DETECTION OF ANTIMICROBIAL RESISTANCE DETERMINANTS IN *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM CULTIVATED OYSTERS AND ESTUARINE WATERS

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การตรวจหาการดื้อยาต้านจุลชีพในวิบริโอ พาราฮีโมลัยติคัส ที่แยกได้จากหอยนางรมและน้ำบริเวณปากแม่น้ำ

นายวินน์ คันต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DETECTION OF ANTIMICROBIAL RESISTANCE DETERMINANTS IN *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM CULTIVATED OYSTERS AND ESTUARINE WATERS
By	Mr. Winn Khant
Field of Study	Veterinary Science and technology
Thesis Advisor	Associate Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

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ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาคุณลักษณะการดื้อยาต้านจุลชีพ และยีนควบคุมปัจจัยในการก่อโรควิบริโอ พาราฮีโมลัยติคัส ที่แยกได้จากหอยนางรมและน้ำบริเวณปากแม่น้ำในภาคใต้ของประเทศไทย วิบริโอ พาราฮีโมลัยติคัสทั้งหมด จำนวน 594 isolates มาจากหอยนางรม 361 isolates และ น้ำกร่อย 233 isolates ตัวอย่างจะเก็บทุกเดือนระหว่างเดือนมีนาคม พ.ศ. 2559 ถึง กุมภาพันธ์ พ.ศ. 2560 จากอำเภอทับปุด จังหวัดพังงา การตรวจยืนยันวิบริโอ พาราฮีโมลัยติคัส โดยการหายีน *tlh* ที่มีความจำเพาะต่อสายพันธุ์ และยีนควบคุมปัจจัยในการก่อโรค *tdh* และ *trh* การตรวจความไวรับต่อยาต้านจุลชีพทั้งหมด 8 ชนิด เพื่อหายีนดื้อยา เชื้อทั้งหมดนำมาตรวจหาการแสดงออกของ SXT และ class 1, 2 และ 3 integrons จากการศึกษาพบว่าวิบริโอ พาราฮีโมลัยติคัสให้ผลบวกกับยีน *tlh* ทั้งหมดมี 4 isolates (0.7%) จากตัวอย่างหอยนางรม 2 isolates และน้ำกร่อยที่ 2 isolates ให้ผลบวกต่อยีน *tdh* และไม่พบตัวอย่างที่ให้ผลบวกกับยีนก่อโรค *trh* ในการศึกษาครั้งนี้พบว่า 34% ของ isolates จะดื้อยาต้านจุลชีพอย่างน้อยหนึ่งชนิด และ 5.1% ดื้อยาต้านจุลชีพหลายชนิดพร้อมกันอย่างน้อย 3 กลุ่ม จำนวน ส่วนใหญ่พบว่ามีการดื้อยา erythromycin (54.2%) ตามด้วย sulfamethoxazole (34.7%), trimethoprim (28.0%) และ ampicillin (11.1%) ตามลำดับ เพียง 0.8% และ 0.5% ของ isolates จะดื้อต่อยา streptomycin และ tetracycline ตามลำดับ และไม่พบการดื้อยา chloramphenicol และ ciprofloxacin ในทุก isolates จากผลการตรวจความไวรับต่อยาต้านจุลชีพพบว่า ความชุกของการดื้อยา tetracycline ในน้ำกร่อยมากกว่าในหอยนางรมอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) โดยรูปแบบการดื้อที่พบมากที่สุด คือ ERY (21%) ลักษณะการดื้อยาต้านจุลชีพมากกว่า 3 กลุ่มที่พบมากที่สุดคือ ERY-SUL-AMP (1.9%) และยีนดื้อยาต้านจุลชีพที่พบมากที่สุด ได้แก่ *qnr* (77.8%) และ *strB* (27.4%) ตามด้วย *tetA* (22.1%) *dfr18* (19.5%) *ermB* (15.2%) และ *sul2* (14.8%) ตามลำดับ อย่างไรก็ตามยีน *dfrA1* (7.4%) และ bla_{TEM} (0.8%) พบได้น้อยมากในการศึกษานี้ การตรวจพบยีน *qnr* และ *dfr18* ที่พบในหอยนางรมและน้ำกร่อยมีความแตกต่างอย่างมีนัยสำคัญทางสถิติ และไม่พบการแสดงออกของยีน SXT (int_{SXT}) และ class 1, 2 และ 3 integrons จากผลการศึกษาแสดงให้เห็นถึงความจำเป็นของการมีสุขอนามัยที่ดี และการบริโภคหอยนางรมที่ปรุงสุก เพื่อความปลอดภัยของการบริโภคด้านอาหารทะเล ลดความเสี่ยงจากโรคภาวะอาหารเป็นพิษ และการติดเชื้อดื้อยาจากการบริโภคอาหารทะเล

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Winn Khant : DETECTION OF ANTIMICROBIAL RESISTANCE DETERMINANTS IN *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM CULTIVATED OYSTERS AND ESTUARINE WATERS. Advisor: Assoc. Prof. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

In this study, we aimed to detect antimicrobial resistance determinants and virulence factor genes in *V. parahaemolyticus* isolates from cultivated oysters and estuarine waters in southern Thailand. A total of 594 *V. parahaemolyticus* isolates were collected from pooled oysters (n=361) and estuarine waters (n=233). The samples were collected monthly between March 2016 and February 2017 from Thap Put district, Phang Nga province in southern Thailand. Confirmation of *V. parahaemolyticus* was carried out by PCR assay detecting species-specific (*tlh*) gene and the presence of virulence genes (*tdh* and *trh*). Antimicrobial susceptibility test was performed in eight antimicrobials, and the occurrence of resistance genes was investigated. All isolates were detected for the presence of SXT elements and class 1, 2, and 3 integrons. The results showed that all presumptive *V. parahaemolyticus* isolates (n=594) were positive to species-specific (*tlh*) gene. Four isolates (0.7%) from pooled oysters (n=2) and estuarine waters (n=2) were positive to *tdh*. None of the *trh*-positive isolates were observed. In this study, 34% of the isolates were resistant to at least one antimicrobial agent and 5.1% of the isolates were multidrug-resistant (MDR). Most of the isolates were resistant to erythromycin (54.2%), followed by sulfamethoxazole (34.7%), trimethoprim (28.0%), and ampicillin (11.1%), respectively. Only 0.8% and 0.5% of the isolates were resistant to streptomycin and tetracycline, respectively, and chloramphenicol and ciprofloxacin resistance were not observed in all isolates. Among the tested antimicrobials, the prevalence of tetracycline resistance in estuarine waters was significantly higher than oyster ($P < 0.05$). The most frequent resistance pattern was ERY (21.0%) and the most common MDR phenotype was ERY-SUL-AMP (1.9%). The most commonly found AMR genes were *qnr* (77.8%) and *strB* (27.4%), followed by *tetA* (22.1%), *dfr18* (19.5%), *ermB* (15.2%), and *sul2* (14.8%), respectively. However, *dfrA1* (7.4%) and bla_{TEM} (0.8%) genes were rarely found AMR genes in this study. The occurrence of *qnr* and *dfr18* genes were significantly different between oysters and estuarine water samples ($P < 0.05$). None of the isolates were possessed SXT integrase gene (int_{SXT}) and class 1, 2, and 3 integrons. Our results highlighted the need for improving good sanitary practices and consumption of adequate cooked oysters to promote seafood safety for consumption and to reduce the risk of seafood-borne illnesses and antimicrobial resistance infection.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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AMP	ampicillin
AMR	antimicrobial resistance
AMU	antimicrobial use
BAM	bacteriological analytical manual
bp	base pair
℃	degree Celsius
CDC	centers for disease control and prevention
CFU	colony forming unit
CHL	chloramphenicol
CIP	ciprofloxacin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia, for example
ERY	erythromycin
etc.	et cetera, and other similar things
et al.	et alii, and others
g	gram (s)
hr	hour (s)
kb	kilobase
KP	Kanagawa phenomenon
LB	Luria-Bertani medium
M	molar
MDR	multidrug resistant
mg	milligram (s)
MHA	Mueller-Hinton agar
MIC	minimum inhibitory concentration
min	minute (s)
ml	milliliter (s)

µg	microgram (s)
µl	microliter (s)
µM	micromolar
MPN	most probable number
NaCl	sodium chloride
NaOH	sodium hydroxide
NSS	normal saline solution
PCR	polymerase chain reaction
pH	the negative logarithm of hydrogen ion concentration
rpm	round per minute
s	second (s)
spp.	species
STR	streptomycin
SUL	sulfamethoxazole
TAE	tris-acetate-ethylenediaminetetraacetic acid
TCBS	thiosulfate citrate bile salt sucrose
tdh	thermostable direct hemolysin
TET	tetracycline
tlh	thermolabile hemolysin
trh	thermostable direct hemolysin-related hemolysin
TRI	trimethoprim
TSA	tryptic soy agar
UV	ultraviolet
%	percentage

CHAPTER I

INTRODUCTION

The increased demand for shellfish products has been recognized widely due to the relatively inexpensive and nutritious source of protein, minerals, and vitamins (Gjedrem et al., 2012). Thailand has been ranked as one of the largest exporters of fish and fishery products among the top 10 countries, with 6.5 million tons including estimated production of bivalves at 210,000 tons in 2014 (FAO, 2014). Shellfish aquaculture in Thailand has been started for over a century and rapidly growing. In Thailand, oyster aquaculture has been developed to serve domestically and internationally. However, most of the shellfish products especially oysters are available for domestic consumption. Until now, people around the world increasingly consume shellfish and preferably raw consumption because they provide several nutrients and minerals, which subsequently result in great health benefits. Additionally, seafood consumption is associated with potential health benefits, including reduced risk of heart disease, strengthen the immune system and promote healing (Iwamoto et al., 2010).

Phang Nga is one of the southern coastal provinces on the shore of the Andaman Sea, which is an abundance of aquatic animals with plentiful natural resources. Moreover, this place has been more recognized for relativity new oyster production area due to lots of oyster seeds, enrich nutrients and proper conditions for growing oysters. Phang Nga bay coast is not only suitable for growing and nursing oysters, but also producing high quality of mature oysters to serve their communities, nearby provinces, and seafood restaurants. The commercially significant oyster species in the Phang Nga bay coast are *Crassostrea lugubris* and *C. belcheri* where they are acquired from natural seeding resources. At the same time, this enriched

bay with mangrove forest has been promoted to be ecotourism spots for sightseeing, canoeing, and boating. Thus, the aggregation of domestic and international travelers and growth in local communities and industries with the need for aquatic and seafood products in this region could potentially threat aquatic animals and estuarine water.

Bivalves, including oysters, are effective filter feeders that can easily concentrate not only nutrients, sediments, and algae, but also toxic substances including bacteria and chemical pollutants from their environment. Thus, the presence of heavy metals and bacterial contaminants in estuarine water can potentially influence the quality of oyster meat. Besides these pollutants, estuarine water can be contaminated with antimicrobial resistant (AMR) bacteria, which can disseminate from the environment, especially through wastewater. This is because wastewater is plentiful with nutrients, antimicrobial substances, and other pollutants such as heavy metals. Such plentiful of nutrients, bacterial contaminants, and warmer temperature in wastewater offer optimal conditions for bacterial growth and the spread of AMR bacteria into the aquatic environment by mutation or horizontal gene transfer (Guyomard-Rabenirina et al., 2017). High concentration of bacterial accumulation in oyster meat may pose a serious public health concern and cause seafood-borne diseases (Jeamsripong et al., 2018). Thus, monitoring of microbial contamination and AMR in both oysters and estuarine waters should be implemented for seafood safety production.

Vibrio parahaemolyticus is one of the important seafood-borne pathogens. Infection of vibrio in human usually occurs through the consumption of raw or insufficiently cooked seafood products, drinking of contaminated water, and direct contact with the contaminated environment via open wounds. The clinical manifestations of these bacteria are ranged from gastroenteritis to septicemia. In

Thailand, the occurrence of *V. parahaemolyticus* was first identified in 1970, however, the incidence of *Vibrio* infection was remarkable after 1996 when a pandemic strain harboring the *tdh* virulence gene and the emergence of O3:K6 serotype (Thongjun et al., 2013). According to the annual epidemiological surveillance report, the diagnostic database of foodborne pathogens was reported that 69 out of 147 (46.9%) patients were infected with *V. parahaemolyticus* annually (Bureau of Epidemiology, 2016).

Many foodborne disease outbreaks associated with raw oyster consumption have been reported worldwide. For example, the largest *V. parahaemolyticus* outbreak affecting 11,000 human cases was reported in Chile in 2005, and the source of this contamination was from shellfish consumption (Fuenzalida et al., 2006). In the United States (U.S.), the outbreak of *V. parahaemolyticus* associated with raw oyster consumption involved in 104 cases and six hospitalizations in 2013 (Newton et al., 2014). Infection of *V. parahaemolyticus* has affected half of the foodborne disease outbreaks in many Asian countries. Apparently, consumption of raw or minimized cooked oysters carrying virulence strains of *V. parahaemolyticus* can pose the serious public health concern.

Foodborne outbreaks associated with *V. parahaemolyticus* usually occur when the existence of virulence genes such as thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*). Hemolysis and cytotoxic activities resulted from the expression of these virulence genes are the major cause of gastroenteritis in humans. In 2012-2013, among 34 human clinical isolates from Maryland, three (8.0%) and four (11.0%) isolates were harbored *tdh* and *trh,* respectively, and 19 (53.0%) isolates were carried both *tdh* and *trh* (Haendiges et al., 2015). Thus, *V. parahaemolyticus* carrying *tdh* and/or *trh* play a major role in human clinical cases associated with foodborne gastroenteritis outbreaks.

AMR has been recognized as an important and urgent health threat issue to public health worldwide. This is because antimicrobial agents have been widely used in different settings such as human, veterinary, agriculture, and aquaculture. The sources of AMR bacteria and their genes have been found in aquatic animals and the environment from sewage and farm wastes through the effluent and stormwater. Multidrug resistance (MDR) bacteria have been increasingly emerged and become a major cause of failure treatment of bacterial infectious diseases. In addition, antimicrobial use (AMU) in livestock farms can lead to an increasing number of MDR bacteria in the environment through soil, manure, wastewater, sewage, and animal effluents. Imprudent use and overuse of antimicrobial agents may increase selection pressure for AMR bacteria and promote distribution of resistance determinants of bacteria including *V. parahaemolyticus.*

Resistance to antimicrobials can occur through either the acquisition of mobile genetic elements such as plasmids, transposons, and integrons or persistent mutations in chromosomal genes. Among several mechanisms of AMR distribution, the integrons are important mechanisms for the acquisition of AMR genes and these elements can transfer either between or within species. Among nine integron types, class 1 integron is the most commonly found that relevant to the AMR presented in MDR pathogens (Rowe-Magnus and Mazel, 2002). Class 1 integron is also recognized as the major source of dissemination and exchanging of resistance genes among bacteria.

Integrative and conjugative elements (ICEs), called SXT elements, contained AMR genes have been reported in *V. parahaemolyticus* (Kitiyodom et al., 2010). ICEs are self-transmissible mobile genetic elements encoded by the integrase gene (int_{SXT}) that can integrate into and excise from the bacterial chromosome using its own integrase gene and can be transferred to a new host by conjugation. Finding of SXT

element has been associated with MDR including chloramphenicol (*floR*), streptomycin (*strA* and *strB*), sulfamethoxazole (*sul2*), trimethoprim (*dfrA1* and *dfr18*), and tetracycline (*tetA*) (Ceccarelli et al., 2006; Kitiyodom et al., 2010).

At present, data on the distribution of AMR and genetic characteristics of *V. parahaemolyticus* including mechanisms underlying AMR and virulence factor genes among oyster meat and estuarine water is still limited in southern Thailand. This information would assist to better understand the emergence and spread of AMR on *V. parahaemolyticus* in the environment. Obtaining on molecular epidemiological data of AMR bacteria from aquatic animals and their environment is very useful for future AMR monitoring and risk assessment in aquaculture settings in Thailand. The linkage between AMR genes, mobile genetic elements and virulence factor genes of *V. parahaemolyticus* will assist for a better understanding of the dissemination of AMR in the aquatic environment. In addition, the results obtained from this study can be used in further studies to demonstrate the association between aquatic animals and the environment on AMR and genetic elements.

