พันธุกรรมการดื้อยาต้านจุลชีพของเชื้อวิบริโอที่แยกได้จากกุ้งทะเลเพาะเลี้ยงในประเทศไทย

นางสาวสิริกร กิติโยดม

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GENETICS OF ANTIMICROBIAL RESISTANCE IN *VIBRIO* SPECIES ISOLATED FROM FARMED MARINE SHRIMPS IN THAILAND

Miss Sirikorn Kitiyodom

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Public Health
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Thesis Title	GENETICS OF ANTIMICROBIAL RESISTANCE IN *VIBRIO* SPECIES ISOLATED FROM FARMED MARINE SHRIMPS IN THAILAND
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สิริกร กิติโยดม : พันธุกรรมการดื้อยาต้านจุลชีพของเชื้อวิบริโอที่แยกได้จากกุ้งทะเลเพาะเลี้ยงในประเทศไทย. (GENETICS OF ANTIMICROBIAL RESISTANCE IN *VIBRIO* SPECIES ISOLATED FROM FARMED MARINE SHRIMPS IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.สพ.ญ.ดร. รุ่งทิพย์ ชวนชื่น, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.สพ.ญ.ดร. เจนนุช ว่องธวัชชัย, 63 หน้า.

ศึกษาพันธุกรรมการดื้อยาต้านจุลชีพของเชื้อวิบริโอจำนวน 83 เชื้อที่แยกได้จากกุ้งทะเลเพาะเลี้ยงในประเทศไทย ประกอบด้วยเชื้อ *V. parahaemolyticus* จำนวน 26 เชื้อ *V. cholera* จำนวน 18 เชื้อ *V. fluvalis* จำนวน 23 เชื้อ และ *V. vulnificus* จำนวน 16 เชื้อ ทำการศึกษาความไวรับของเชื้อต่อยาปฏิชีวนะจำนวน 10 ชนิด ตรวจหาการปรากฏและศึกษาลักษณะของ class 1, 2 และ 3 integrons ในเชื้อทุกตัว ตรวจหาการปรากฏของยีน *tet(K), tet(L), tet(M), tet(O)* และ *tet(S)* ในเชื้อทุกตัว และตรวจการกลายพันธุ์ในส่วน Quinolone Resistance Determining Regions (QRDRs) ของยีน *gyrA* และ *parC* ในเชื้อที่ดื้อต่อ ciprofloxacin และ/หรือ enrofloxacin จำนวน 17 ตัว ผลการวิจัยพบว่ามีเชื้อวิบริโอที่ดื้อต่อยาปฏิชีวนะอย่างน้อยหนึ่งชนิด 74 เชื้อ (89 %) และมีเชื้อที่ดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน 17 เชื้อ (21%) โดยเชื้อวิบริโอดื้อต่อยา ampicillin มากที่สุด (62%) และมีรูปแบบการดื้อยาที่พบมากที่สุดคือ AMP-SMX-TMP (4.82%) พบเชื้อที่มีการปรากฏของ class 1 integrons คิดเป็น 6% และไม่พบการปรากฏของ class 2 และ 3 integrons ในเชื้อตัวใด โดยมีเชื้อ *V. cholera* จำนวน 1 เชื้อที่มีการปรากฏของ gene cassettes จากผลการถอดรหัสพันธุกรรมพบว่า gene cassettes ที่แทรกตัวอยู่คือบางส่วนของยีน *rumA* และเชื้อทุกตัวที่มีการปรากฏของ class 1 integrons ไม่พบมีการปรากฏของส่วน typical 3'conserved segment เชื้อทุกตัวไม่พบมีการปรากฏของยีน *tet* ที่ทำการศึกษา เชื้อที่ดื้อต่อ ciprofloxacin และ/หรือ enrofloxacin มีการกลายพันธุ์ในส่วน QRDR ของยีน *gyrA* คือ G248T (59%) ซึ่งเปลี่ยนกรดอะมิโน serine เป็น isoleucine ที่ตำแหน่ง 83 และไม่พบว่าเชื้อตัวใดมีการกลายพันธุ์ในส่วนของยีน *parC* จากผลการวิจัยครั้งนี้แสดงให้เห็นว่า ควรมีการศึกษาพันธุกรรมการดื้อยาของเชื้อวิบริโอที่แยกได้จากการเพาะเลี้ยงกุ้งทะเลเพิ่มเติมต่อไป

ภาควิชา สัตวแพทยสาธารณสุข..... ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2551..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : *Vibrio* spp. / Vibriosis / Multidrug resistance / Integrons / *gyrA*

SIRIKORN KITIYODOM : GENETICS OF ANTIMICROBIAL RESISTANCE IN *VIBRIO* SPECIES ISOLATED FROM FARMED MARINE SHRIMPS IN THAILAND. ADVISOR : ASST. PROF. RUNGTIP CHUANCHUEN, CO-ADVISOR : ASSOC. PROF. JANENUJ WONGTAVATCHAI, 63 pp.

A total of 83 *Vibrio* isolates from farmed marine shrimps, comprising *V. parahaemolyticus* (*n*=26), *V. cholera* (*n*=18), *V. fluvalis* (*n*=23) and *V. vulnificus* (*n*=16) were included in this study. Susceptibilities to 10 antimicrobials were determined. The occurrence and characteristics of class1, 2 and 3 integrons were investigated. The presence of *tet(K)*, *tet(L)*, *tet(M)*,*tet(O)* and *tet(S)* genes were examined. Seventeen isolates resistant to ciprofloxacin and/or enrofloxacin were examined for mutations the Quinolone Resistance Determining Regions (QRDRs) of *gyrA* and *parC* genes. Seventy-four isolates (89%) were resistant to at least one antibiotic and 17 isolates (21%) were multidrug-resistant. Most of the *Vibrio* isolates (62 %) were resistant to ampicillin and the most common resistance pattern was the AMP-SMX-TMP (4.82%). As class 1 integrons were identified in 6%, no class 2 and 3 integrons were detected. Only one class 1 integrons in a *V. cholera* isolate carried gene cassettes. Nucleotide sequencing analysis revealed that the inserted gene cassette was the partial *rumA* gene. All of class 1 integrons did not harbor the typical 3'conserved segment. None of the isolates possessed *tet* genes tested. As Ser-83-Ile substitution in GyrA was the major mutation identified in the fluoroquinolone-resistant isolates (59%), no mutation in ParC was observed. The results warranted further studies to investigate other mechanisms underlying resistance to antibiotics in the *Vibrio* isolates from farmed marine shrimps.

