaemolyticus* in seafood and water environment in the Mekong Delta, Vietnam

In the period from 2015 to 2016, a total of 449 samples including 385 seafood samples and 64 water samples were examined for the prevalence of *V. parahaemolyticus* associated with human foodborne disease. Of 385 seafood samples, 332 (86.2%) samples were contaminated with *V. parahaemolyticus* and 25 (6.5%) samples were pathogenic *V. parahaemolyticus* carrying *tdh* and/or *trh* gene. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (5.7%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 5 (1.3%) samples. Of 25 pathogenic *V. parahaemolyticus* strains, two strains harbored both *tdh* and *trh* genes and the other 23 strains carried either *tdh* or *trh* gene. Of 64 water samples from aquaculture farms, 50 (78.1%) samples were contaminated with *V. parahaemolyticus*. No *V. parahaemolyticus* harboring *tdh* gene were found; meanwhile, *V. parahaemolyticus* harboring *trh* gene was detected in 1 (1.6%) sample. Twenty-six pathogenic *V. parahaemolyticus* strains

isolated were classified into 6 types of O antigen, in which the serotype O3:K6 was detected in 4 strains. These results indicate that human pathogenic *V. parahaemolyticus* seems to be prevalent widely in seafood and water environment in the Mekong Delta of Vietnam.

Chapter 2: Isolation of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) of shrimp in shrimp, molluscan shellfish and water environment in the Mekong Delta, Vietnam

A total of 481 samples including 330 retail shrimp and molluscan shellfish, 87 farming shrimp and molluscan shellfish and 64 water samples collected in 2015, 2016 and 2017 were examined for the prevalence of pathogenic *V. parahaemolyticus* related to AHPND in shrimp. The pir^{vp} gene positive strains were found in 2 of 298 (0.7%) retail molluscan shellfish samples, 7 of 71 (9.9%) farming shrimp samples and 2 of 42 (4.8%) water samples from shrimp ponds. These strains belonged to 2 types of O serogroup including O1 and O3 in which O1 was predominant. These results indicate that AHPND *V. parahaemolyticus* seems to be present widely in environment in the Mekong Delta of Vietnam.

Chapter 3: Genetic and biological characteristics of human pathogenic and AHPND *V. parahaemolyticus* strains originated in the Mekong Delta, Vietnam

Several genetic and biological characteristics of human pathogenic *V. parahaemolyticus* and AHPND *V. parahaemolyticus* were examined. Regarding to human *V. parahaemolyticus*, those strains showed resistance to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%). Twenty-five of 26 strains showed resistance to one or more antimicrobial agents. All 4 O3:K6 strains were GS-PCR positive showing pandemic *V. parahaemolyticus*. Those strains

performed a diversity of PFGE patterns and showed close genetic relationship to the Chinese strain although they were genetically far from those originated from Japan. Regarding to AHPND *V. parahaemolyticus*, almost all AHPND strains were resistant to colistin (100.0%), ampicillin (93.8%) and streptomycin (87.5%). All (100.0%) AHPND strains showed multidrug resistance. Five strains examined caused AHPND in shrimp by experimental challenge.

In this study, *V. parahaemolyticus* associated with foodborne illness in human and AHPND in shrimp seems to be prevalent widely in environment in the Mekong Delta. The human pandemic *V. parahaemolyticus* serotype O3:K6 presents in the Mekong Delta, indicating a risk of human *V. parahaemolyticus* infection in this area. Moreover, most of human pathogenic and shrimp pathogenic *V. parahaemolyticus* strains showed resistance to several antimicrobial agents and multidrug resistance. These findings can be used for understanding the risk of human pathogenic and shrimp pathogenic *V. parahaemolyticus* in the Mekong Delta and in developing preventive measures against human and shrimp *V. parahaemolyticus* infection.