Objectives of the study

1. To detect the virulence factor genes of *V. parahaemolyticus* isolated from cultivated oysters and estuarine waters in southern Thailand.

2. To determine the prevalence of AMR among *V. parahaemolyticus* isolated from cultivated oysters and estuarine waters in southern Thailand.

3. To detect AMR determinants including AMR genes, class 1, 2 and 3 integrons, and integrative and conjugative elements (ICEs) of *V. parahaemolyticus* isolated from cultivated oysters and estuarine waters in southern Thailand.

CHAPTER II

LITERATURE REVIEW

1. General characteristics and pathogenesis of *V. parahaemolyticus*

Vibrio spp. belongs to the family *Vibrionaceae,* which is a Gram-negative, halophilic (grows in 0.5-10% NaCl), curved or rod-shaped, facultatively anaerobic, oxidase positive, sucrose negative, and non-spore forming bacteria. Vibrios are approximately 0.5-0.8 μm in diameter and 1.4-2.4 μm long. *V. parahaemolyticus* composed of two types of flagella, a polar flagellum which facilitates high motility in liquid media and lateral flagella that assists to move through the semi-solid surface by swarming (Yeung and Boor, 2004). This bacterium naturally presents in marine creatures, crustaceans, planktons, bivalves, fish, coastal waters, and estuary environments.

In general, *V. parahaemolyticus* grows at a wide range of temperatures between 15°C and 44°C. The pH for growth of *V. parahaemolyticus* is between 4.8 and 11.0 within an optimum pH of 7.8-8.6. Thiosulfate citrate bile salts sucrose (TCBS) agar has been recommended as a selective medium for the isolation of *V. parahaemolyticus*, which can be identified as a green color colony in this medium (Kaysner and DePaola, 2004). However, the typical colonies of *V. parahaemolyticus* in TCBS agar plate are very difficult to distinguish visually from the colonies of other *Vibrio* spp., because they might be covered by a yellow color produced by sucrose fermenting bacteria (Hara-Kudo et al., 2001). CHROMagar™ *Vibrio* (CHROMagar; Paris, France) is another selective medium which allows for the isolation and identification of *Vibrio* spp. by using chromogenic technology, resulting in *V. parahaemolyticus* can be distinguished as mauve colonies from other bacteria (Figure 1).

A B

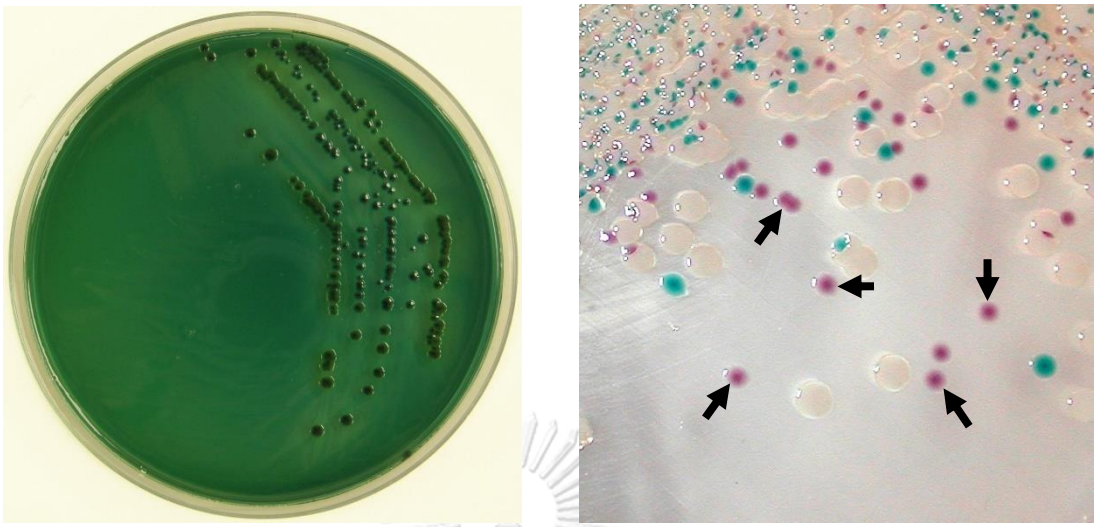

Figure 1: Colonies of *V. parahaemolyticus* in different agar (A) green colonies on TCBS agar and (B) mauve colonies on CHROMagar *Vibrio* agar

According to its pathogenicity, strains of *V. parahaemolyticus* can be classified by serotyping, and the serotypes can be determined by somatic (O) and capsular (K) antigens patterns. Currently, 13 O serotypes and 71 K serotypes have been identified (Li et al., 2016; Han et al., 2017). The predominant serotype of *V. parahaemolyticus* is O3:K6 which is distributed globally. The O3:K6 serotype was also responsible for a major outbreak, which affected 13,607 acute diarrheal patients in Kolkata, India in 1996. The existence serovariants of O3:K6 consisting of O1:K25, O1:K41, O1:KUT, O4:K12, and O4:K68 which were responsible for regional outbreaks presented in South and South East Asia (Bhunia, 2018). In southern Thailand during 1999, the pandemic serovar O3:K6 and three serovars of O1:K25, O1:K41, and O4:K12 was reported in 76% of the clinical *V. parahaemolyticus* strains (n=317) isolated from stool samples of diarrhea patients in Songklanagarind and Hat Rai hospitals (Chowdhury et al., 2000; Laohaprertthisan et al., 2003; Nair et al., 2007).

Infection of *V. parahaemolyticus* that harboring *tdh* and/or *trh* can cause acute gastroenteritis in humans. These virulence genes can be transmitted to humans through the consumption of contaminated seafood products such as oysters, clams, mussels, crabs, shrimps, and fish. The infective dose is approximately 10^6 organisms. Clinical signs of *V. parahaemolyticus* infection in humans are gastroenteritis, acute diarrhea, abdominal cramps, headache, nausea, fever, vomiting, and septicemia. Major clinical syndromes of vibrio infection are approximately 60-80% gastroenteritis, 34% of wound infections, and 5% septicemia (Elbashir et al., 2018). In fatal cases of the infection of *V. parahaemolyticus*, septicemia may occur in patients with immunocompromised or pre-existing medical conditions which including cancer, heart disease, recent gastric surgery, liver disease, and diabetes. Non-toxigenic *V. parahaemolyticus* strains, however, can cause gastroenteritis (Ottaviani et al., 2012).

2. Occurrence and epidemiology of *V. parahaemolyticus*

Infection of *V. parahaemolyticus* in humans is primarily transmitted by the fecal-oral route, indirectly through contaminated seafood products, raw consumption, and water supplies. Seafood poisoning from *V. parahaemolyticus* infection generally occurs during the summer (June to October) and is strongly associated with raw consumption or partially cooked of contaminated seafood products including shellfish, shrimps, crabs, lobster, and fish (Wang et al., 2015).

V. parahaemolyticus is the most common pathogen that has been found in the aquatic environment and isolated from seafood. The bacterium was found to be prevalent among oysters (48.8-100%), clams (63.9-100%), scallops (55.0-60.0%), mussels (34.0-68.1%), cockles (7.5-62.0%), shrimps (7.1-57.8%), crabs (20%), and fish (2.9-45.1%) (Odeyemi and Stratev, 2016). According to a meta-analysis of the

occurrence of *V. parahaemolyticus* from 2003 to 2015, the overall prevalence was 63.4% in oysters, 52.9% in clams, 51.0% in fish, 48.3% in shrimps, and 28.0% in mussel, scallop, and periwinkle, respectively (Odeyemi, 2016). In Thailand, the prevalence of *V. parahaemolyticus* was isolated from raw oysters were greater than 70% up to 100%, and 12% of the samples were present in Ang Sila coast, Chon Buri Province (Changchai and Saunjit, 2014). Additionally, the prevalence of *V. parahaemolyticus* was observed 100% from the samples isolated from oyster meat with an average of 8.5×10^7 ($\pm 3.8 \times 10^6$) MPN/g of oyster and estuarine water with an average of 3.8×10^5 ($\pm 4.7 \times 10^4$) MPN/100 ml (Jeamsripong et al., 2018).

In estuarine water, the occurrence of *V. parahaemolyticus* is influenced by seasons, and the high prevalence of vibrios found in the warmer months (Nelapati et al., 2012). In aquatic ecosystems, oysters, clams, and mussels which are filter-feeding shellfish can concentrate vibrios in their guts during filtration (Dame, 2016; Froelich and Noble, 2016).

The outbreaks of *V. parahaemolyticus* occur in different parts of the world. In the U.S., *V. parahaemolyticus* was first reported as an etiological agent after three outbreaks with 425 cases, and the main route of infection is the consumption of undercooked crabs (Molenda et al., 1972). In 1997, the largest outbreak of *V. parahaemolyticus* associated with raw oyster consumption occurred in British Columbia and Washington, California, and Oregon in the U.S. (U.S centers for disease control and prevention (CDC), 1998). Later in 1998, two *V. parahaemolyticus* outbreaks from the raw oyster consumption were reported in Texas (416 cases) and Washington (43 cases) (Letchumanan et al., 2014). Most recently, multistate outbreaks of *V. parahaemolyticus* involving 104 cases with six hospitalizations caused by consuming raw shellfish (Newton et al., 2014).

V. parahaemolyticus was first discovered as a causative agent of the largest foodborne outbreak in Japan by Tsunesaburo Fujino in 1950 with 272 illnesses and 20 deaths after ingestion of shirasu or the fry of sardines *(*Chen et al., 2017*).* Currently, the infection of *V. parahaemolyticus* is considered as a typical cause of seafood-borne pathogen in many Asian countries. During 1996-1998, *V. parahaemolyticus* was a major cause of food poisoning affected 24,373 cases in Japan, which were accounted for 78.4% of all foodborne disease outbreaks in Taiwan from 1999 to 2008 (Chang et al., 2011). *V. parahaemolyticus* has been responsible for 20-30% of foodborne infection in Japan (Jahangir Alam et al., 2002). From 2006 to 2010, *V. parahaemolyticus* infection occurred up to 17.1% of all bacterial foodborne disease outbreaks in Korea (Kim et al., 2012). During 2003 to 2008, a total of 322 outbreaks of *V. parahaemolyticus* involved 9,041 cases with 3,948 hospitalizations in China (Wu et al., 2014). Sporadic cases (n=319) of *V. parahaemolyticus* outbreak were reported in Thailand due to the consumption of bloody clams, and the outbreak was increasingly observed during summer with the highest prevalence of *V. parahaemolyticus* in August (FAO/WHO, 2011).

3. Virulence factor genes in *V. parahaemolyticus*

Different virulence factor genes of *V. parahaemolyticus* are *tdh*, *trh*, *tlh*, and type III secretion systems (T3SS1 and T3SS2) (Wang et al., 2015). Among these virulence factors, *tdh* and *trh* are considered as major virulence factor genes of *V. parahaemolyticus* infection. The *tlh* gene is considered as a species-specific marker for all clinical and environmental samples. The prevalence of *tdh* was reported 90-95% of human clinical cases (Wang et al., 2015). The *tdh* acts as a pore-forming toxin in the enterocyte plasma membrane to facilitate entry of Ca^{2+}, Na^{+}, and Mn^{2+}, and to trigger secretory diarrhea. If the absence of these virulence factor

genes containing hemolysins, *V. parahaemolyticus* remains pathogenic indicating that the existence of other virulence traits. The pathogenesis of *trh* and *tdh* found in *V. parahaemolyticus* infection is very similar. These genes can be phenotypically detected by the presence of ß-hemolysis ring on Wagatsuma blood agar, which is known as the Kanagawa Phenomenon (KP). The presence of *tdh* in *V. parahaemolyticus* from seafood products can pose a serious public health threat. In India, 55.3% (94/170) of the *V. parahaemolyticus* isolates obtained from fish and shellfish samples were positive to *tdh* (Das et al., 2016). The prevalence of *tdh* has been reported 10-12% in seafood. In Brazil, two out of 23 (8.7%) of *V. parahaemolyticus* isolated from oyster samples were detected *tdh* (Rojas et al., 2011). In the UK, a total of 12% (n=6/49) of *V. parahaemolyticus* isolated from shellfish were positive *tdh* (Wagley et al., 2008). However, the high prevalence of *tdh* and *trh* (52%, n=75/144) had observed in *V. parahaemolyticus* derived from sediment, seawater and shrimp samples (Velazquez-Roman et al., 2012).

The high prevalence of these virulent genes has been increasingly reported in clinical isolates than those from environmental samples. In China, 83.5% of clinical isolates and 22.0% of sea fish isolates were positive to *tdh (*Hongping et al., 2011*).* In the Maryland Chesapeake Bay, *V. parahaemolyticus* isolated from estuarine water *(*n=33*)*, and oyster samples *(*n=33*)* showed that 13% of estuarine waters and 20% of oysters contained *tdh* (Parveen et al., 2008*).* In Poland, approximately 2.9% *(*n=3*/*104*)* of *V. parahaemolyticus* isolates from raw shellfish originated from various countries were identified as *tdh* positive *(*Lopatek et al., 2018*).*

In Japan, a total of 24.3% (n=52/215) of *V. parahaemolyticus* isolates from diarrhea patients were found *trh* in the clinical samples (Shirai et al., 1990). During 2006 to 2010, *V. parahaemolyticus* isolates (n=776) from clinical cases were reported $tdh^{+}trh^{-}$ (63.3%), $tdh^{+}trh^{+}$ (4.4%), and $tdh^{-}trh^{+}$ (1.0%) from Hat Yai hospital in Thailand.

According to the finding of these studies, the distribution of *tdh* and/or *trh* virulence factor genes in *V. parahaemolyticus* were usually lower in environmental and food samples than those of the clinical samples.

4. Antimicrobial resistance of *V. parahaemolyticus*

Over the past few decades, AMR has been recognized as an important public health threat. Antimicrobials are used in farm animals for several reasons as therapeutics, metaphylaxis, prophylactics, and growth promotion. Misuse or overuse of antimicrobials in food-producing animals and humans can lead to selection pressure and can spread resistant bacteria (Woolhouse et al., 2015). AMR and MDR strains can disseminate into the environment due to the imprudent use of antimicrobial drugs in human, veterinary, community, agriculture, and aquaculture settings. The sources of AMR bacteria and their resistance genes in the aquatic environment can come from the farm wastes and sewage through wastewater, effluent, and stormwater (Watts et al., 2017). Once AMR bacteria, which harboring resistance genes are disseminated into the estuary environment, oysters can easily be contaminated with these bacteria and resistance genes during their filtration of estuarine water. The possible pathways for the spread of AMR bacteria and their resistance genes into the aquatic environment are illustrated in Figure 2.

The U.S. Centers for Disease Control and Prevention (CDC) recommends aminoglycosides (amikacin and gentamicin), cephalosporin (cefotaxime and ceftazidime), fluoroquinolones (levofloxacin), and folate pathway inhibitors (trimethoprim-sulfamethoxazole) for treatment of *Vibrio* infection in humans (Letchumanan et al., 2015). These antimicrobial agents are highly susceptible to many clinical cases of humans infection with *V. parahaemolyticus*.