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Academic Year 2008..... Co-Advisor's Signature

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CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
DNA	deoxyribonucleic acid
DW	distilled water
e.g.	exempli gratia, for example
et al.	et alibi, and others
g	gram (s)
h	hour (s)
i.e.	id est, that is
M	molar
µl	microgram (s)
µg	microliter (s)
mg	milligram (s)
ml	milliliter (s)
NSS	normal saline solution
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
rpm	round per minute
sec	second (s)
TAE	Tris-Acetate-EDTA
U	Unit
V. cholera	*Vibrio cholera*
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. vulnificus	*Vibrio vulnificus*
V. fluvialis	*Vibrio fluvialis*

CHAPTER I

INTRODUCTION

Marine shrimp farming in Thailand began in the 1970s and the country has exported the cultivated marine shrimps since 1984. Thailand has currently become one of the largest shrimp suppliers among 30 shrimp-producer countries, of which the top importing partners include the United States, the European Union and Japan (National food institute, Thailand, 2008). Total shrimp production of Thailand is estimated 500,000 tons per year and, approximately, 350,000 tons per year is designated for exporting purpose. In 2007, the estimated value of exporting marine shrimps was 80,000 million Baht, which was accounted for 1.5 % of total exporting income of the country (National food institute, Thailand, 2008).

As marine shrimps are one of the important export products of Thailand, they have faced several devastating disease outbreaks, for examples; Vibriosis, Yellow-head and White spot syndrome (OIE/ FAO/WHO, 2004; 2006). Vibriosis has been known as one of the most frequent bacterial diseases contributing to major loss in the marine shrimp culture (Lightner, 1993). *V. parahaemolyticus*, *V. alginolyicus* and other *Vibrio* spp. have been associated with outbreaks in many countries including Thailand (Direkbusarakom et al., 1992; Lighner and Redman 1998), Indonesia (Hisbi et al., 2000), Philippines (Tendencia et al., 1997), Taiwan (Lee et al., 1996) and China (Sudheesh et al., 2001). Clinical signs of Vibriosis in shrimps include septicemia, necrosis of appendage, brown spot and red disease syndrome (Lightner, 1993). As *V. harveyi*, a luminous *Vibrio* strain, is an important etiological agent of Vibriosis in shrimps, disease outbreaks attributed to the species have been observed in Thailand (Flegel et al., 1999), Indonesia (Hisbi et al., 2000), Taiwan (Lee et al., 1996), Philippines (Lavilla-Pitogo et al., 1990), India (Karunasagar et al., 1994) and Ecuador (Austin et al., 1998). Clinical signs of *V. harveyi* infection usually include hepatopancreatic degeneration, larvae bioluminescent and mass mortality (Lightner, 1993; Austin et al., 1998).

Vibrio species are generally considered opportunistic pathogens causing diseases when shrimps are stress (Lightner, 1993; Dalsgaad et al., 1995). Some species i.e. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. fluvialis* are also foodborne and zoonotic pathogens with the clinical manifestations ranging from gastroenteritis to septicemia and wound infection (Daniels and Shafaie, 2000; Elliot et al., 1998). Human infections are usually associated with consumptions of raw or insufficiently-cooked seafood especially shellfish. Human infections due to drinking of contaminated water and exposure of open wounds to *Vibrio*-contaminated environment have been reported (Charles et al., 2004; Codex, 2005a; Croci et al., 2008). *V. cholera* and *V. parahaemolyticus*, the common food-borne enteric pathogens in Asia are responsible for approximately half of the food poisoning and gastroenteritis outbreaks in Thailand, Taiwan, Japan, and several Southeast Asian countries (Codex, 2005a; Gopal et al., 2005). They have been implicated in numerous outbreaks of seafood-borne gastroenteritis in the United States and Europe (Elliot et al., 1998; Falbo et al., 1999; Charles et al., 2004). In Thailand, *V. parahaemolyticus* and *V. cholerae* are particularly concerned due to the increasing number of populations at risk for outbreaks. Among *Vibrio* spp. associated outbreaks, infection with *V. parahaemolyticus* occurs at the highest rate up to 78 % of all food poisoning cases (Codex, 2005a; Sriwanna, 2007). Infections of *V. cholera* had high morbidity rate of 1.57 per 100,000 populations and are mostly reported in the Southern region with of Thailand (Bureau of epidemiology, 2007). *V. vulnificus* and *V. fluvalis* infections have happened at lower incidence. However, *V. vulnificus* usually causes severe infections and ends up with death, of which mortality rate is up to 75% of total seafood-borne patients (Sriwanna et al., 2006). *V. fluvialis* outbreaks have occurred at much less frequency and clinical signs are like those of cholera (Srifuengfung et al., 2004). Taken together, widespread of *Vibrio* spp. in humans and marine shrimp culture could affect both public health and economy of the country.

As fast-growing of the marine shrimp culture industry promptly calls for the need to intensify farming practices to maximize profits, production of marine shrimp culture is mostly depressed by bacteria infections. In the past, antibiotics were administered

during farming primarily to prevent and treat bacterial infections. The antibiotics in use include fluoroquinolones (especially enrofloxacin), florfenicol, oxytetracycline, trimetroprim and sulfonamide (Serrano, 2005; OIE/FAO/WHO, 2006). In aquaculture, the mostly-used antimicrobials are fluoroquinolones and tetracycline that are also drugs of choice for treatment of *Vibrio* infections in human (Okuda et al., 1999; Elliot et al., 1998). Therefore, improper and over-use of antimicrobials in aquaculture can select for antimicrobial resistant bacteria, especially *Vibrio* species (OIE/FAO/WHO, 2004; Serrano, 2005; OIE/FAO/WHO, 2006). Currently, incidence of antimicrobial-resistant *Vibrio* spp. has been increasing worldwide and the pathogen can transfer resistance genes to other bacteria intra- and inter-species (Codex, 1995b; Dalsgaad et al., 1995; 2000). Resistance to antimicrobials can occur from low to high resistance levels through persistent mutations in chromosomal genes or/and through the acquisition of mobile genetic elements e.g. bacteriophages, plasmids, transposons or integrons (Codex, 1995b; Fluit et al., 2004; OIE/FAO/WHO, 2006).

As a result, international organizations have implemented projects and conferences to discontinue the abuse of antibiotics. Codex Alimentarius Commission has issued code of practice to minimize and contain antimicrobial resistance. The main idea of this code is to plan for reduce antimicrobial resistance in bacteria with the scientific supports studies of mechanisms of antimicrobial resistance and molecular epidemiology of antimicrobial resistance in bacteria (Codex, 2005b). Joint of Food and Agriculture Organization (FAO), World organization for animal health (OIE) and World Health Organization (WHO) have discussed on the topic of antimicrobial use in aquaculture and antimicrobial resistance (OIE/ FAO/WHO, 2006). These conferences have intended to control the abuse of antimicrobials and to decrease the incidence of antimicrobial-resistant bacteria.

In aquaculture, various antimicrobials have been widely used for treatment and prevention of disease. Such antimicrobial use may increase selection pressure for antimicrobial resistance and promote distribution of resistance determinants in *Vibrio* spp.. As seen in other bacterial strains, *Vibrio* species have several mechanisms

of antimicrobial resistance. Integrons are important mechanisms for the acquisition of antibiotic resistance genes among Gram-negative bacteria. They are mobile genetic elements that can transfer between and within species. There are 9-integron types and class 1 integrons is the most commonly identified type. Class 1 Integrons are able to contain one or more antibiotic resistance genes that can be mobilized to other bacteria (Mazel, 1998; Fluit et al., 2004). Resistance to tetracycline in bacteria can occur via two primary mechanisms. Two major mechanisms include the energy-dependent efflux systems and ribosomal protection protein (RPP) (Robert et al., 2001; Song et al., 2006a). Additional mechanism responsible for resistance to tetracycline in bacteria is an enzyme that inactivates the tetracycline molecule. Thirty-nine different tetracycline resistance *tet* genes have been characterized (Robert et al., 2001; Suzuki et al., 2002). The majority of *tet* genes in bacteria have been associated with conjugative or mobilization elements. These mobile elements have enabled the *tet* genes to intra- and inter- genus/species transfer (Robert et al., 1996; 2001). The acquisition of fluoroquinolone resistance is mainly due to chromosomal mutations, although a plasmid mediating fluoroquinolone resistance.

While data regarding molecular epidemiology and mechanisms of antimicrobial resistance has been increasingly required to control and reduce dissemination of resistant *Vibrio* spp., this data in Thailand is still limited. Such information from other countries cannot be totally applied to situations in Thailand because type of antimicrobial use is different in different countries. To date, studies of genetics of antimicrobial resistance in *Vibrio* spp. have been conducted in many countries e.g. India (Amita et al., 2003), China (Song et al., 2006a) and Italy (Falbo et al., 1999). However, in Thailand, there are only two published articles, one in *V. cholera* isolated from hospitalized patients (Dalsgaad et al., 1999a; 2000) and the other associated with the strains isolated from marine shrimps (Dalsgaad et al., 2000). These two studies only concentrated on class 1 integrons. Genetics of antibiotic resistance in other *Vibrio* species isolated from marine shrimp culture have never been reported in Thailand.

Therefore, this study, the molecular mechanisms underlying resistance to tetracycline and fluoroquinolones, drugs of choice for treatment of *Vibrio* infections were determined. Class 1, 2 and 3 integrons that are mobile-genetic elements and can transfer multiple drug resistance to other bacteria intra- and inter-species were characterized. Transferability of integrons among *Vibrio* species was determined. The species of *Vibrio* included in this study were *V. cholera, V. parahaemolyticus, V. vulnificus* and *V. fluvialis* that could inflict diseases in both humans and shrimps.

Results from this study will help us to understand molecular mechanisms of antimicrobial resistance in *Vibrio* spp. isolated from cultured marine shrimp in Thailand. The data obtained can be used as part of antimicrobial resistance monitoring. It can be also used to demonstrate the link of antimicrobial resistance *Vibrio* spp. between aquatic animals and humans when combined with molecular epidemiological data from humans and aquaculture products. In addition, the data can be applied in risk analysis of antimicrobial resistance and facilitate further studies of molecular mechanisms of antimicrobial resistance in *Vibrio* spp.

CHAPTER II

REVIEW LITERATURES

1. General characteristics and pathogenesis of *Vibrio* spp.

Vibrio spp. is a gram-negative bacterium in Vibrionaceae family. Its shape is straight or curved rod like "comma" with 0.5-0.8 µm width and 1.4-2.6 µm length. *Vibrio* spp. has single flagella with membrane, therefore it can motile. The bacterium is facultative anaerobe, produces no spore and is oxidase-positive. The optimal growth temperature is 37°C. It can grow well at pH 8.95 and also in salinity water especially in sea water surface, therefore; is classified as a halophilic bacterium. *Vibrio* species are generally able to grow on the selective medium thiosulfate-citrate bile salt-sucrose agar (TCBS) (Daniels and Shafaie, 2000; Charles et al., 2004).

Vibrio species are the major causative agents for most serious diseases in shrimp particularly in stressful condition. The organisms are pathogenic to humans and have been implicated in food-borne diseases and zoonosis. They can be isolated from infected patients, aquatic animals and environment. Clinical signs and severity of the disease depend on bacterial species and strains (Elliot et al., 1998; Daniels and Shafaie, 2000).

A. B.

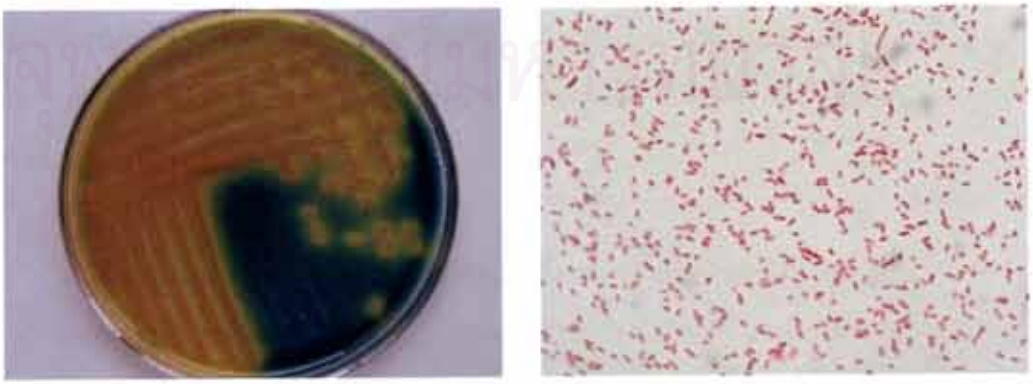


Figure 1: *Vibrio cholera* A. Colony on TCBS agar and B. Gram stain

V. cholera, which causes gastroenteritis and diarrhetic syndrome, is a non invasive bacterium. The target organ is small intestine with the infective dose of 10^8 organisms. *V. cholera* O1 and O139 strains cause cholera epidemic and large outbreak of cholera (Daniels and Shafaie, 2000; Charles et al., 2004). They produce a heat-sensitive cholera enterotoxin (CT) that is the primary virulence factor of the cholera disease. The typical characteristic cholera symptoms include diarrhea with rice water stool, mild to acute diarrhea, vomiting and dehydration. However, *V. cholera* non-O1 and non-O139 strains are sporadically involved in cholera-like diarrheal disease. Clinical sign are mild gastroenteritis and septicemia (Elliot et al., 1998; Daniels and Shafaie, 2000).

V. parahaemolyticus is an invasive bacterium. Clinical signs of *V. parahaemolyticus* infection are gastroenteritis, acute diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and septicemia. The infective dose is 10^6 organisms. Most clinical strains of *V. parahaemolyticus* produce thermostable direct hemolysin (TDH) and/or a TDH-related hemolysin (TRH) (Daniels and Shafaie, 2000; Charles et al., 2004).