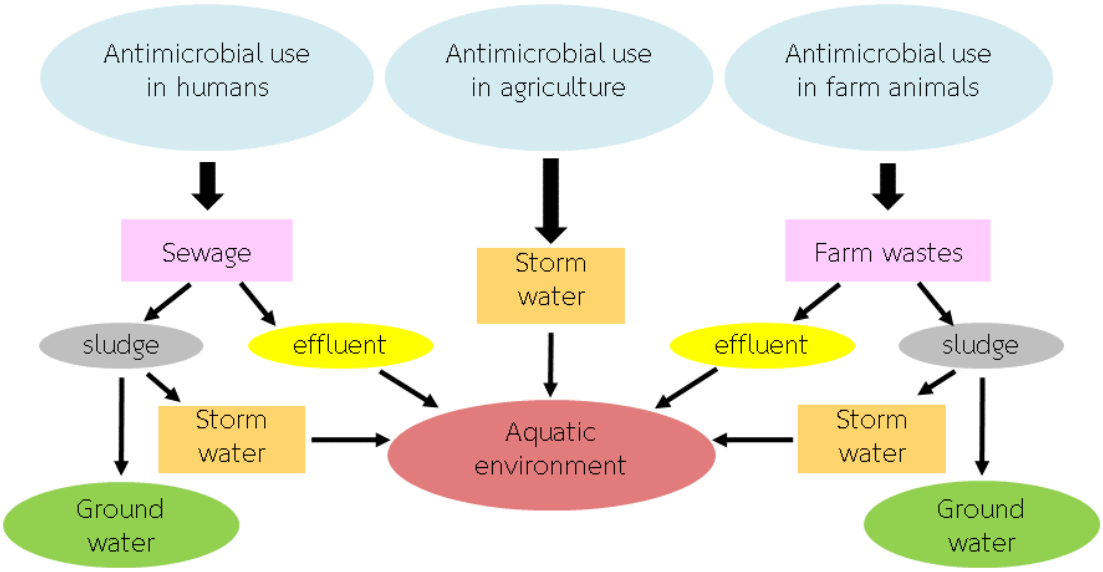

Figure 2: Possible pathways for the spread of multidrug resistant bacteria and resistance genes into the aquatic environment (source: FAO, 2017)

AMR of *V. parahaemolyticus* isolates has been reported from different sources such as in the environment, oysters, and human clinical cases. For example, *V. parahaemolyticus* isolates (n=39) from fresh oysters in Brazil were resistant to vancomycin (97.4%), penicillin (92.3%), ampicillin (48.6%), and erythromycin (15.4%), respectively, while resistance rates among frozen oysters (n=48) were vancomycin (100%), penicillin (100%), ampicillin (95.8%), and erythromycin (33.3%), respectively. In addition, MDR bacteria was also observed 94.9% of fresh oysters and 100% of frozen oysters and the most frequent of MDR patterns were vancomycin-penicillin-ampicillin (Costa et al., 2015).

In the previous study from northeastern Thailand, the high prevalence of AMR to ampicillin were observed in clinical isolates from diarrheal patients (97.3%), while a few isolates were resistant to norfloxacin (4.1%), tetracycline (4.1%), trimethoprim-sulfamethoxazole (4.1%), gentamicin (2.7%), ofloxacin (2.7%), and cefotaxime (1.4%), respectively. However, cockle samples were resistant only to ampicillin (83.8%) (Mala

et al., 2016a). For environmental samples, *V. parahaemolyticus* isolates from surface water from the Chesapeake Bay and the Maryland Coastal Bays were found that 68.0% and 53.0% resistance to penicillin and ampicillin, respectively (Shaw et al., 2014).

During 2001 and 2002 in Thailand, a total of 95 *V. parahaemolyticus* isolates from acute diarrhea patients showed that 52% of isolates were resistant to ampicillin and sulfisoxazole, while all isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole (Serichantalergs et al., 2007). A recent study in Poland reported that *V. parahaemolyticus* isolates (n=104) from imported raw shellfish samples were resistant to ampicillin (75.0%), streptomycin (68.3%), and gentamicin (12.5%), however, one isolate was resistant to ciprofloxacin and all isolates were susceptible to tetracycline and chloramphenicol (Lopatek et al., 2018). In Egypt, all *V. parahaemolyticus* isolates (n=36) from shrimp and crab samples were resistant to ampicillin, ampicillin-sulbactam, and tetracycline, whereas most of the isolates were resistant to ceftazidime (97.2%), ciprofloxacin (91.7%), cefotaxime (91.7%), trimethoprim-sulfamethoxazole (75%), kanamycin (72%), nalidixic acid (69.4%), chloramphenicol (61.1%), gentamicin (50%), and amikacin (30.6%), respectively (Ahmed et al., 2018).

5. Integrons in *V. parahaemolyticus*

Integrons are mobile genetic elements that can capture, integrate and express the exogenous genes. These integrons can play an important role in the distribution and dissemination of AMR genes among Gram-negative bacteria. There are two groups of integrons which are super-integrons and resistance integrons. Super-integrons are primarily found in the chromosomes of the bacteria, while resistance

integrons are located on transposons or plasmids. Among the nine classes of integrons, class 1 integron is the most commonly reported (Fluit and Schmitz, 2004). The structure of class 1 integrons consists of two conserved regions (CS), 5'-CS and 3'-CS. The 5' conserved segment contains the *intI* gene, which is responsible for the insertion and excision of the gene cassettes, a promoter region, and an *attI1* site is responsible for the integration of the gene cassettes. The 3' conserved segment consists of *qacEΔ1* encoding the genes resistance to quaternary ammonium compounds and sulfonamides (*sul1*) (Deng et al., 2015). Between 5' and 3' conserved segment of the integron, a variable region contains one or more gene cassettes encoding AMR genes. In Angola, class 1 integron was detected in *V. parahaemolyticus* isolates obtained from human clinical cases, which carried *dfrA15* gene cassettes (Domingues et al., 2015). During 2009 and 2010 in China, all *V. parahaemolyticus* isolates (n=87) from sea cucumbers samples were possessed class 1 integrons, while none of the isolates were positive to class 2 and 3 integrons (Jiang et al., 2014).

For class 2 integrons, 5' conserved segment contains *intI2* and a variable region containing gene cassettes and open reading frame (*orfX*) of unknown function. It has the transposition (*tns*) responsible for transposase binding in mobility of transposon. Class 2 integrons were previously reported in only two isolates of *V. cholerae*, and one isolate derived from seafood samples or environmental samples in Bangladesh (Ahmed et al., 2006). Class 3 integrons have never been reported in *V. parahaemolyticus.* These integrons contain 5'-CS where an *intI3* gene can be isolated, and a variable region contains gene cassettes and open reading frame (*orfX*) of unknown function (Cao et al., 2014). The genetic structure of class 1, 2, and 3 integrons are shown in Figure 3.

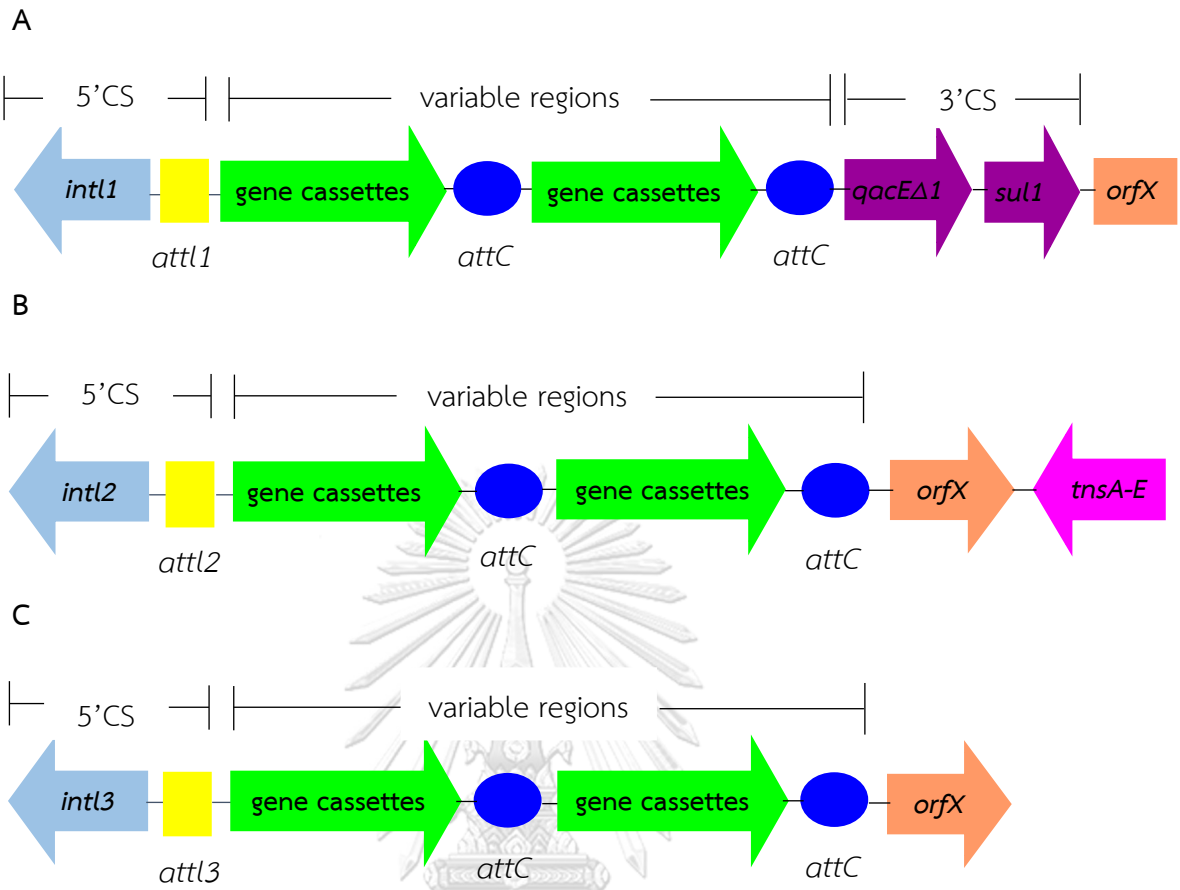

Figure 3: Genetic structure of integrons (A) class 1 integrons, (B) class 2 integrons, and (C) class 3 integrons. The type 1, 2, and 3 integrase enzymes are encoded by the *intI1*, *intI2*, and *intI3* genes, respectively. The *attI1*, *attI2*, and *attI3* are the integron-associated recombination sites which are responsible for the site-specific integration of gene cassettes. Gene cassettes are inserted into an array through recombination between attI and the cassette-associated *attC* recombination sites. The *tnsA-E* is transposition genes and the *orfX* is an open reading frame of unknown function (modified from Kaushik et al., 2018)

6. Role of integrative and conjugative elements (ICEs) in *V. parahaemolyticus*

Integrative and conjugative elements (ICEs) encoding the integrase gene (int_{SXT}) are self-transmissible mobile genetic elements that belong to the SXT/R391 family. These elements had described in *V. cholera* O139, and they can mobilize plasmid and genomic DNA from one to another strain (Spagnoletti et al., 2014). SXT element has been associated with MDR such as chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline (Marin et al., 2014).

In previous studies, the SXT element had been detected in *V. parahaemolyticus* and *V. cholera* strains derived from clinical and environmental samples carrying AMR genes related with the SXT^{MO10} or R391, which were encoded with florfenicol (*floR*), sulfonamides (*sul2*) and streptomycin (*strA* and *strB*) (Kitiyodom et al., 2010; Mala et al., 2016b). Another study in South Africa, SXT elements encoding resistance to florfenicol (*floR*), streptomycin (*strB*), sulfonamides (*sul2*), trimethoprim (*dfrA1* and *dfr18*), and tetracycline (*tetA*) of *V. parahaemolyticus* isolates had reported in wastewater samples (Okoh and Igbinosa, 2010). In Mozambique, three out of six *V. parahaemolyticus* isolates from estuarine water and shellfish samples were positive to SXT integrase gene (Taviani et al., 2008).

CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 3 phases; phase 1: Confirmation of *V. parahaemolyticus* and detection of virulence genes, phase 2: Phenotypic characterization of AMR, and phase 3: Genotypic characterization of AMR isolated from oyster meat and estuarine water samples. The research design is shown in Figure 4.

Vibrio parahaemolyticus isolates

A total of 594 presumptive *V. parahaemolyticus* isolates derived from pooled oyster meat samples (n=361) and estuarine waters (n=233) were received from the stock isolates of the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. Pooled oyster meat and estuarine water samples were collected between March 2016 and February 2017 from Thap Put district, Phang Nga province in southern Thailand. The location of the sampling site is shown in Figure 5.

Vibrios were isolated followed by the standard methods as described in the United States Food and Drug Administration (U.S. FDA) Bacteriological Analytical Manual (Kaysner and DePaola, 2004). All bacterial strains were stored at -80°C in 20% glycerol stocks for further studies.

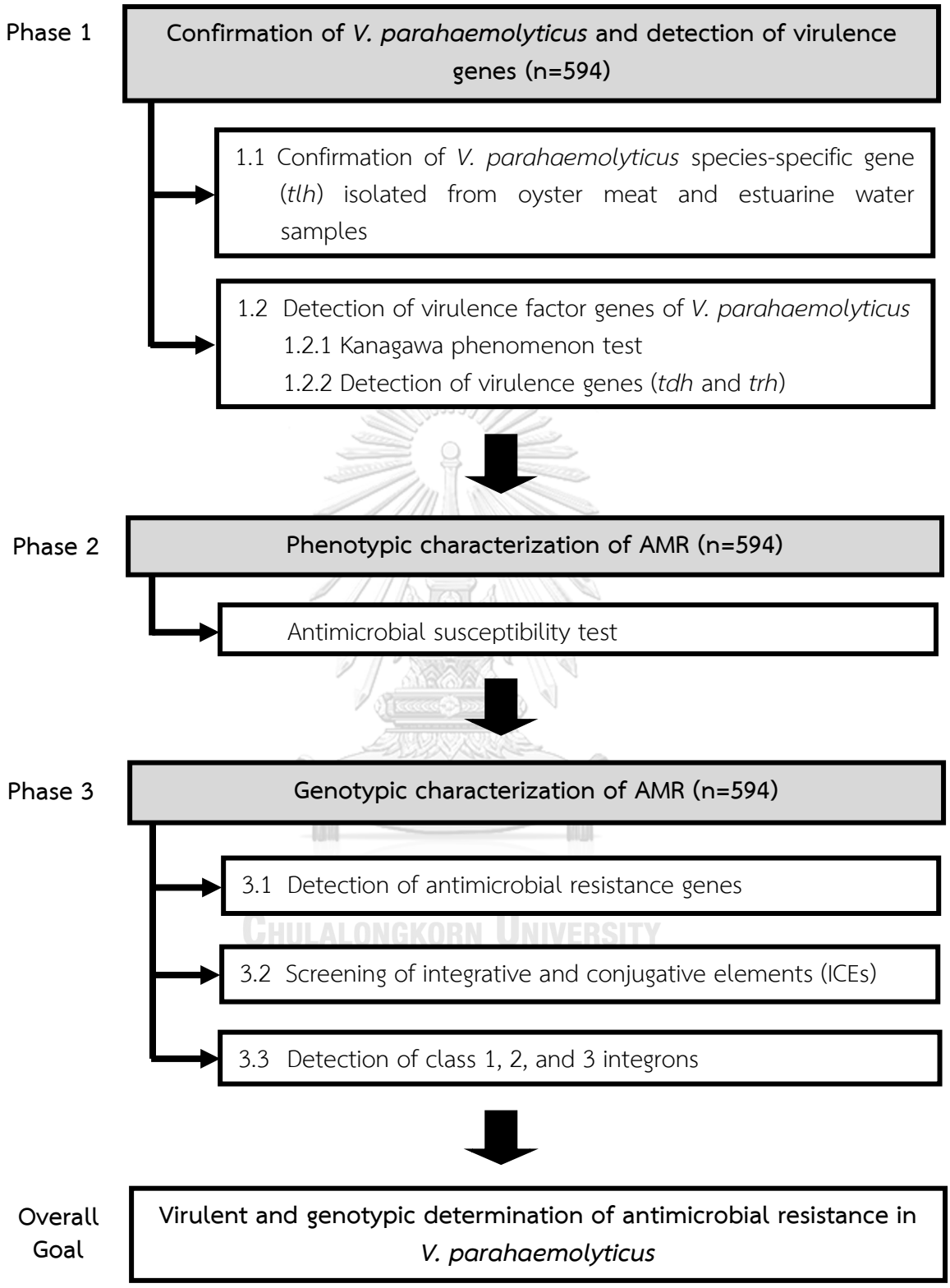

Figure 4: The research design of this experiment

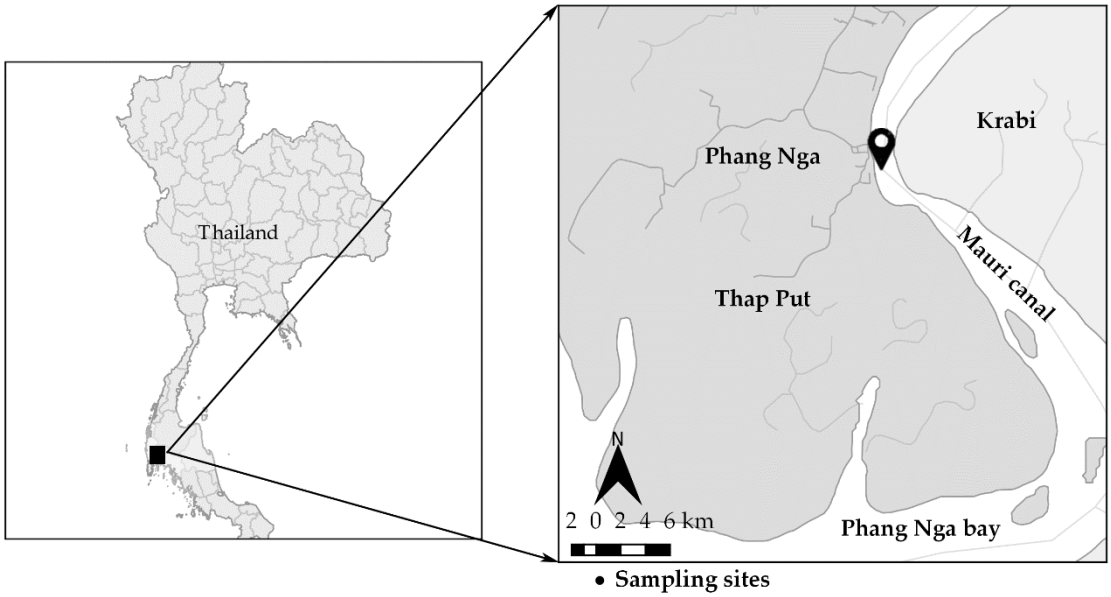

Figure 5: Map of the location of the sampling site at Thap Put district in Phang Nga province

Phase 1: Confirmation of *V. parahaemolyticus* and detection of virulence factor genes

1.1 Confirmation of *V. parahaemolyticus* species-specific gene (*tlh*) isolated from oyster meat and estuarine water samples (n=594)

Confirmation of *V. parahaemolyticus* was performed by PCR amplification method using species-specific gene (*tlh*) with specific primers as previously described (Bej et al., 1999). The primer pairs used for the confirmation of *V. parahaemolyticus* are listed in Table 1.