V. vulnificus causes wound infection, gastroenteritis or a syndrome known as primary septicemia. Clinical signs of *V. vulnificus* infection in healthy individuals are gastroenteritis and wound infection. The infection is usually severe and ends up with death in persons who are immunocompromised, especially in people with some type of chronic underlying illness e.g. liver disease, diabetes, cirrhosis, leukemia or a compromised immune system. Invasion of bacteria is characterized by occurrence of blister like skin lesion and rapidly-spreading necrosis (Elliot et al., 1998; Charles et al., 2004).

V. fluvialis strains are the sporadic causes of diarrhea like cholera. The clinical signs include dehydration, vomiting, fever, abdominal pain and diarrhea (Elliot et al., 1998; Daniels and Shafaie, 2000). *V. fluvialis* produces an enterotoxin similar to non-O1 *V. cholera* inducing fluid accumulation. This enterotoxin is known as "the

vacuolating cytotoxin" that contains cytotoxic and vacuolating activity on cells. This toxin may play a role in the pathogenicity of *V. fluvialis* (Chakraborty et al., 2005).

2. Occurrence and epidemiology of antimicrobial resistance in *Vibrio* spp.

Vibriosis in humans is transmitted primarily by the fecal-oral route, indirectly through contaminated raw seafood and water supplies (Charles et al., 2004). *V. parahaemolyticus* and *V. cholera*, common food-borne enteric pathogens in Asia, cause approximately half of the food poisoning and gastroenteritis outbreaks in Thailand, Taiwan, Japan and India (Elliot et al., 1998; Wong et al.,1999; Gopal et al., 2005).

Among *Vibrio* spp. associated outbreaks in Thailand, infections with *V. parahaemolyticus* occur at the highest rate up to 78 % of food poisoning cases (Codex, 2005a; Sriwanna, 2007). *V. cholera* infections in Thailand is the morbidity rate of 1.57 per 100,000 populations and case fatality rate 0.71%. They are mostly reported in the Southern region with the rate of 3.6 per 100,000 populations (Bureau of epidemiology, 2007). *V. vulnificus* and *V. fluvalis* infections occur at lower incidence. *V. vulnificus* usually causes severe infection and ends up with death, of which the mortality rate is accounted for up to 50% of total seafood-borne patients (Charles et al., 2004; Sriwanna, 2006). The rates of *V. fluvialis* outbreaks are much less and the clinical sign is similar to cholera (Srifuengfung et al., 2004; Chakraborty et al., 2005).

Previous studies showed that *Vibrio* spp. strains resistant to antimicrobial are increasing around the world (Yamamoto et al., 1995; Dalsgaard et al., 1999a, 2000). Reported of Cholera cases in Thailand in 2007, *V. cholera* serogroup O1, serotype Ogawa strain were resistant to tetracycline (100%) and susceptible to norfloxacin (100%) (Bureau of epidemiology, 2007). Clinical and environmental *V. cholera* strains isolated in Thailand in 1982-1995 were resistant to gentamicin, kanamycin, tobramycin, streptomycin, spectinomycin, β-lactams and trimetroprim (Dalsgaad et al., 1995; 2000). *V. cholera* non-O1 and non-O139 strains isolated during the cholera-like epidemic

among the Cambodia in 1982 were resistant to trimethoprim-sulfamethoxazole with a high percentage (92%) (Dalsgaad et al., 1995). *V. cholera* strains isolated from Vietnam during 1979-1996 were resistant to sulfonamides and streptomycin (Dalgaard et al., 1999b). In Varanasi, India before 1992, *V. cholera* isolates were resistant to cotrimoxazole, trimetroprim, streptomycin, furazolidone and ampicillin (Mohapatra et al., 2008). Later, *V. cholera* strains that re-emerged in India and Bangladesh after 1992 were resistant to sulfamethoxazole, trimethoprim and streptomycin (Amita et al., 2003). *V. cholera* strains isolated in Africa (Thunggapathra et al., 2002), India (Columbo et al., 2006a; 2006b) and Thailand (Dalsgaard et al., 2000) were resistant to β-lactams, trimetroprim, sulfonamides and streptomycin.

3. Genetics of antimicrobial resistance in *Vibrio* spp.

3.1 Integrons in *Vibrio* spp.

Integrons are important mechanisms for the acquisition of antibiotic resistance genes among bacteria. They are mobile genetic elements that can transfer between and within species. There are 9-integron types and class 1 integrons is the most commonly identified type that plays an important role in transfer and distribution of antibiotic resistance particularly in gram-negative bacteria (Mazel, 1998; Fluit et al., 2004).

Genetic organization of class 1 integrons is shown in figure 2A. The integrons have 2 conserved regions (CS), 5'CS and 3'CS. The 5'-CS contains the *intI1* gene, which encodes the type 1 integrase responsible for site-specific insertion and excision of gene cassettes. The region also contains the *attI1* site, which is responsible for recombination of the site specific integration of the gene cassettes into the integron structure. The 3'-CS is usually characterized by the fused structure of *qacEΔ1* and *sul1* genes encoding resistance to quaternary ammonium compounds and sulfonamide, respectively (Mazel, 1998; Dalgaard et al., 2000). The integrons have internal variable regions, which usually contain gene cassettes encoding antibiotic resistance (Dalsgaad et al., 1995; Hall and Collis, 1995). Class 1 integrons were also previously determined in

clinical and environmental *V. cholera* strains isolated in Thailand. They were found to harbor the gene cassettes *aadB*, *aadA2*, *blaP1*, *dfrA1* and *dfrA15* that encode resistance to gentamicin, kanamycin and tobramycin, streptomycin and spectinomycin, β-lactams and trimetroprim, respectively (Dalsgaad et al., 1995; 2000). A study in Vietnam showed that *V. cholera* carried the *aadA2* gene cassette in the integrons (Dalgaard et al., 1999b). *V. cholera* strains isolated in Africa (Columbo et al., 2006a; 2006b), India (Thunggapathra et al., 2002) and Thailand (Dalsgaard et al., 2000) carried the *blaP1*, *dfrA15 and aadA2* gene cassettes. *V. cholera* isolated in Albania and Italy carried the *aadA1* gene cassettes (Falbo et al., 1999). Class 1 integrons were previously determined in other *Vibrio* spp.. *V. parahaemolyticus* isolated in South Africa carried the *dfrA15* gene cassettes (Columbo et al., 2006b) and *V. fluvialis* isolated in India carried the *dfrA15*, *aac(3)-Id* and *aadA7* gene cassettes that encode resistance to trimetroprim, gentamicin, streptomycin and spectinomycin, respectively (Srinivasan et al., 2006). Class 1 integrons can be founded on chromosome and/or plasmid. If these integrons are founded on chromosome, they cause the permanent drug resistance without antibiotic pressure. Those integrons carried on plasmid could promote distribution of antibiotic resistance genes by intra- and inter- genus/species transfer (Hall and Collis, 1995; Fluit et al., 2004).

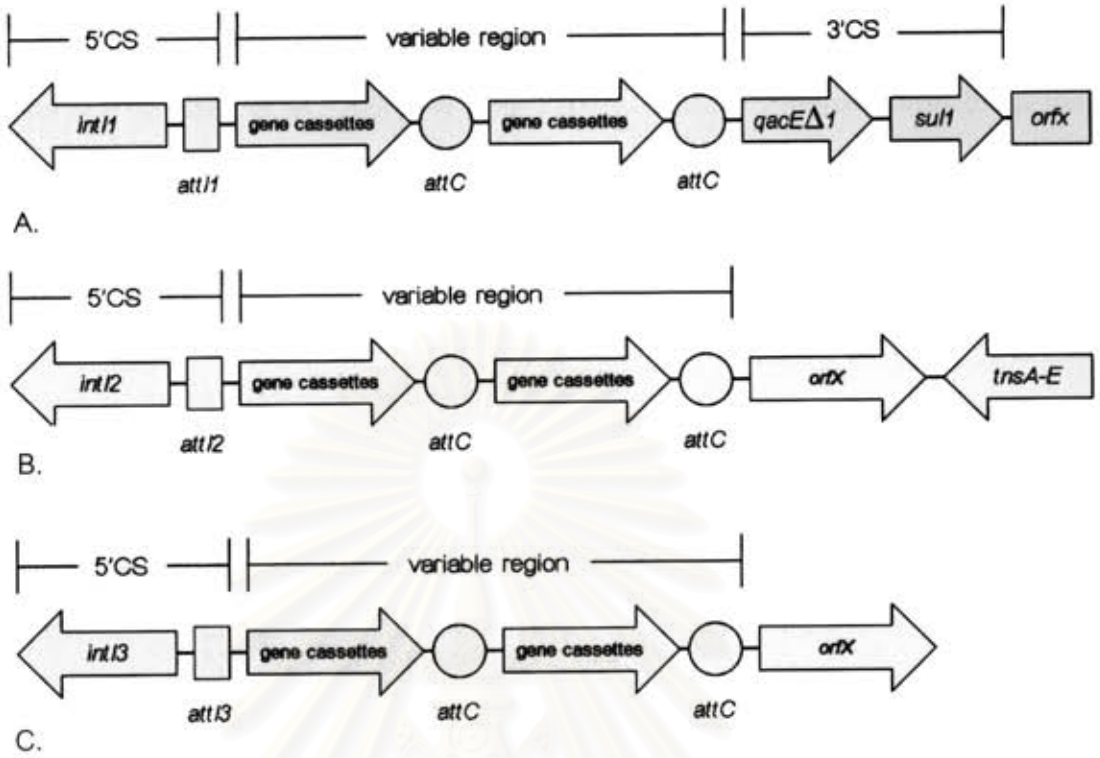


Figure 2: Genetic organization of (A) class 1 integrons, (B) class 2 integrons and (C) class 3 integrons. The *IntI1*, *intI2* and *intI3* genes encode the type 1, 2 and 3 integrase enzymes, respectively. The *attI1*, *attI2* and *attI3* sites are responsible for recombination of the site specific integration of gene cassettes. The *orfX* is an open reading frame of unknown function. The *tnsA-E* is transposition genes responsible for transposase binding.

Class 2 integrons was previously identified in only two isolates of *V. cholera*. One was a clinical isolate in India and the other was an environmental isolate in Bangladesh (Ahmed et al., 2006). They were found to carry the *dfrA1*, *sat1* and *aadA1* gene cassettes that encode resistance to trimetroprim streptothricin and streptomycin/spectinomycin, respectively (Ahmed et al., 2006). Genetic organization of class 2 integrons is shown in figure 2B. The integrons have 5'-CS that contains the *intI2* gene and a variable region containing gene cassette(s) and open reading frame of unknown function. It has the transposition (*tns*) genes responsible for transposase binding in mobility of transposon (Hansson et al., 2002).

Class 3 integrons (Figure 2C) have never been identified in *Vibrio* spp.. To date, they have been only identified in *Serratia marcescens*, *Klebsiella pneumonia* and *Delftia* spp. (Duarte et al., 2003; Xu et al., 2007). Class 3 integrons have 5'-CS that contains the *intI3* gene and a variable region containing gene cassette(s) and open reading frame of unknown function (Duarte et al., 2003).

3.2 Tetracycline resistance

Tetracyclines exhibit activity against a wide range of gram-positive and gram-negative bacteria. Tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Resistance to tetracycline in bacteria can occur via two primary mechanisms. Two major mechanisms include the energy-dependent efflux systems that extrude drugs out of the cells and ribosomal protection protein (RPP) that protects the ribosome from tetracycline binding (Robert et al., 2001; Song et al., 2006b). Additional mechanism responsible for resistance to tetracycline in bacteria is an enzyme that modifies and inactivates the tetracycline molecule (Robert et al., 2001; Suzuki et al., 2002)

Thirty-nine different tetracycline resistance (*tet*) genes have been characterized. The *tet* genes encode for efflux pumps e.g. *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(K)* and *tet(L)*. A previous study demonstrated that *tet(A-E)* and *tet(G)* are widespread among Gram-negative bacteria isolated from marine animals (Song et al., 2006a; 2006b). The *tet(H-L)* genes have been present in less frequency (Robert et al., 2001; Suzuki et al., 2002; Jeong et al.,2008). The *tet* genes encode for ribosomal protection proteins e.g. *tet(M)*, *tet(O)*, *tet(S)* and *tet(W)*. A previous study demonstrated that *tet(M)*, *tet(O)* and *tet(S)* genes have been present in less frequency among gram-negative bacteria (Robert et al., 2001; Suzuki et al., 2002; Barton et al.,2007). Little is still known about the RPP-encoding genes. Up to date, only the *tet(M)* and *tet(S)* genes have been identified in *Vibrio* spp. (Suzuki et al., 2004). The *tet (34)* gene encode an enzyme, xanthine-guanine phosphoribosyltransferase, which inactivates the tetracycline molecule (Robert et al., 2001; Suzuki et al., 2002; Jeong et al., 2007). The majority of *tet* genes in

bacteria have been associated with conjugative or mobilization elements e.g. mobile plasmids, conjugative transposons and integrons. These mobile elements have enabled the *tet* genes to intra- and inter- genus/species transfer (Robert et al., 1996; 2001).