For DNA preparation, DNA template was prepared by the whole cell boiled lysate method as previously described (Scarano et al., 2014). *V. parahaemolyticus*

isolates were grown on tryptic soy agar (TSA; Difco, MD, USA) plate supplemented with 2% NaCl. A single colony was inoculated into two ml of Luria Bertani broth (LB; Difco) containing 3% NaCl. After incubation at 37°C for 24 hr, one ml of broth culture was centrifuged at 13,000 x g for 5 min, and the pellet was resuspended in 500 µl of sterile distilled water. The samples were boiled for 10-15 min to facilitate cell lysis and immediately placed on ice for 10 min. Then, the cell lysate was recentrifuged at 5,000 x g for 5 min. A total of 200 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube and stored at -20°C until used.

PCR assays were performed in a final volume of 25 µl using TopTaq Master Mix Kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. Each PCR reaction contained 2 µl of DNA template, 1.0 µl of each forward and reverse primer at 10 µM, 12.5 µl of master mix, 2.5 µl of coralload PCR buffer, and 6 µl of sterile distilled water to make a final volume of 25 µl. PCR amplification for *tlh* was carried out on a PCR Tpersonal combi model (Biometra®, Göttingen, Germany) using the following thermal cycling conditions: an initial denaturation 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, and extension at 72°C for 1 min. One cycle of the final extension was conducted at 72°C for 5 min. The gels were stained by using RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology®, Sungnam, Korea). The separation of PCR products was conducted by electrophoresis on 1.5% agarose gel (Sigma-Aldrich®, St. Louis, MO, USA) in 1×Tris-acetate/EDTA (1×TAE) buffer. Visualization of PCR products was carried out under UV light by the Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, Ventura, CA, USA).

1.2 Detection of virulence factor genes of *V. parahaemolyticus*

1.2.1 Kanagawa phenomenon test

V. parahaemolytius isolates were examined for phenotypic determination of thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) virulence factor genes on Kanagawa phenomenon (KP) test as previously described (Honda et al., 1980). For each isolate, a single colony from TSA (Difco) plate supplemented with 3% NaCl was dropped on Wagatsuma agar plates (HiMedia, Mumbai, India) containing 2% fresh sheep red blood cells. After overnight incubation at 37°C, the isolates that produce ß-hemolytic zone around the colony were recorded as positive to KP. The positive KP isolate containing both *tdh* and *trh* was used as a positive control.

1.2.2 Detection of virulence factor genes (*tdh* and *trh*)

Multiplex PCR was conducted to detect the virulence genes (*tdh* and *trh)* of *V. parahaemolyticus* as previously described (Bej et al., 1999). All primers used for the multiplex PCR are listed in Table 1. The multiplex PCR reaction contained 12.5 µl of master mix, 2 µl of DNA template, 1.0 µl of each forward and reverse primer at 10 µM, 6 µl of sterile distilled water, and 2.5 µL of coralload PCR buffer.

Table 1: PCR primers used in this study

Primer	Sequence (5'-3')	Gene	Amplicon Size (bp)	References
Confirmation of *V. parahaemolyticus*				
tlhF	AAAGCGGATTATGCAGAAGCACTG	*tlh*	450	(Bej et al., 1999)
tlhR	GCTACTTTCTAGCATTTTCTCTGC			
Virulence factor genes				
tdhF	GTAAAGGTCTCTGACTTTTGGAC	*tdh*	269	(Bej et al., 1999)
tdhR	TGGAATAGAACCTTCATCTTCACC			
trhF	TTGGCTTCGATATTTTCAGTATCT	*trh*	500	(Bej et al., 1999)
trhR	CATAACAAACATATGCCCATTTCCG			
Antimicrobial resistance genes				
bla_{TEM}F	ATAAAATTCTTGAAGAC	bla_{TEM}	1075	(Letchumanan et al.,
bla_{TEM}R	TTACCAATGCTTAATCA			2015)
qnrF	TCTCGCTAAGGCTCGTAGC	*qnr*	902	(Poirel et al., 2005)
qnrR	TTCCTCGTCGAGGTTATTCG			
ermBF	AGACACCTCGTCTAACCTTCGCTC	*ermB*	640	(Raissy et al., 2012)
ermBR	TCCATGTACTACCATGCCACAGG			
strBF	GGCAGCATCAGCCTTATAATTT	*strB*	470	(Mala et al., 2016b)
strBR	GTGGATCCGTCATTCATTGTT			
sul2F	TGCGGATGAAGTCAGCTCC	*sul2*	623	(Mala et al., 2016b)
sul2R	GGGGGCAGATGTGATCGAC			
tetAF	GTAATTCTGAGCACTGTCGC	*tetA*	957	(Mala et al., 2016b)
tetAR	CTGCCTGGACAACATTGCTT			
dfrA1F	CAAGTTTACATCTGACAATGAGAACGTAT	*dfrA1*	277	(Mala et al., 2016b)
dfrA1R	ACCCTTTTGCCAGATTTGGTA			
dfr18F	ACTGCCGTTTTCGATAATGTGG	*dfr18*	389	(Mala et al., 2016b)
dfr18R	TGGGTAAGACACTCGTCATGGG			
Integrons				
int1F	CCTGCACGGTTCGAATG	*intl1*	497	(Kitiyodom et al.,
int1R	TCGTTTGTTCGCCCAGC			2010)
int2F	GGCAGACAGTTGCAAGACAA	*intl2*	247	(Kitiyodom et al.,
int2R	AAGCGATTTTCTGCGTGTTT			2010)
int3F	CCGGTTCAGTCTTTCCTCAA	*intl3*	155	(Kitiyodom et al.,
int3R	GAGGCGTGTACTTGCCTCAT			2010)
Integrative and conjugative elements				
intSXTF	GCTGGATAGGTTAAGGGCGG	int_{SXT}	592	(Kitiyodom et al.,
intSXTR	CTCTATGGGCACTGTCCACATTG			2010)

PCR amplification for *tlh, tdh,* and *trh* were conducted on a PCR Tpersonal combi model (Biometra®) using the following cycling conditions: an initial denaturation 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, extension at 72°C for 1 min, and a final extension at 72°C for 5 min, respectively. The PCR products were separated on 1.5% agarose gel (Sigma-Aldrich®, Steinheim, Germany) electrophoresis in 1×Tris-acetate/ EDTA (TAE) buffer applying 90 voltages for 45 min. The gels were stained with RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology®) and visualized by the Bio-Rad Gel-Documentation system (Bio-Rad Laboratories).

Phase 2: Phenotypic characterization of antimicrobial resistance

Antimicrobial susceptibility test

V. parahaemolyticus were examined for their susceptibilities to eight antimicrobial agents: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TRI). Antimicrobial susceptibility test was determined by Minimum Inhibitory Concentration (MIC) using the two-fold agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI M45, 2016) with slight modification. Antimicrobial agents were purchased from Sigma-Aldrich®. Different concentrations of antimicrobial agents were prepared with appropriate diluents. The diluents, concentrations, and breakpoints of antimicrobial agents are shown in Table 2.

Table 2: Solvents, concentration ranges, and breakpoints of tested antimicrobial agents

Antimicrobials	Solvent	Concentration ranges (µg/ml)	Breakpoint (µg/ml)		
			S	I	R
ampicillin[a]	sterile distilled water	0.25-512	≤8	16	≥32
chloramphenicol[a]	95% ethanol	0.25-1024	≤8	16	≥32
ciprofloxacin[a]	0.1 M NaOH and sterile distilled water	0.016-64	≤1	2	≥4
erythromycin[b]	95% ethanol	0.062-128	≤0.5	1-4	≥8
streptomycin[c]	sterile distilled water	1-1024	≤32	-	≥64
sulfamethoxazole[a]	0.1 M NaOH and sterile distilled water	1.19-1216	≤38	-	≥76
tetracycline[a]	70% ethanol	0.062-512	≤4	8	≥16
trimethoprim[a]	dimethyl acetamide	0.125-128	≤2	-	≥4

[a] MIC interpretative standard for *Vibrio* spp.

[b] MIC interpretative standard for *Enterococcus* spp.

[c] NARMS interpretative standard when CLSI standard was not available.

S, susceptible; I, intermediate; R, resistance

V. parahaemolyticus isolates were grown in Mueller-Hinton agar (MHA; Difco) and incubated at 37°C for 24 hr. The single colony was suspended in 0.85% NaCl solution (NSS), and turbidity of the cell suspension was adjusted to 0.5 McFarland standard approximately 10^8 or 0.5-1 × 10^8 CFU/ml. Then, the suspension was ten-fold diluted in 0.85% NSS to achieve a final cell count at 10^7 CFU/ml. The cell suspensions were transferred into the microtiter plates and inoculated onto the MHA plates containing proper concentrations of antimicrobials. After overnight incubation at 37°C, the MIC values were recorded as susceptible or resistant based on their breakpoints. MDR was defined as the isolates that are resistant to at least three or more different classes of antimicrobial agents. *Escherichia coli* (ATCC 25922*)* and *Staphylococcus aureus* (ATCC 29213) were used as quality control strains. When CLSI standards were not available for some antimicrobial agents such as the breakpoints described in the National Antimicrobial Resistance Monitoring System (NARMS, 2015) was applied.

Phase 3: Genotypic characterization of antimicrobial resistance

3.1 Detection of antimicrobial resistance genes

All *V. parahaemolyticus* isolates were detected for the presence of AMR genes based on their resistance phenotypes by using conventional and multiplex PCR (Table 3). PCR assays were performed in a final volume of 25 µl using TopTaq Master Mix (Qiagen®) containing 12.5 µl of master mix, 6 µl of sterile distilled water, 2 µl of DNA template, 1 µl of each forward and reverse primer at the final concentration of 10 µM and coralload PCR buffer to reach the final volume. The PCR primers used for the detection of AMR genes are listed (Table 1).

Multiplex PCR amplifications for *strB, sul2, tetA, dfrA1,* and *dfr18* were conducted on a PCR Tpersonal combi model (Biometra®) with slight modification (Mala et al., 2016b). Briefly, the thermal cycling conditions for multiplex PCR amplification was conducted with an initial denaturation at 95°C for 15 min, followed by 35 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 57°C for 1.5 min, primer extension at 72°C for 1.5 min and one cycle of final extension at 72°C for 10 min.

The thermal cycling conditions for PCR amplification of ampicillin resistance gene (bla_{TEM}) was started with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min, respectively. The final extension step was performed at 72°C for 6 min.

For PCR amplification of erythromycin resistance gene (*ermB*), it was performed as previously described (Raissy et al., 2012) using the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 40 s, extension at 72°C for 40 s and final extension at 72°C for 7 min.

The following PCR conditions were used for the detection of quinolones resistance gene (*qnr*) as previously described (Ma et al., 2018): an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 15 s, extension at 72°C for 50 s and final extension at 72°C for 7 min.

The PCR products were separated on 1.5% agarose gel electrophoresis in the 1×TAE buffer. The gels were stained with RedSafe nucleic acid staining solution (Intron Biotechnology) and visualization of PCR products was carried out under ultraviolet light by using the Bio-Rad Gel-Documentation system.

Table 3: Antimicrobial resistance phenotypes and their corresponding resistance encoding genes tested

Resistance phenotype	Resistance genes
Ampicillin	bla_{TEM}
Quinolones	*qnr*
Erythromycin	*ermB*
Streptomycin	*strB*
Sulfamethoxazole	*sul2*
Tetracycline	*tetA*
Trimethoprim	*dfrA1, dfr18*

3.2 Screening of integrative and conjugative elements (ICEs)

Isolates of *V. parahaemolyticus* were observed for the presence of SXT element by determining SXT integrase (int_{SXT}) using PCR amplification method (Kitiyodom et al., 2010). The primers used for screening of SXT element are listed (Table 1). Briefly, PCR assays were carried out in a final volume of 25 µl containing 12.5 µl of master mix, 2 µl of DNA template, 1 µl of each primer at 10 µM, 6 µl of sterile distilled water, and 2.5 µl of coralload PCR buffer. The following thermal cycling conditions were included: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were separated on 1.5% agarose gel electrophoresis in the 1×TAE buffer. The gels were stained with Red Safe nucleic acid staining solution (Intron Biotechnology®) and visualized under UV light using Gel-Documentation system (Bio-Rad Laboratories).

3.3 Detection of class 1, 2 and 3 integrons

The presence of class 1, 2, and 3 integrons in *V. parahaemolyticus* isolates were detected by multiplex PCR determining the integrase genes *intI1, intI2,* and *intI3* as previously described with a slight modification (Kitiyodom et al., 2010). The PCR primers for detection of integrons are listed in Table 1. Multiplex PCR amplification was conducted on a PCR Tpersonal combi model (Biometra®) using the following thermal cycling conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of amplification with denaturation at 95°C for 45 s, primer annealing at 52°C for 45 s, primer extension at 72°C for 1 min and a final extension at 72°C for 8 min. The PCR products were separated on 1.5% agarose gel (Sigma-Aldrich®) in the 1×TAE buffer and visualized under UV light by Gel-Documentation system (Bio-Rad Laboratories).

STATISTICAL ANALYSIS

The presence of AMR phenotype and genotype, and the occurrence of virulence genes, integrons, and SXT element were described using descriptive statistics. Pearson's Chi-square test was used to determine the association between oysters and estuarine waters on the prevalence of AMR rate and resistance genes. A *P*-value < 0.05 was considered as statistically significant different under the two-sided hypothesis test. Logistic regression analysis was performed to test the association between AMR genes in oysters and estuarine waters. An odds ratio greater than one (OR > 1) with *P*-value < 0.05 was considered as significance association. All statistical analysis was conducted using SPSS software Version 22.0 (IBM Corp, Armonk, NY, USA).

CHAPTER IV

RESULTS

1. Confirmation of *V. parahaemolyticus* and detection of virulence genes

1.1 The occurrence of *V. parahaemolyticus* species-specific gene (*tlh*) isolated from oyster meat and estuarine water samples (n=594)

The presumptive colonies of *V. parahaemolyticus* isolates (n=594) were confirmed species-specific (*tlh*) gene together with the detection for the presence of virulence genes (*tdh* and *trh*) by multiplex PCR. All isolates (100%) were positive to *tlh,* which yielded the amplicon size of 450 bp when the isolates were amplified with the specific primers. The PCR amplicons of samples and positive control of *tlh* are shown in Figure 6.