The *tet*(A) gene has been previously identified in many *Vibrio* species, including *V. cholera* (Dalsgaard et al., 2001), *V. harveyi*, *V. tubiashii* (Barton et al., 2007), *V. splendidus*, *V. tasmaniensis* (Song et al., 2006a; 2006b). The *tet*(B) gene has been reported previously in *V. splendidus*, *V. tasmaniensis* (Song et al., 2006a; 2006b) and *Vibrio* spp. (Robert et al., 2001; Song et al., 2006a; Jeong et al., 2007). The *tet*(C) gene has been previously identified in *V. cholera* (Olsvik et al., 1995) and many of *Vibrio* spp. (Robert et al., 2001). The *tet*(D) gene has been previously found in *V. splendidus*, *V. tasmaniensis* (Song et al., 2006a; 2006b) and other unidentified *Vibrio* spp. (Robert et al., 2001). The *tet*(E) gene has been identified in *V. mimicus* (Barton et al., 2007). The *tet*(G) gene has been previously determined in several *Vibrio* species. (Robert et al., 2001). The *tet*(M) gene has been previously identified in *V. mimicus* (Barton et al., 2007) and other *Vibrio* spp. (Suzuki et al., 2004, Jeong et al., 2007). The *tet(S)* gene has been found in *Vibrio* spp. at low frequency (Suzuki et al., 2004, Jeong et al., 2007). In this paper, the species of *Vibrio* was not identified.

3.3 Fluoroquinolones resistance

The fluoroquinolones are broad spectrum antibacterial agents. The first and third generations of quinolones, i.e. nalidixic acid and levofloxacin, have greater activity against gram positive bacteria. The second generation of quinolones, i.e. ciprofloxacin and norfloxacin, has excellent activity against gram negative bacteria (Vila, 2005). The bacterial targets of fluoroquinolones are the DNA gyrase (topoisomerases II) and topoisomerase IV. They function by inhibition of enzyme DNA gyrase or the topoisomerase IV enzyme in DNA synthesis. DNA gyrase enzyme consists of 2 subunits, GyrA and GyrB encoded by the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV enzyme consists of 2 subunits, ParC and ParE encoded by the *parC* and *parE* genes, respectively. Both DNA gyrase and topoisomerase IV require ATP to complete the

function. DNA gyrase is the enzyme responsible for the generation of negative supercoiling of DNA and is necessary for initiation and propagation of the DNA replication fork. Topoisomerase IV plays a major role in decatenating daughter replicons following DNA replication (Okuda et al., 1999; Ruiz, 2003; Vila, 2005)

The acquisition of fluoroquinolones resistance is mainly due to chromosomal mutations, although a plasmid mediating fluoroquinolones resistance i.e. *qnr* gene. The chromosomal mutations can be distributed into two groups: First, mutations in topoisomerase genes i.e. *gyrA, gyrB, parC* and *parE*. Second, mutations causing reduced drug accumulation, by a decreased uptake or by increased efflux (Ruiz, 2003; Vila, 2005). The mutations in topoisomerase genes, resistance to fluoroquinolones arises from amino acid substitution in a target region of the corresponding topoisomerase called the "Quinolone Resistance Determining Regions: QRDRs" located within the DNA-binding domain on the surface of gyrase and topoisomerase protein. The mutations may alter the interaction between enzymes and drugs resulting in the reduced sensitivity to fluroquinolones (Ruiz, 2003; Vila, 2005).

In *Vibrio* spp., mutations in *gyrA* and par*C* have been shown to confer decreased sensitivity of DNA gyrase to fluoroquinolones. Previous studies showed that Mutation in *gyrA*, *V. parahaemolyticus* (Okuda et al., 1999), *V. cholera* (Kundu et al., 2002), *V. fluvialis* (Srinivisan et al., 2006) and *V. anguillarum* (Colquhoun et al., 2007, Aoki et al., 2008) strains resistant to fluoroquinolones were shown to contain a single base pair change in the *gyrA* sequence responsible for a serine to isoleucine change at residue position 83 (Ser-83-Ile). *V. cholera* had an additional mutation in the *gyrA* sequence responsible for a aspartic acid to tyrosine change at residue position 100 (Asp-100-Tyr) (Kundu et al., 2002). Mutation in *parC*, *V. parahaemolyticus* (Okuda et al., 1999), *V. cholera* (Kundu et al., 2002), and *V. anguillarum* (Colquhoun et al., 2007; Aoki et al., 2008) strains resistant to fluoroquinolones were shown to contain a single base pair change in the *parC* sequence responsible for a serine to leucine change at residue position 85 (Ser-85-Leu)

CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 3 phases: phase I, test for antimicrobial susceptibility; phase II, determination of genetics of antimicrobial resistance and phase III, test for transferability of integrons. The conceptual framework is shown in Figure 3.

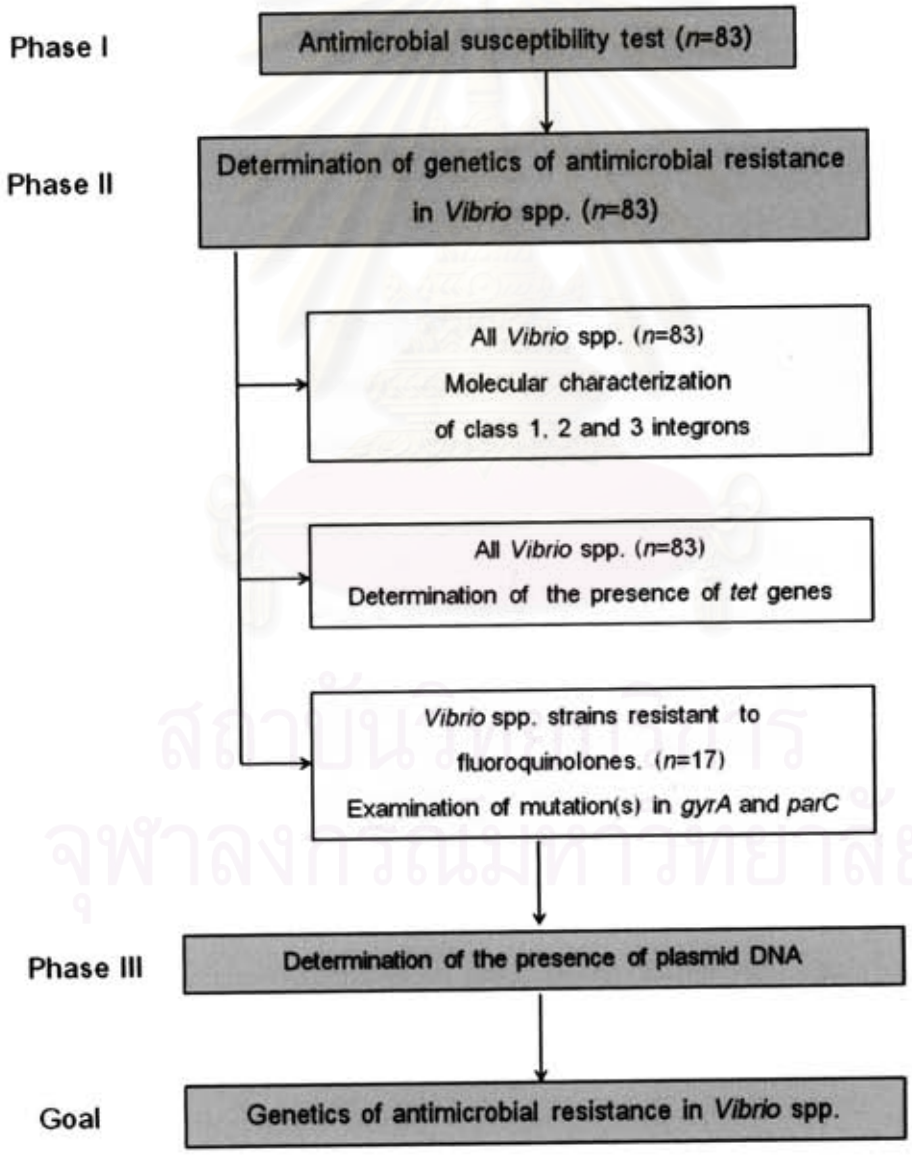


Figure 3: The conceptual framework in this study

Vibrio isolates

A total of 83 *Vibrio* isolates comprising *V. parahaemolyticus* (*n*=26), *V. cholera* (*n*=18), *V. fluvalis* (*n*=23), and *V. vulnificus* (*n*=16) were included in this study. All of the isolates were obtained from the strain collection of Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. These strains were isolated from clinical cases of diseased shrimps in farmed-marine shrimps in Thailand during 2001-2002. All the strains were isolated using the standard methods as described in FDA Bacteriological Analytical Manual (Elliot et al., 1998) and tested for their biochemical characteristics with API 20E (Biomerieux, France) in previous studies (Wongtavatchai et al., 2006). All of the bacterial strains were stored as 20% glycerol stocks at -80°C and sent to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for further studies as follows.

Phase I Test for antimicrobial susceptibility

All of the *Vibrio* isolates were tested for their antimicrobial susceptibility by determining Minimum Inhibitory Concentrations (MICs) using a serial two-fold agar dilution technique. The procedures are in accordance with the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS). The antimicrobials include ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), enrofloxacin (ENR), kanamycin (KAN), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMP). The *Vibrio* isolates were grown on Tryptic Soy Agar (TSA; Difco, MD, USA) supplemented with 1% NaCl. After incubation at 37°C for 18-24 h, the colonies were transferred to 0.85% NaCl solution (NSS) and the cell density was adjusted to Mcfarland standard 0.5 or approximately 10^8 CFU/ml. Then, the suspension was ten-fold diluted in NSS, giving a final cell count of approximately 10^7 CFU/ml. The suspension was inoculated onto the Muller-Hinton agar (MHA; Difco, MD, USA) containing appropriate concentrations of antimicrobials by using a multi-point inoculator. The serial two-fold dilutions of antimicrobials were dissolved in appropriate solvents. The solvents and antimicrobial concentrations used are shown in Table 1.

After 18-24 hr. incubation, the MIC was recorded as the lowest concentration of an antimicrobial yielding no visible growth of bacteria. Breakpoints are the discriminating concentrations used for the interpretation of results of susceptibility testing to define isolates as susceptible or resistant with the interpretation guidelines established by CLSI and shown in Table 2. Multidrug resistance (MDR) was defined as isolates being resistant to 3 or more different classes of antimicrobials. *Escherichia coli* ATCC 25922 and *Staphylococus aureus* ATCC 29213 were used as quality control organisms.

Table 1: Solvents and concentrations of antimicrobials

Antimicrobials	Solvent	Concentration range (µg/ml)
ampicillin	Distilled water	0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128
chloramphenicol	95% ethanol	0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128
ciprofloxacin	0.1N NaOH	0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32
enrofloxacin	0.1N NaOH	0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32
erythromycin	methanol	0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32
kanamycin	Distilled water	0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256
streptomycin	Distilled water	0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256
sulfamethoxazole	0.1N NaOH	0.59, 1.19, 2.38, 4.75, 9.5, 19, 38, 76, 152, 304, 608
tetracycline	70% ethanol	0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64
trimethoprim	dimethylacetamide	0.125, 0.25, 0.5, 1, 2, 4, 8, 16

Table 2: Breakpoints used in this study

Antimicrobials	Breakpoint (µg/ml)
ampicillin[a]	32
chloramphenicol[a]	32
ciprofloxacin[b]	4
enrofloxacin[b]	4
erythromycin[c]	8
kanamycin[b]	64
streptomycin[b]	16
sulfamethoxazole[a]	76
tetracycline[a]	16
trimethoprim[a]	4

[a] MIC Interpretative standard for *V. cholera*

[b] MIC Interpretative standard for Enterobacteriaceae

[c] MIC Interpretative standard for *Enterococcus* spp.

Phase II Determination of genetics of antimicrobial resistance in *Vibrio* spp.

The experiments in this phase included

1. Characterization of class 1, 2 and 3 integrons
2. Determination of the presence of *tet* genes
3. Examination of mutation(s) of the QRDRs in *gyrA* and *parC* genes.

For all steps, DNA template was prepared by the whole cell boiled lysate procedure (Dalgaard, 1999[b]). Bacteria were grown on TSA supplemented with 1% NaCl and incubated at 37°C for 24 h. A single colony was suspended in 50 µl of sterile distilled water and heated in a boiling water bath for 10 minutes. The suspension was centrifuged at 12,000 rpm for 5 minutes. The supernatant was removed to a new eppendorf tube and stored at -20°C until used.

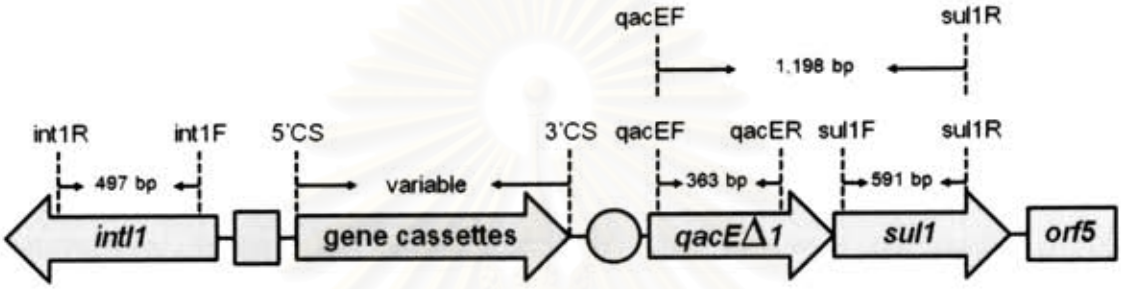
All primers used in this study are listed in Table 3.