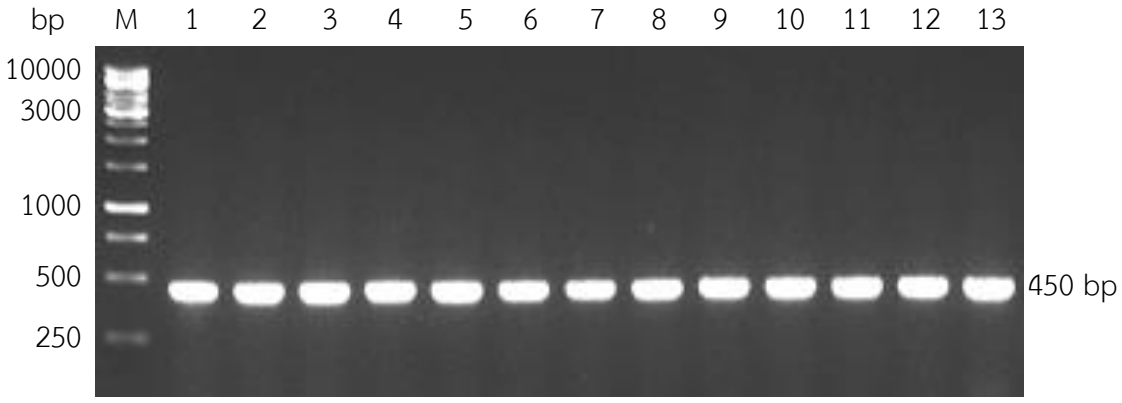

Figure 6: PCR amplicons of species-specific *tlh* gene. Lane M, 1 kb marker; Lane 1-12, *trh*-positive *V. parahaemolyticus* isolates; Lane 13, a positive control

1.2 The distribution of virulence factor genes in *V. parahaemolyticus*

1.2.1 Kanagawa phenomenon

Kanagawa phenomenon test was performed in 594 isolates of *V. parahaemolyticus* using Wagatsuma agar, supplemented with 2% sheep erythrocytes to detect the presence of *tdh* and *trh* virulence factor genes phenotypically. All isolates did not show ß-hemolytic zone around the bacterial colonies, meaning that none of the Kanagawa phenomenon positive was observed in this study.

1.2.2 Prevalence of virulence factor genes (*tdh* and *trh*)

Among *V. parahaemolyticus* isolates, four isolates were positive to *tdh* from pooled oyster meat (0.6%) and estuarine water (0.9%) samples (Table 4). PCR amplicons of *tdh* are shown in Figure 7. None of the isolates harboring *trh* gene was detected in this study.

Table 4: Distribution of *tdh* and *trh* virulence factor genes in *V. parahaemolyticus* isolated from pooled oysters and estuarine waters

Samples	Number of isolates	Number of positive genes (%)		
		tdh	*trh*	*tdh/trh*
Pooled oyster meat	361	2 (0.6%)	0	0
Estuarine water	233	2 (0.9%)	0	0
Total	594	4 (0.7%)	0	0

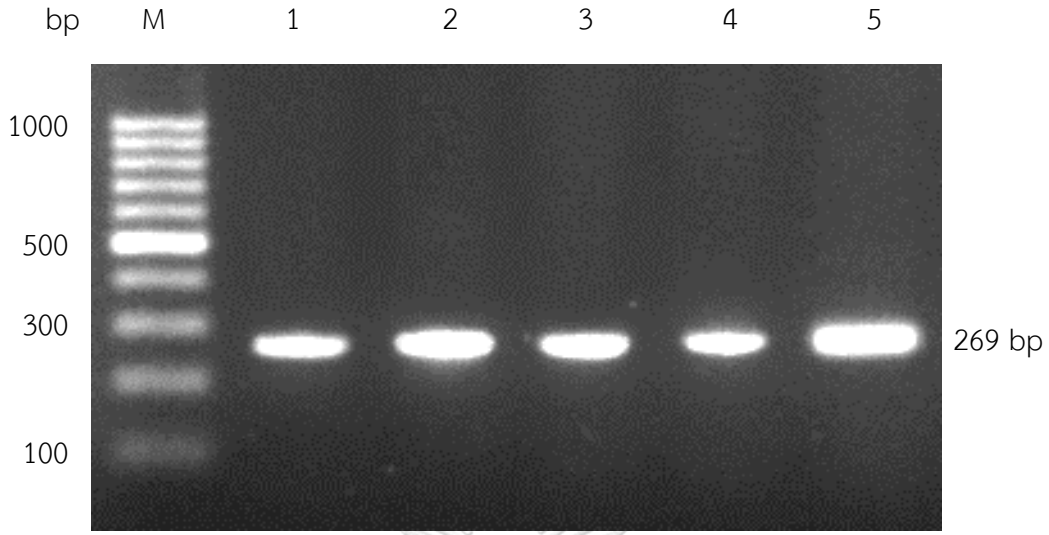

Figure 7: PCR amplicons of *tdh* gene. PCR amplifications were performed using primers tdhF and tdhR that yielded the amplicon size of 269 bp. Lane M, 100 bp marker; Lane 1-2, *tdh*-positive isolates from oysters; Lane 3-4, *tdh*-positive isolates from estuarine waters; Lane 5, a positive control isolate

2. Phenotypic characterization of antimicrobial resistance

Antimicrobial susceptibility test

The distribution of the antimicrobial susceptibility test was analyzed in all *V. parahaemolyticus* isolates (n=594) (Figure 8). *V. parahaemolyticus* isolates were resistant to erythromycin (54.2%), followed by sulfamethoxazole (34.7%). trimethoprim (28.0%), and ampicillin (11.1%), respectively. Only 0.8% resistant to streptomycin and 0.5% resistant to tetracycline. No chloramphenicol and ciprofloxacin resistance were detected in all isolates. Thirty-four percent of the isolates were resistant to at least one antimicrobial agent, while 155 isolates (26.0%) were susceptible to all antimicrobials tested. Multidrug-resistant bacteria were found on 5.1% of the isolates which was resistant to at least three different classes of antimicrobial agents.

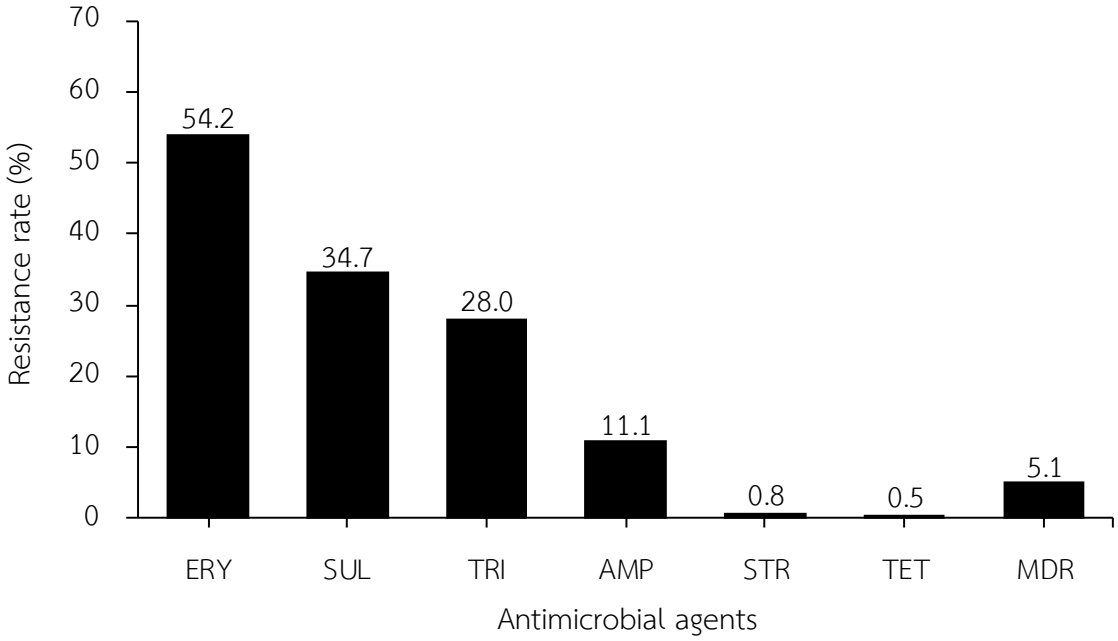

Figure 8: Distribution of antimicrobial resistance among *V. parahaemolyticus* isolates (n=594). Abbreviations: ERY, erythromycin; SUL, sulfamethoxazole; TRI, trimethoprim; AMP, ampicillin; STR, streptomycin; TET, tetracycline; MDR, multidrug-resistant

In oyster meat samples, *V. parahaemolyticus* isolates were resistant to erythromycin (53.7%) followed by sulfamethoxazole (33.5%), trimethoprim (27.4%), ampicillin (10.2%), and streptomycin (1.4%), respectively. The resistant rates of estuarine waters were found in erythromycin (54.9%), sulfamethoxazole (36.5%), trimethoprim (28.8%), ampicillin (12.4%), and tetracycline (1.3%). Antimicrobial susceptibilities to chloramphenicol and ciprofloxacin were observed in both oysters and estuarine waters. Among the *V. parahaemolyticus* isolates, MDR rates were found on 5.3% in pooled oyster and 4.7% in estuarine water samples, respectively (Figure 9).

Pearson's chi-square test was performed to compare the association of AMR rate between oyster meat and estuarine water samples (Table 5). In general, no marked differences in the percentages of resistance rate among the tested antimicrobials were observed between oysters and estuarine water samples ($P > 0.05$). This means that the prevalence of AMR rate was similarly distributed in both oysters and estuarine waters. No statistical analysis was performed on the AMR rate of chloramphenicol, ciprofloxacin, streptomycin, and tetracycline between oysters and estuarine waters due to no AMR.

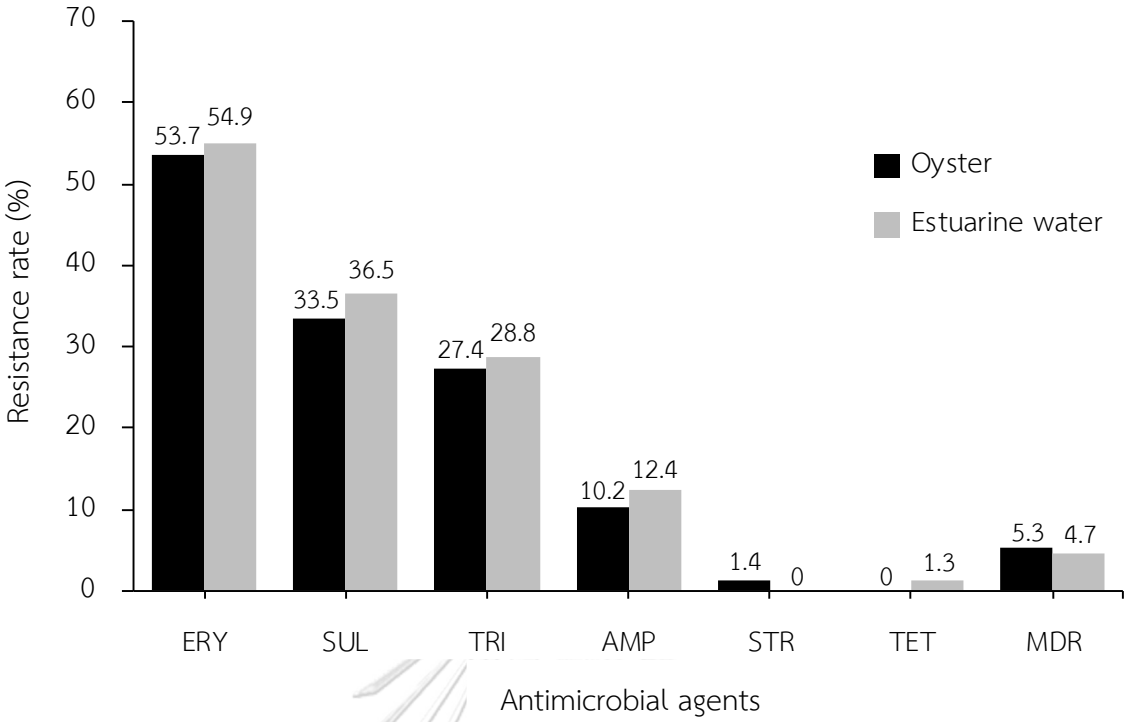

Figure 9: Distribution of antimicrobial resistance among *V. parahaemolyticus* isolates from pooled oyster meat (n=361) and estuarine water (n=233) samples. Abbreviations: ERY, erythromycin; SUL, sulfamethoxazole; TRI, trimethoprim; AMP, ampicillin; STR, streptomycin; TET, tetracycline; MDR, multidrug-resistant

Table 5: The association between oysters and estuarine water samples on the distribution of antimicrobial resistance rate in *V. parahaemolyticus*

Antimicrobials	Samples	Number of isolates	Resistance isolates (%)	OR (C.I.)[a]	*P*-value
Ampicillin	Oyster	361	37 (10.2%)		
	Estuarine water	233	29 (12.4%)	0.80 (0.48-1.35)	0.405
	Total	594	66 (11.1%)		
Erythromycin	Oyster	361	194 (53.7%)		
	Estuarine water	233	128 (54.9%)	0.95 (0.69-1.33)	0.775
	Total	594	322 (54.2%)		
Sulfamethoxazole	Oyster	361	121 (33.5%)		
	Estuarine water	233	85 (36.5%)	0.88 (0.62-1.24)	0.459
	Total	594	206 (34.7%)		
Trimethoprim	Oyster	361	99 (27.4%)		
	Estuarine water	233	67 (28.8%)	0.94 (0.65-1.35)	0.724
	Total	594	166 (28.0%)		

[a] Odds ratio (95% confidence interval)

Twenty different AMR patterns of *V. parahaemolyticus* were presented in Table 6. The most common resistance pattern was ERY (21.9%), followed by ERY-SUL (12%), ERY-SUL-TRI (8.4%), and TRI (6.9%), respectively. The most predominant MDR phenotype was ERY-SUL-AMP (1.9%) and ERY-SUL-TRI-AMP (1.2%).

AMR phenotypes of *V. parahaemolyticus* isolates were classified into 19 and 16 different patterns in oyster meat and estuarine water samples, respectively (Table 7). For oyster meat samples, the most common resistance pattern was ERY (21.0%) and the most common MDR phenotype was ERY-SUL-AMP (2.8%). For estuarine water isolates, the most common AMR pattern was ERY (23.2%) while the most predominant MDR phenotype was ERY-SUL-TRI-AMP (1.3%).

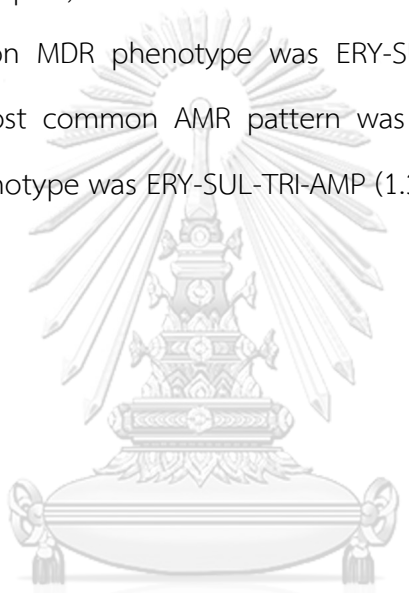

Table 6: Antimicrobial resistance patterns of *V. parahaemolyticus* isolates (n=594)

	Resistance patterns	Number of *V. parahaemolyticus* isolates (%)	
1	ERY	130	(21.9)
2	SUl	21	(3.5)
3	TRI	41	(6.9)
4	AMP	9	(1.5)
5	STR	1	(0.2)
6	ERY-SUL	70	(12.0)
7	ERY-TRI	25	(4.2)
8	ERY-AMP	19	(3.2)
9	ERY-STR	1	(0.2)
10	SUL-TRI	28	(4.7)
11	SUL-AMP	9	(1.5)
12	TRI-AMP	4	(0.7)
13	TRI-STR	1	(0.2)
14	ERY-SUL-TRI	50	(8.4)
15	ERY-SUL-STR	2	(0.3)
16	ERY-SUL-AMP	11	(1.9)
17	ERY-TRI-AMP	3	(0.5)
18	SUL-TRI-AMP	4	(0.7)
19	ERY-SUL-TRI-AMP	7	(1.2)
20	ERY-SUL-TRI-TET	3	(0.5)
	Total	439	(73.9)

Abbreviations: ERY, erythromycin; SUL, sulfamethoxazole; TRI, trimethoprim; AMP, ampicillin; STR, streptomycin; TET, tetracycline

Table 7: Antimicrobial resistance patterns of *V. parahaemolyticus* isolates among pooled oyster meat (n=361) and estuarine water (n=233) samples

	Resistance patterns	Number of isolates (%)	
		Pooled oyster (n=361)	Estuarine water (n=233)
1	ERY	76 (21.0)	54 (23.2)
2	SUl	13 (3.6)	8 (3.4)
3	TRI	29 (8.0)	12 (5.2)
4	AMP	2 (0.6)	7 (3.0)
5	STR	1 (0.3)	0 (0.0)
6	ERY-SUL	43 (11.9)	27 (11.6)
7	ERY-TRI	19 (5.3)	6 (2.6)
8	ERY-AMP	8 (2.2)	11 (4.7)
9	ERY-STR	1 (0.3)	0 (0.0)
10	SUL-TRI	12 (3.3)	16 (6.9)
11	SUL-AMP	7 (1.9)	2 (0.9)
12	TRI-AMP	3 (0.8)	1 (0.4)
13	TRI-STR	1 (0.3)	0 (0.0)
14	ERY-SUL-TRI	28 (7.8)	22 (9.4)
15	ERY-SUL-STR	2 (0.6)	0 (0.0)
16	ERY-SUL-AMP	10 (2.8)	1 (0.4)
17	ERY-TRI-AMP	2 (0.6)	1 (0.4)
18	SUL-TRI-AMP	1 (0.3)	3 (1.3)
19	ERY-SUL-TRI-AMP	4 (1.1)	3 (1.3)
20	ERY-SUL-TRI-TET	0 (0.0)	3 (1.3)
	Total	262 (72.6)	177 (76.0)

Abbreviations: ERY, erythromycin; SUL, sulfamethoxazole; TRI, trimethoprim; AMP, ampicillin; STR, streptomycin; TET, tetracycline

3. Genotypic characterization of antimicrobial resistance

3.1 The presence of antimicrobial resistance genes

All *V. parahaemolyticus* isolates (n=594) were analyzed for the presence of eight AMR genes including bla_{TEM}, *dfrA1, dfr18, ermB, qnr, sul2, strB,* and *tetA* (Figure 10-13).

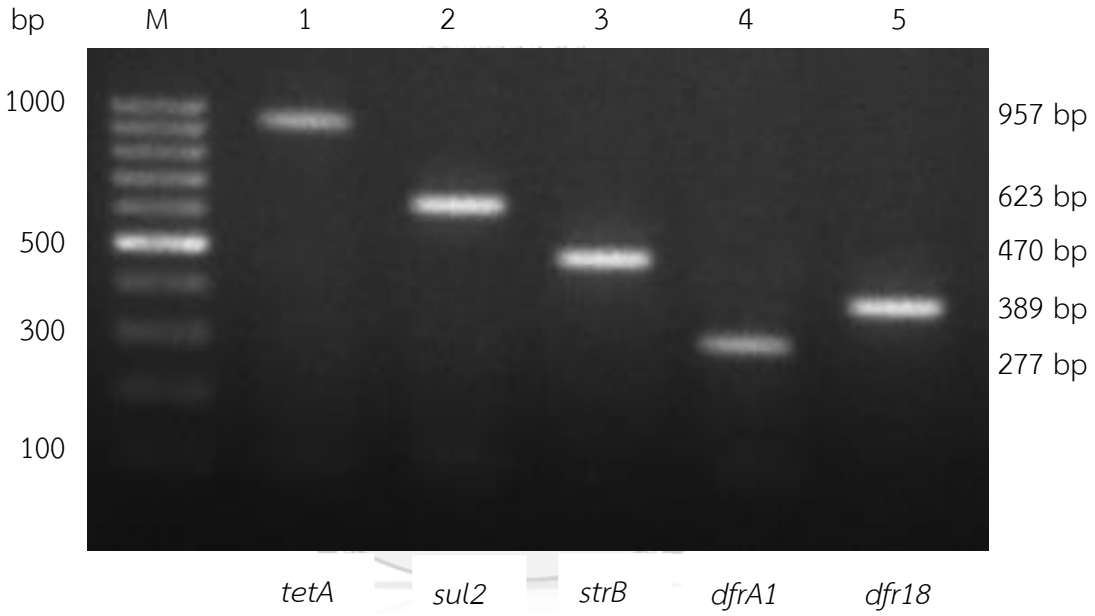

Figure 10: PCR amplicons of tetracycline, sulfamethoxazole, streptomycin, and trimethoprim resistance genes. Lane M, 100 bp marker; Lane 1-5, the result of the PCR reaction amplifying *tetA*, *sul2*, *strB*, *dfrA1*, and *dfr18*

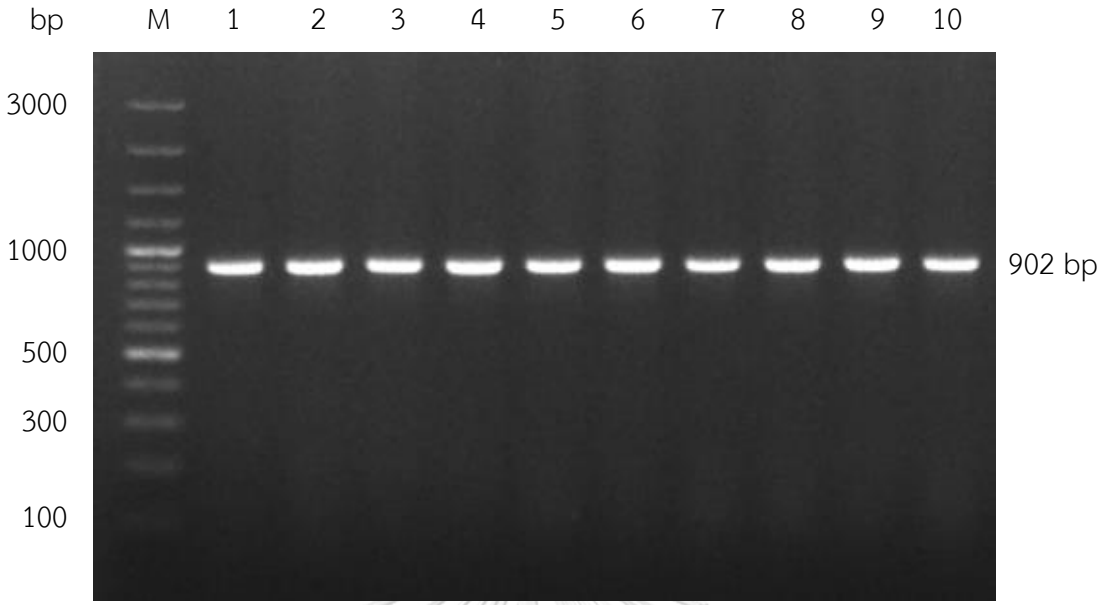

Figure 11: PCR amplicons of quinolone resistance gene. Lane M, 100 bp plus marker; Lane 1-9, *qnr*-positive *V. parahaemolyticus* isolates; Lane 10, positive control isolate

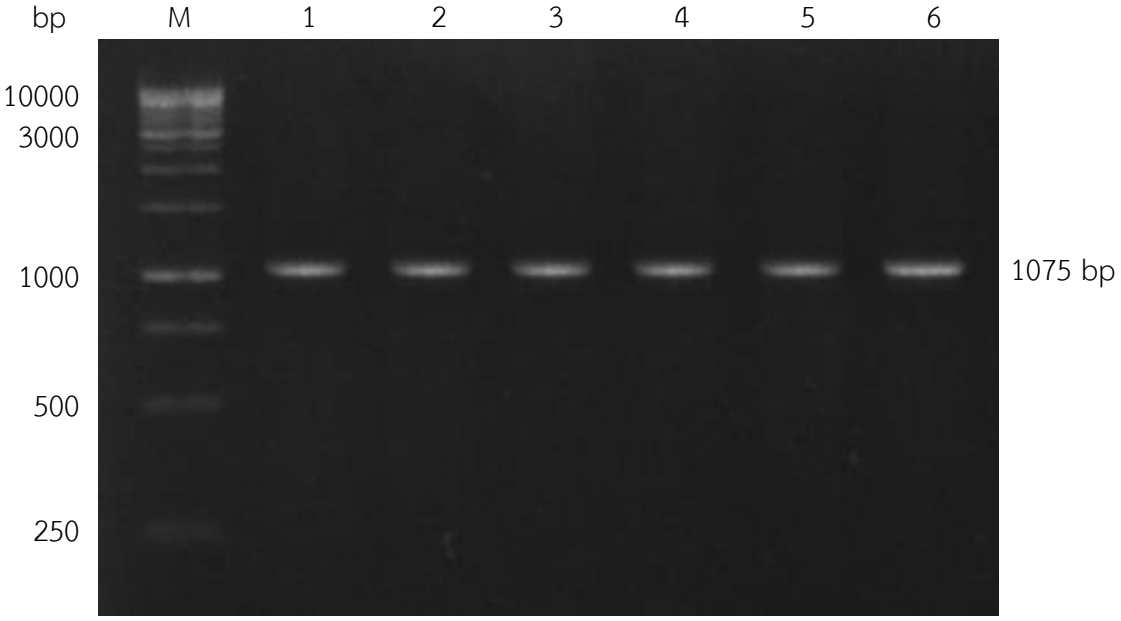

Figure 12: PCR amplicons of ampicillin resistance gene. Lane M, 1 kb marker; Lane 1, bla_{TEM}-positive isolate from estuarine water; Lane 2-5, bla_{TEM}-positive isolates from oyster meat; Lane 6, a positive control isolate

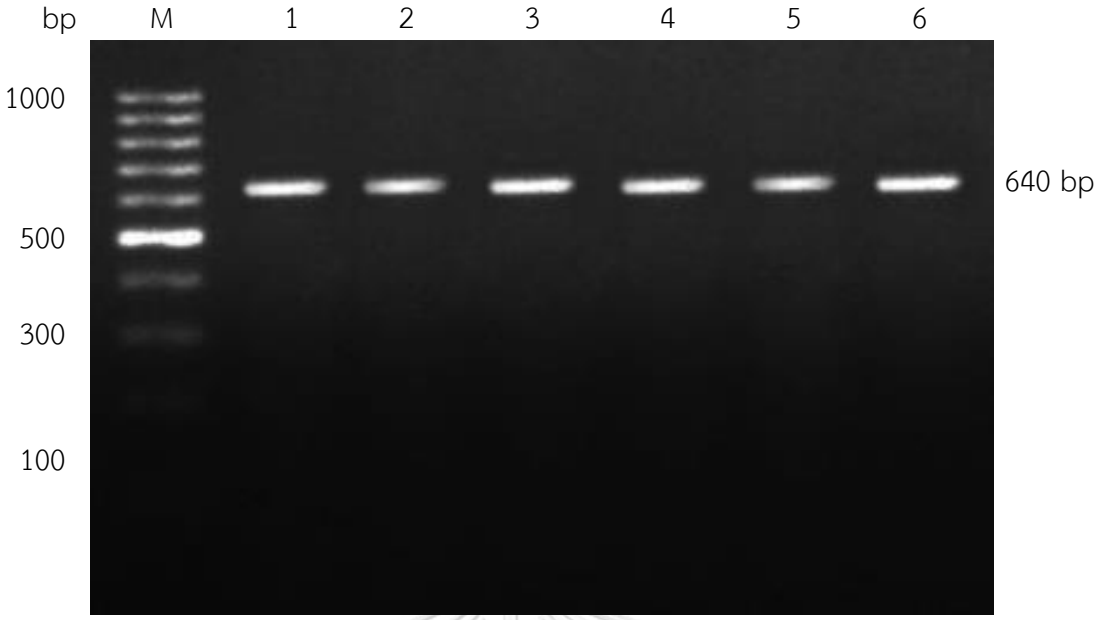

Figure 13: PCR amplicons of erythromycin resistance gene. Lane M, 100 bp marker; Lane 1-5, *ermB*-positive *V. parahaemolyticus* isolates; Lane 6, a positive control isolate

The overall distribution of AMR genes in *V. parahaemolyticus* isolated from oysters and estuarine waters were shown in Figure 14. The PCR result showed that the *qnr* gene was the highest prevalence (77.8%) of all *V. parahaemolyticus* isolates followed by *strB* gene (27.4%). Out of 594 isolates, 131 (22.1%) were positive for *tetA* gene, 116 (19.5%) for *dfr18* gene, 90 (15.2%) for *ermB* gene, 88 (14.8%) for *sul2* gene, 44 (7.4%) for *dfrA1* gene, and 5 (0.8%) for bla_{TEM} gene, respectively.

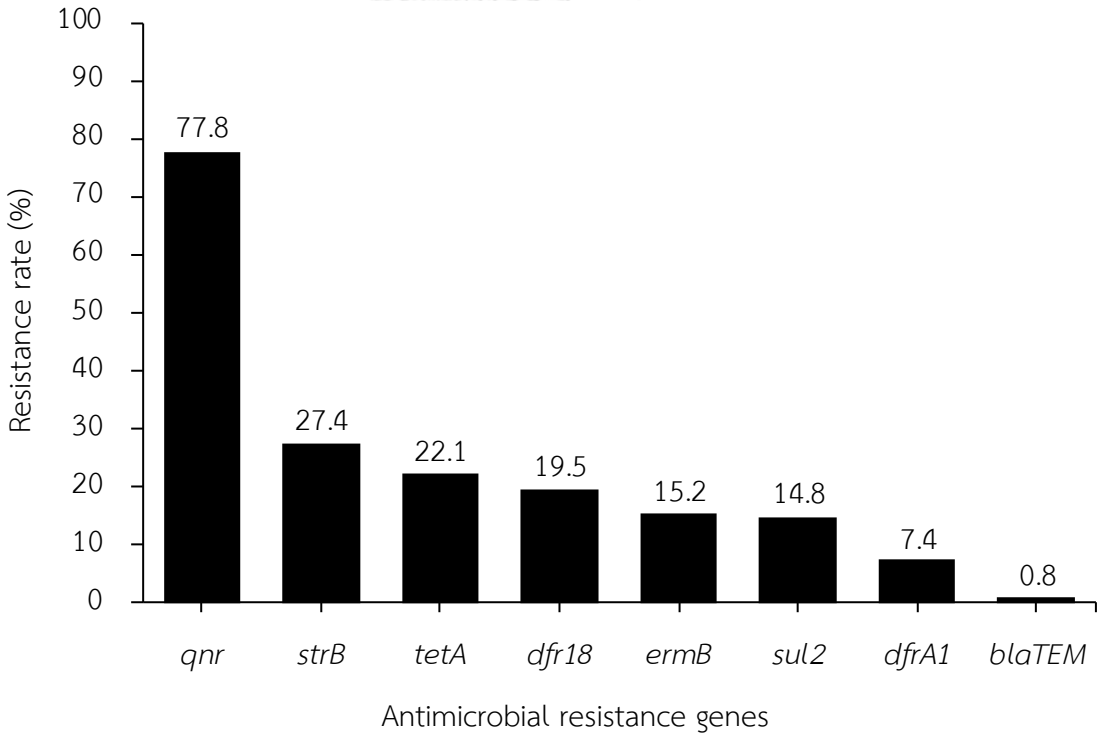

Figure 14: Distribution of antimicrobial resistance genes in *V. parahaemolyticus* isolates (n=594).

In Figure 15, the prevalence of AMR genes in *V. parahaemolyticus* isolates from oyster meat samples were detected to *qnr* (81.2%) followed by *strB* (27.1%), *tetA* (23.3%), *dfr18* (16.6%), *ermB* (16.3%), *sul2* (13.6%), *dfrA1* (8.3%), and bla_{TEM} (1.1%), respectively. Among the estuarine water samples, the presence of AMR genes was observed in *qnr* (72.5%) followed by *strB* (27.9%), *dfr18* (24.0%), *tetA* (20.2%), *sul2* (16.7%), *ermB* (13.3%), *dfrA1* (6.0%), and bla_{TEM} (0.4%), respectively.

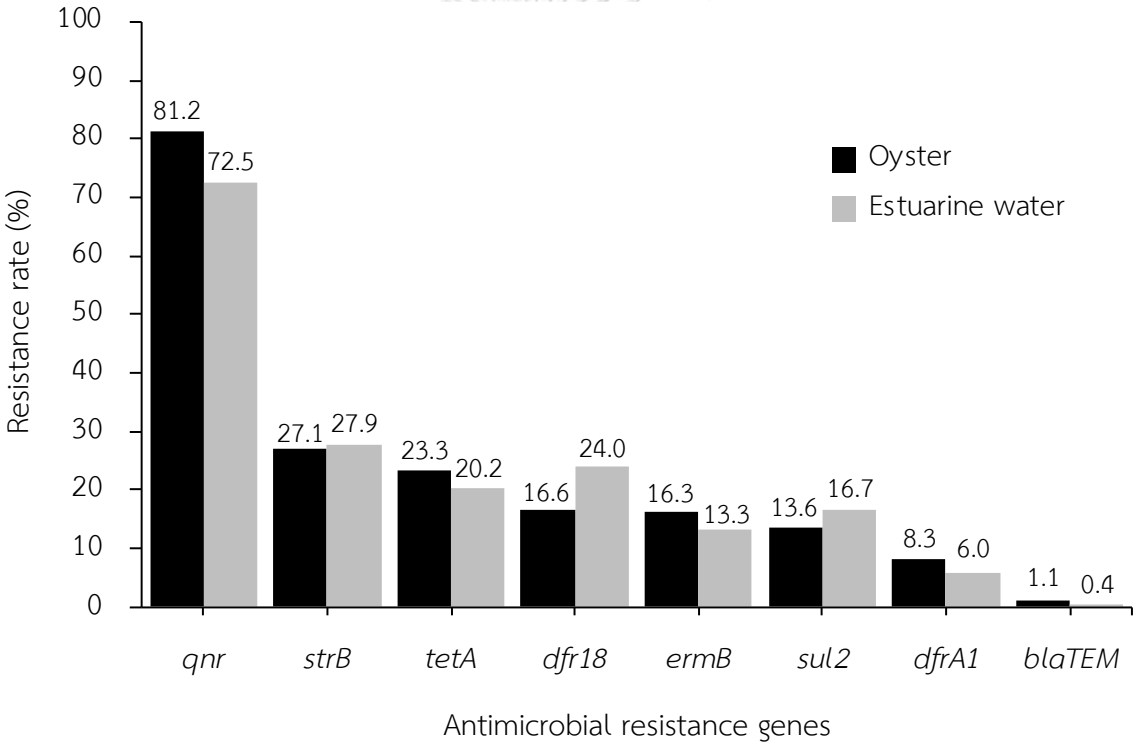

Figure 15: Distribution of antimicrobial resistance genes in *V. parahaemolyticus* isolates among pooled oyster meat (n=361) and estuarine waters (n=233).

The distribution of eight AMR genes was also analyzed by using Pearson 's chi-square test to observe the association between AMR genes and the samples (Table 8). The result showed that no statistically significant difference of bla_{TEM}, *ermB, strB, sul2, tetA,* and *dfrA1* genes among oysters and estuarine water samples were observed. This means that the AMR genes are equally distributed among oysters and estuarine waters. However, *qnr* and *dfr18* were observed different between oyster meat and estuarine water samples. The presence of quinolones resistance gene (*qnr*) was significantly higher in oysters than those of estuarine waters ($P < 0.05$). In contrast, higher prevalence of trimethoprim resistance gene (*dfr18*) was observed in estuarine water samples than oysters ($P < 0.05$).

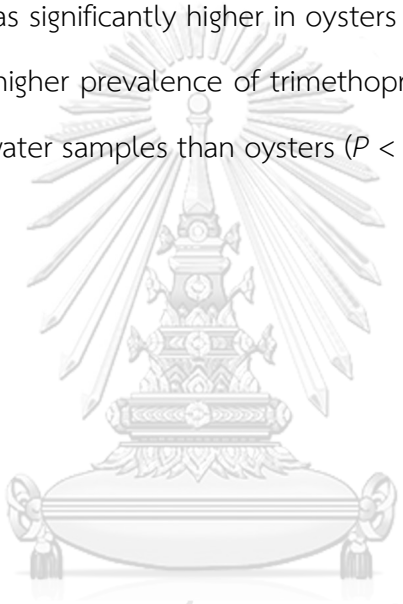

Table 8: The association between oysters and estuarine water samples on the distribution of antimicrobial resistance genes in *V. parahaemolyticus*

Resistance genes	Samples	Number of isolates (number)	AMR genes positive isolates (%)	OR (C.I.)[a]	*P*-value
bla_{TEM}	Oyster	361	4 (1.1%)		
	Estuarine water	233	1 (0.4%)	2.60 (0.29-23.40)	0.377
	Total	594	5 (0.8%)		
qnr	Oyster	361	293 (81.2%)		
	Estuarine water	233	169 (72.5%)	1.63 (1.10-2.41)	0.013
	Total	594	462 (77.8%)		
ermB	Oyster	361	59 (16.3%)		
	Estuarine water	233	31 (13.3%)	1.27 (0.80-2.04)	0.313
	Total	594	90 (15.2%)		
strB	Oyster	361	98 (27.1%)		
	Estuarine water	233	65 (27.9%)	0.96 (0.67-1.39)	0.841
	Total	594	163 (27.4%)		
sul2	Oyster	361	49 (13.6%)		
	Estuarine water	233	39 (16.7%)	0.78 (0.50-1.23)	0.289
	Total	594	88 (14.8%)		
tetA	Oyster	361	84 (23.3%)		
	Estuarine water	233	47 (20.2%)	1.20 (0.80-1.80)	0.374
	Total	594	131 (22.1%)		
dfr18	Oyster	361	60 (16.6%)		
	Estuarine water	233	56 (24.0%)	0.63 (0.42-0.95)	0.026
	Total	594	116 (19.5%)		
dfrA1	Oyster	361	30 (8.3%)		
	Estuarine water	233	14 (6.0%)	1.42 (0.74-2.74)	0.296
	Total	594	44 (7.4%)		

[a] Odds ratio (95% confidence interval)

3.1.1 Logistic regression model for the association between antimicrobial resistance genes in *V. parahaemolyticus* isolates from oysters (n=361) and estuarine waters (n=233)

Logistic regression model was used to find the association between AMR genes of *V. parahaemolyticus* isolated from oyster and estuarine water samples (Table 9 and 10). The significant association ($P < 0.05$) with odds ratio (OR > 1) between *qnr* and *strB*, and *qnr* and *dfr18* in oyster samples. The presence of *dfr18* in *V. parahaemolyticus* isolates from oysters was 2.7 times more likely to contain in *qnr* positive isolates than those of *qnr* negative isolates. Similarly, *V. parahaemolyticus* isolates from oysters that harboring *strB* was 2.08 times more likely to contain in *qnr* positive isolates than *qnr* negative isolates. The odds of the presence of *ermB*, *tetA*, *sul2*, and *dfrA1* in oysters were not significantly different from that of the presence of *qnr*. In estuarine water samples, the significant association was observed between *qnr* and *dfr18*. In contrast with oysters, the presence of *dfr18* in *V. parahaemolyticus* isolates from estuarine waters was 2.70 times more likely to contain in *qnr* negative isolates than that in *qnr* positive isolates. The odds of the presence of *ermB*, *tetA*, *strB*, *sul2*, and *dfrA1* were not significantly different from that of the *qnr* in estuarine waters.

Table 9: Logistic regression model for the association among antimicrobial resistance genes in oyster meat samples (n=361)

qnr	Odds ratio	S.E.	*P*-value	95% C.I.
ermB	0.61	0.35	0.154	0.31 to 1.20
tetA	1.59	0.36	0.193	0.79 to 3.22
strB	2.08	0.36	0.041	1.03 to 4.21
sul2	1.04	0.41	0.934	0.46 to 2.33
dfrA1	1.57	0.57	0.427	0.52 to 4.77
dfr18	2.70	0.48	0.037	1.06 to 6.83
bla_{TEM}	0.10	1.09	0.028	0.01 to o.78
Constant	3.17	0.19	<0.0001	

S.E., Stardard Error; C.I. Confidence Interval

Table 10: Logistic regression model for the association among antimicrobial resistance genes in estuarine water samples (n=233)

qnr	Odds ratio	S.E.	*P*-value	95% C.I.
ermB	0.93	0.44	0.865	0.39 to 2.19
tetA	1.17	0.42	0.715	0.51 to 2.65
strB	1.68	0.37	0.165	0.81 to 3.49
sul2	0.85	0.41	0.692	0.38 to 1.89
dfrA1	2.20	0.80	0.322	0.46 to 10.53
dfr18	0.37	0.33	0.003	0.19 to 0.70
Constant	2.94	0.22	<0.0001	

S.E., Standard Error; C.I. Confidence Interval

3.2 The distribution of integrative and conjugative elements (ICEs) and class 1, 2, and 3 integrons

All *V. parahaemolyticus* isolates were detected for the presence of SXT integrase gene (int_{SXT}) for the integrative and conjugative elements (ICEs) and *intI1*, *intI2*, and *intI3* genes for class 1, 2, and 3 integrons. The result showed that *V. parahaemolyticus* isolates were negative to these genes. The PCR amplicons of the positive control of class 1 integron were 497 bp in size when primers set for *intI1*, *intI2*, and *intI3* were used. The amplicon size of the positive control used for the SXT element was 592 bp.

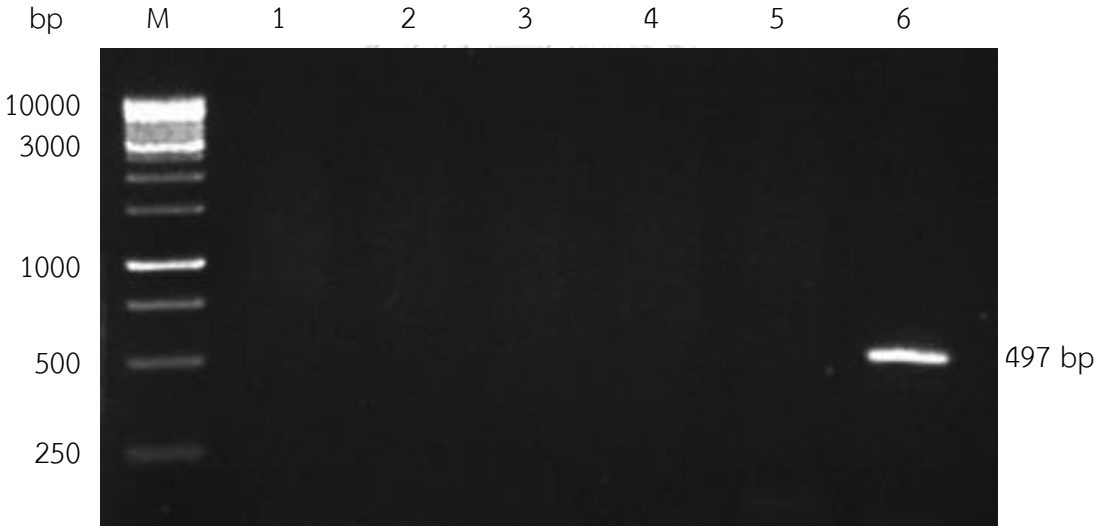

Figure 16: Electrophoresis of PCR products for the detection of class 1, 2, and 3 integrons. Lane M, 1 kb marker; Lane 1-5, *intI1*, *intI2*, and *intI3*-negative *V. parahaemolyticus* isolates; Lane 6, positive control isolate for class 1 integron

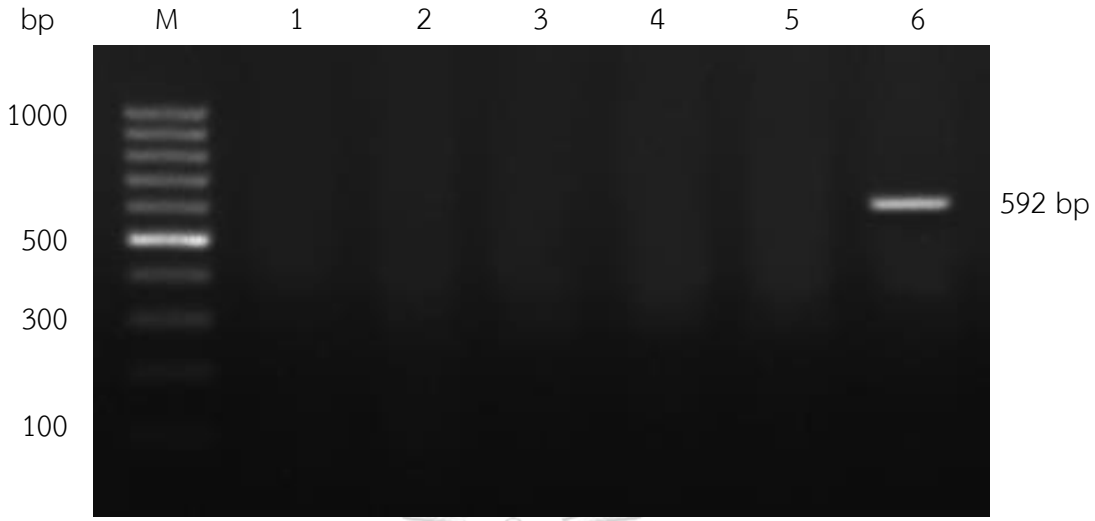

Figure 17: Electrophoresis of PCR products for the screening of integrative and conjugative elements (ICEs). Lane M, 100 bp marker; Lane 1-5, int_{SXT}-negative *V. parahaemolyticus* isolates; Lane 6, a positive control for SXT integrase gene

CHAPTER V

DISCUSSION

As oyster is one of the most nutritious source and beneficial seafood products with the need of both domestic and international consumers, *V. parahaemolyticus* is a commonly found pathogen in oysters, which has been a major cause of seafood-borne infection in humans. Antimicrobial agents have been widely used in different settings including human and veterinary medicine, agriculture and aquaculture. Antimicrobial agents are applied for many purposes such as treatment and prevention of infectious diseases in humans and animals and growth promoter in food-producing animals as well. It has been known that improper use of antimicrobials could result in the development of MDR bacteria, transference of resistance determinants and reduction of efficacy of treatment to bacterial infections.

In this study, all oyster and estuarine water samples were positive to *V. parahaemolyticus*. A previous study from Thailand showed that *V. parahaemolyticus* was found in cockles collected from three provinces at Surat Thani, Nong Khai, and Khon Kaen in Thailand (Mala et al., 2016a). The prevalence of *V. parahaemolyticus* in our study was consistent with other studies in Thailand (Kanjanasopa et al., 2011; Changchai and Saunjit, 2014). High prevalence of *V. parahaemolyticus* in oysters were also reported in China (89.3%) and in Brazil (100%) (Chen and Ge, 2010; Sobrinho Pde et al., 2011). The high prevalence of *V. parahaemolyticus* in oyster meat samples obtained from this study highlighted the importance of *V. parahaemolyticus* contamination in both oysters and estuarine waters that would be harmful to the consumers. The occurrence of *V. parahaemolyticus* in oysters and estuarine waters depending on harvest condition, transportation, and the environment (Hsiao et al., 2016). Inappropriate storage during

post-harvest processing and marketing, especially cooling system is the potential cause of oyster meat samples being exposed to an ambient temperature causing bacterial growth (Mudoh et al., 2014). Additionally, *Vibrio* infections from raw oyster consumption are more common in tropical and temperate regions. The temperature of the coastal water of the Southeast Asian region is always warm throughout the year, hence it may be the main contributing factor influencing the abundance of *V. parahaemolyticus* in the estuary environment in Thailand. The symptoms of vibriosis include watery diarrhea, abdominal cramping, nausea, vomiting, fever, and chills and these symptoms usually begin within 24 hours of exposure. Many studies reported that fatal cases of septicemia may occur in immunocompromised patients or those with a pre-existing medical condition (such as liver disease, cancer, heart disease, recent gastric surgery, antacid use, or diabetes) (Daniels et al., 2000; Butt et al., 2004; Payinda, 2008).

The presence of major virulence factor genes (*tdh* and *trh*) was examined in all *V. parahaemolyticus* (n=594) isolates from oyster meat and estuarine water samples. Despite the high occurrence of *V. parahaemolyticus*, *trh* was not observed in the samples. The occurrence of *tdh* was found 0.7% (4/594), which was lower than previous studies found in Thailand (29.7%), Malaysia (8.5%), China (8.2%), and New Zealand (3.4%), respectively (Kirs et al., 2011; Mala et al., 2016a; Tan et al., 2017; Yang et al., 2017). However, our finding was in agreement with Sobrinho Pde et al. (2010) that the detection of *V. parahaemolyticus* was 0.04% (1/2243), samples originated from oysters harvested in the southern coast of Sao Paulo State in Brazil. Our results were similar with the previous reports on the distribution of *tdh* and *trh* genes showed that these virulence factor genes were rarely observed in *V. parahaemolyticus* strains from environmental sources in Spanish Mediterranean coast and in the eastern province of Saudi Arabia (Lopez-Joven et al., 2015; Ghenem

and Elhadi, 2018). However, pathogenic *V. parahaemolyticus* contaminated with seafood can cause serious disease to humans and enormous economic losses in the seafood industry.

Generally, *Vibrio* is considered to be highly susceptible to most clinically used antimicrobials (Oliver et al., 2013). However, a higher frequency of AMR *V. parahaemolyticus* has been reported in many studies (Okoh and Igbinosa, 2010; Hua and Apun, 2013). In this study, most of *V. parahaemolyticus* isolates were resistant to erythromycin approximately 54.2%. This finding was similar to *V. parahaemolyticus* isolates from raw shellfish in Poland, raw oysters in Spain and the tropical estuary and adjoining traditional prawn farm along the southwest coast in India (Lozano-Leon et al., 2003; Lopatek et al., 2015; Silvester et al., 2015). In Asia, most of the *V. parahaemolyticus* isolates were resistant to this antimicrobial (Vaseeharan et al., 2005; Liu et al., 2009).

In this study, *V. parahaemolyticus* isolates from oysters and estuarine waters were resistant to sulfamethoxazole (34.7%) and trimethoprim (28.0%), respectively. In contrast, a previous study reported a sensitivity of *V. parahaemolyticus* isolates from a shrimp farm in the southwest coast of India towards trimethoprim (Devi et al., 2009). In another study from Tunisia, more than 70% of the isolates of *Vibrio* spp. showed susceptible to trimethoprim and sulfamethoxazole (Lajnef et al., 2012). In our study, chloramphenicol and ciprofloxacin resistance were not detected in any isolates. The previous studies showed that up to 100% of *V. parahaemolyticus* isolates from environmental sources were resistant to chloramphenicol in northern England, although no resistant rate was observed in Italy and 31% in Hong Kong (Ottaviani et al., 2001; Daramola et al., 2009; Wong et al., 2012). These inconsistencies may be because of different geographical location, antimicrobial usage, and different population. In this study, a low level of ampicillin resistance was

also detected, which was in agreement with the previous report in northern England (Daramola et al., 2009). In this study, tetracycline resistance was found only 0.5%, which was consistent with the previous report in China that 4.6% of *V. parahaemolyticus* isolates from cultured sea cucumbers were resistant to tetracycline (Jiang et al., 2014). However, the prevalence of streptomycin resistance (0.8%) was much lower than the previous finding from China that 43.7% of the environmental *V. parahaemolyticus* isolates from sea cucumbers were resistant to streptomycin (Jiang et al., 2014).

In this study, 34% of the isolates were resistant to at least one antimicrobial agent, while 155 isolates (26.0%) were susceptible to eight antimicrobials tested. Khan et al. (2007) determined the susceptibility of 27 strains of *V. parahaemolyticus* isolated from cultured shrimp at Khulna in Bangladesh, and it was suggested that tetracycline was the best choice for controlling infectious diseases caused by enteric bacterial infection, including *V. parahaemolyticus* (Costa et al., 2015). Many classes of antimicrobial agents were previously detected in environmental samples such as sewage, effluents, surface water, and wastewater treatment plants (Lundborg and Tamhankar, 2017). For example, the previous studies from the U.S., Malaysia, Brazil, and Jordan reported that pathogenic *V. parahaemolyticus* was isolated from estuary and coastal water contained high prevalence of AMR (Baker-Austin et al., 2008; Alaboudi et al., 2016; Drais et al., 2016; Menezes et al., 2017). These findings indicated that animal effluents and wastewater come from various sources may play an important role in the dissemination of AMR in the environment that can impact on oysters and estuarine water quality.

One of the important findings, MDR bacteria were found 5.1% of the isolates, which was resistant to at least three different classes of antimicrobial agents. Our findings reveal that most *V. parahaemolyticus* isolates from oysters and estuarine

waters contained multiple drug resistance. However, there was a difference in the patterns of AMR between oysters and estuarine water samples. Such differences in the percentage of resistance to various antimicrobials may be due to the history of antimicrobial usage in different fields, co-existence of resistant bacteria, and the contamination of MDR strains into the estuary environment.

In this study, all *V. parahaemolyticus* isolates exhibited multiple resistances to seven antimicrobials. The AMR to the *qnr* gene was the highest prevalence (77.8%) in all *V. parahaemolyticus* isolates. Our finding was higher than a previous study from the U.S that 24.7% of *V. parahaemolyticus* isolates from imported shrimps were observed *qnr* gene (Nawaz et al., 2015). Thus, the high level of quinolone resistance in *V. parahaemolyticus* may be due to the mutations and the involvement of active efflux pumps. The widespread occurrence of quinolone-resistant bacteria in the environment might lead to increase the difficulty in treating bacterial infections.

The *dfr18* and *dfrA1* genes coding for trimethoprim resistance and *sul2* gene coding for sulfamethoxazole resistance were observed among the *Vibrio* isolates in this study. These genes have also been found from the previous studies in Thailand and India (Dalsgaard et al., 2000; Thungapathra et al., 2002). The prevalence of *ermB* gene was detected 15.2% of the isolates in this study, which was in the range approximately 7.0-32.3% observed in *Vibrio* isolates (Raissy et al., 2012; Shakerian et al., 2017). Interestingly, the presence of bla_{TEM} (0.8%) in our study was much lower than that reported in China found all isolates from sea cucumbers carried this gene and all *V. parahaemolyticus* isolates from oysters and mussels were harbor bla_{TEM} gene in Brazil (Rojas et al., 2011; Jiang et al., 2014). However, the low prevalence of this gene is still unclear. It is may be due to geographical distribution and differ in the application of antimicrobials in different locations.

In this study, the significant association ($P < 0.05$) with odds ratio (OR > 1) was observed between *qnr* and *strB*, and *qnr* and *dfr18* in oyster samples. These findings were consistent with other studies reported that the presence of *qnr* gene is associated with other classes of antimicrobials including aminoglycosides and sulfonamides (Wang et al., 2003; Robicsek et al., 2006; Strahilevitz et al., 2009). One of the possible reasons is that *qnr* genes are plasmid-mediated quinolone resistance that can be transmitted horizontally (through bacterial conjugation) and can carry additional genes that cause resistance to other antibiotic classes (Aldred et al., 2014). This might explain that some AMR genes from different classes of antimicrobials are locating on the same mobile genetic elements such as plasmids or transposons, harboring co-selective and co-transfer of these determinants together.

The integrons play an important role in rapid evolution and dissemination of AMR. Class 1, 2 and 3 integrons are the most common integrons related to AMR present in multiple AMR pathogens (Rowe-Magnus and Mazel, 2002). *V. parahaemolyticus* isolates were detected for the presence of class 1, 2, and 3 integrons. In this study, none of the isolates was found to carry class 1 integrase gene *intI1,* which was discordant with the findings of previous studies in Thailand and China (Kitiyodom et al., 2010; Jiang et al., 2014), but this finding was similar to a previous study in Chile (Dauros et al., 2010). However, none of the *V. parahaemolyticus* isolates was found to carry class 2 and 3 integrase genes encoding *intI2* and *intI3,* which was consistent with the previous findings in Angola and Thailand (Ceccarelli et al., 2006; Kitiyodom et al., 2010). Ahmed et al. (2006) reported that class 2 integrons were identified in only two *V. Cholera* isolates from human clinical cases in India and an environmental wastewater isolate from Bangladesh. Class 3 integrons were not detected in *V. parahaemolyticus.* Currently, class 3 integrons have been observed in *Klebsiella pneumonia*, *Serratia marcescens*,

and *Delftia* spp. (Correia et al., 2003; Xu et al., 2007). The absence of class 1, 2, and 3 integrons among *V. parahaemolyticus* strains highlighted that there is no selective pressure for this type of genetic element in this environmental setting. The possible explanation could be that AMR genes in this area were distributed by other resistance mechanisms, such as conjugative plasmids, active efflux pumps, and mutations.

A newer and larger integrative and conjugative elements (ICEs) also carrying AMR genes, called the SXT element, has been found in *V. parahaemolyticus* (Kitiyodom et al., 2010). In this study, none of the isolates was found to carry the SXT integrase gene (int_{SXT}). The previous study reported that *V. parahaemolyticus* isolates from wastewater in South Africa carried the SXT element harbored AMR genes (Okoh and Igbinosa, 2010). Nonetheless, no positive amplification for the element in *V. parahaemolyticus* isolates was observed in our study. This indicated that the distribution of AMR in *V. parahaemolyticus* isolates from oyster meat and estuarine waters were not related to integrons and SXT integrase gene.

In conclusion, this study illustrated the evidence of the presence of potentially pathogenic strains of *V. parahaemolyticus* in the Phang Nga area. The result of antimicrobial susceptibility tests revealed the high resistance of *V. parahaemolyticus* to clinically important antibiotics. These findings highlighted the need to reduce and prevent the dissemination of AMR and MDR bacteria into the aquatic environment from the imprudent usage of antimicrobials. Moreover, *V. parahaemolyticus* isolates from this designated area might develop AMR under selective pressure of antimicrobials, which are used for the treatment and prevention of bacterial infections in humans, veterinary, and aquaculture. Therefore, continuous monitoring of antimicrobial susceptibility profiles of *V. parahaemolyticus* is of the importance to ensure the safe consumption of the oysters and to identify and

implement proper public health measures for the control and prevention of disease caused by pathogenic vibrios.

CONCLUSION AND SUGGESTIONS

The main findings of this study were the distribution of AMR bacteria and AMR genes among *V. parahaemolyticus* from pooled oyster meats and estuarine waters, which were considered as environmental samples. The prevalence of virulence genes (*tdh* and *trh*) was low in the environment among oyster and estuarine water samples. *V. parahaemolyticus* carrying both resistance and virulence genes can potentially threat to human infection for those who consume raw or insufficiently cooked of oysters. The high rate of erythromycin, sulfamethoxazole, and trimethoprim resistance were observed among oysters and estuarine waters, indicating that AMR strains of *V. parahaemolyticus* have been already disseminated through the environment. This finding revealed that the various sources of contamination such as sewage, animal effluents, wastewater, farm wastes, stormwater, and runoff water may play an important role in the dissemination of AMR into the aquatic environment.

Multidrug resistant strains of *V. parahaemolyticus* were observed in both oysters and estuarine waters. This confirmed that MDR strains from different sources were spread by both horizontal transfer and clonal dissemination through the environment that might pose threat to public health. In the present study, class 1, 2, and 3 integrons and SXT elements in *V. parahaemolyticus* did not play an important role in the dissemination of AMR. Therefore, restrictive policies on the use of antimicrobials in human, veterinary, agriculture and aquaculture can assist to reduce the dissemination of AMR and MDR in the environment, especially in the area of oyster cultivation. Increasing of sanitary practice and consuming of adequate cooked oysters are also recommended to promote seafood safety and to reduce the risk of seafood-borne illnesses.

The genetic data of AMR and virulence factors among *V. parahaemolyticus* isolates from oysters and estuarine waters can be applied for further studies as follows:

1. In this study, most of resistant *V. parahaemolyticus* isolates did not harbor the resistance determinants including integrons and SXT elements, revealing the existence of other resistance mechanisms. Investigation of other resistance mechanisms should be performed e.g. multidrug efflux systems and chromosomal mutation.

2. The pathogenicity of *V. parahaemolyticus* isolates was not carried out in this study. To investigate pathogenicity and epidemiology of *V. parahaemolyticus* strains, serotyping is needed to perform. Therefore, further studies are recommended to determine the relationship between serotypes and virulence factors in *V. parahaemolyticus* strains from cultivated oysters and estuarine waters.

3. Data on the genetics of AMR in *V. parahaemolyticus* along the food chain will assist to create monitoring and surveillance programs to reduce AMR. However, these data are still limited, especially in developing countries. Therefore, studies of genetics on AMR in *V. parahaemolyticus* isolates from other sources such as human clinical cases, aquatic products, and the environment are recommended.

4. Study of genetic relatedness in *V. parahaemolyticus* isolates from various sources should be conducted. This will prove the link of AMR among human, aquatic animals, and the environment.

Data on AMR and resistance determinants obtained from this study could be beneficially used as follows:

1. The occurrence and distribution of AMR and virulence traits could be included as a part of national and regional AMR monitoring program.

2. The findings of this study could be applied in risk assessment of the distribution of AMR in *V. parahaemolyticus*.

3. The epidemiological data on *V. parahaemolyticus* obtained from this study such as the prevalence of AMR and resistance genes in oysters and estuarine waters could be used for better understanding of the dissemination of AMR in the environment.

4. The results from this study could be used to explain the association between AMR phenotype and genotype among *V. parahaemolyticus* isolates from aquatic animals and estuarine waters.

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APPENDICES

APPENDIX A

Bacterial growth media

1. Luria-Bertani Agar (LB) (Difco)

- Tryptone	10.0	g
- Yeast extract	5.0	g
- Sodium chloride	10.0	g
- Agar	15.0	g

2. Luria-Bertani Broth (LB) (Difco)

- Tryptone	10.0	g
- Yeast extract	5.0	g
- Sodium chloride	10.0	g

3. Mueller Hinton Agar (MHA) (Difco)

- Beef extract powder	2.0	g
- Acid digest of casein	17.5	g
- Starch	1.5	g
- Agar	17.0	g

4. Tryptic Soy Agar (TSA) (Difco)

- Pancreatic digest of casein	15.0	g
- Enzymatic digest of soybean meal	5.0	g
- Sodium chloride	5.0	g
- Agar	15.0	g

5. Wagatsuma Agar (HiMedia®)

- Peptic digest of animal tissue	10.0	g
- Yeast extract	3.0	g
- Sodium chloride	70.0	g
- Dipotassium phosphate	5.0	g
- Mannitol	10.0	g
- Crystal violet	0.001	g
- Agar	15.0	g

APPENDIX B

Reagents

Reagents for PCR assay

1. TopTaq PCR Maste Mix (2X) (Qiagen®) contains

    - *Taq* DNA polymerase in reaction buffer 0.05 units/µl
    - $MgCl_2$ 3 mM
    - dNTPs (dATP, dCTP, dGTP, dTTP) 400 µM of each

2. CoralLoad PCR buffer (Qiagen®)

Reagents for agarose gel electrophoresis

1. 50X TAE (Tris-Acetate buffer) 1000 ml contains

    - Tris base 242.0 g
    - Glacial acetic acid 57.1 g
    - 0.5 M EDTA (pH 8.0) 100.0 ml
    - Distilled deionized water 1,000.0 ml

2. Agarose gel (Sigma-Aldrich®)

    - Agarose (ultra-pure) 1.5 g
    - Adjusted 1X TAE buffer to 100 ml

3. RedSafe™ nucleic acid staining solution (iNtRON)

4. GeneRuler 100 bp DNA ladder (Thermo Scientific™) contains

    - 100 bp DNA ladder 100 µl/50µg
    - 6X TriTrack DNA loading dye 1 ml

OUTPUTS

The results from this study were presented as a poster presentation at the 17th Chulalongkorn University Veterinary Conference (CUVC 2018), which was held on 25-27 April 2018 at IMPACT Forum Building, Nonthaburi, Thailand. Our research was published in the proceeding of the Thai Journal of Veterinary Medicine as follows:

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