Table 3: PCR primers

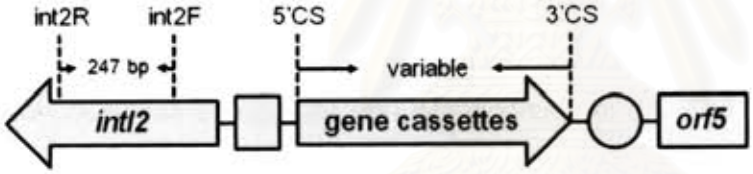
Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
Mutations in QRDRs				
gyrA	VGYR-2	AA(C/T)GA(C/T)TGGAA(C/T)AA(A/G)(G/C)C	203	Okuda et al., 1999
	VGYR-4	TC(A/C/G/T)GT(A/G)TA(A/C/G/T)C(G/T)CAT(A/C/G/T)GC		
parC	VGYR-3	AA(A/G)TA(C/T)CA(C/T)CC(A/C/G/T)CA(C/T)G	106	Okuda et al., 1999
	VGYR-4	TC(A/C/G/T)GT(A/G)TA(A/C/G/T)C(G/T)CAT(A/C/G/T)GC		
Antibiotic resistance gene				
tetK	tetKF	CAATACCTACGATATCTA	352	Klare et al., 2007
	tetKR	TTGAGCTGTCTTGGTTCA		
tetL	tetLF	TGGTCCTATCTTCTACTCATTC	385	Werner et al., 2003
	tetLR	TTCCGATTTCGGCAGTAC		
tetM	tetMF	GGTGAACATCATAGACACGC	401	Werner et al., 2003
	tetMR	CTTGTTCGAGTTCCAATGC		
tetO	tetOF	AGCGTCAAAGGGGAATCACTATCC	1,723	Klare et al., 2007
	tetOR	CGGCGGGGTTGGCAAATA		
tetS	tetSF	ATCAAGATATTAAGGAC	573	Gervers et al., 2003
	tetSR	TTCTCTATGTGGTAATC		
Integrons				
IntI1	Int1F	CCTGCACGGTTCGAATG	497	Chuanchuen et al.,2007
	Int1R	TCGTTTGTTCGCCCAGC		
IntI2	Int2F	GGCAGACAGTTGCAAGACAA	247	Chuanchuen et al.,2008a
	Int2R	AAGCGATTTTCTGCGTGTTT		
IntI3	Int3F	CCGGTTCAGTCTTTCCTCAA	155	Chuanchuen et al.,2008a
	Int3R	GAGGCGTGTACTTGCCTCAT		
variable regions	5'CS	GGCATCCAAGCAGCAAG	variable	Levesque et al., 1995
	3'CS	AAGCAGACTTGACCTGA		
qacEΔ1	qacEF	TAAGCCGTACACAAATTGGGAGATAT	363	Chuanchuen et al.,2007
	qacER	GCCTCCGCAGCGACTTCCACG		
sul1	sul1F	CGGACGCGAGGCCTGTATC	591	Chuanchuen et al.,2007
	sul1R	GGGTGCGGACGTAGTCAGG		
qacEΔ1-sul1	qacEF	TAAGCCGTACACAAATTGGGAGATAT	1,198	Chuanchuen et al.,2007
	sul1R	GGGTGCGGACGTAGTCAGG		

1. Molecular characterization of class 1, 2 and 3 integrons

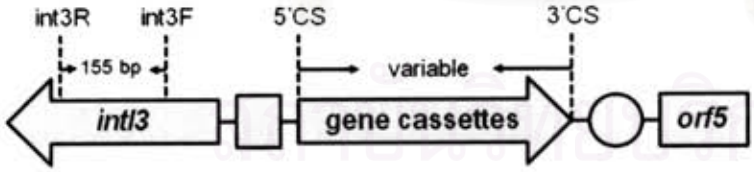
First, the presence of *intI1*, *intI2* and *intI3* genes was identified. The *intI1*, *2* and/or *3*-positive strains were, then, investigated for the presence of inserted gene cassettes that were later characterized. Finally, the presence of typical 3' conserved segment of the *intI1*-positive strains was examined. Locations of primers used for integron analysis are shown in Figure 4. Experiments are described in detail below.



A. Class 1 integrons



B. Class 2 integrons



C. Class 3 integrons

Figure 4: Localization of primers used in characterization of class 1 integrons. Primers int1F and int1R were used for amplification of the *intI1* gene. Primer 5'CS and 3'CS were used for amplification of inserted gene cassettes. Primer pairs qacEF-qacER and sul1F-sul1R were used to amplify *qacEΔ1* and *sul1*, respectively. The fused structure of *qacEΔ1-sul1* was confirmed using primer pair qacEF-sul1R. Primer int2F and int2R were used for amplification of the *intI2* gene. Primer int3F and int3R were used for amplification of the *intI3* gene. The arrows indicate the direction of primers. The vertical-dashed lines indicate locations of primers.

All PCR assays were carried out in a final volume of 25 µl using PCR Master Mix (Fermentas®, Mainz, Germany) according to the manufacturer's instruction. Each PCR reaction consisted of 12.5 µl of Fermentas® MasterMix, 5.5 µl of sterile-distilled water, 1.0 µl of each primer at 10 µm and 5 µl of DNA template. PCR amplifications were conducted on a PCR Tpersonal combi model® (Biometra®, Germany). Thermal cycling conditions included of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 1 min at 94°C, 1 minute at 50°C, 1 minute at 72°C and a final step of 72°C for 5 minutes. The PCR products obtained were separated on gel electrophoresis in 1.5% agarose gel (Agarose, Sigma–Aldrich®, USA) and Tris-acetate/EDTA buffer and applying 100 V for 30-45 minutes. The gels were stained in ethidium bromide solution (Sigma Aldrich Inc.) and visualized by the Bio-Rad Gel-Documentation system (Bio-Rad Laboratories, Ventura, CA, USA). The PCR amplicons were gel purified using Nucleospin Gel Extraction kit (Nucleospin®, Gutenberg, France). The DNA representatives were submitted for sequencing at Macrogen Inc. (Seoul, South Korea). Nucleotide sequence analysis was performed using the Blast algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). *Pseudomonas aeruginosa* P90 (Chuanchuen et al., 2007), *Salmonella* Paratyphi B *var Java* (van Essen-Zandbergen et al., 2007) and pAV3.5 (Xu et al., 2007) were used as positive controls for *intI1*, *intI2* and *intI3* genes, respectively.

All the isolates containing class 1 integrase were assayed for the presence of inserted gene cassettes using 5'CS and 3'CS primers as previously described (Levesque et al., 1995). PCR reaction conditions were an initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 95°C, primer annealing for 1 minute at 54°C, and extension for 3 minutes at 72°C and a final extension at 72°C for 5 minutes.

All the strains containing class 1 integrase gene were examined for the presence of the 3'conserved regions using qacEF and sul1R primers as previously described (Chuanchuen et al., 2007). Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at

95°C, primer annealing for 1 minute at 54°C, and extension for 3 minutes at 72°C and a final extension at 72°C for 5 minutes.

2. Determination of the presence of *tet* genes

All the *Vibrio* isolates were screened for the presence of *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(S)* by PCR using specific primers. PCR reaction conditions for *tet* genes were a predenaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 second at 55°C, and extension for 45 seconds at 72°C and a final extention at 72°C for 5 minutes. PCR products were gel purified and the representative DNA samples were submitted for nucleotide sequencing. *Campylobacter coli* CAC041 was used as positive control for *tet(O)* gene (Chuanchuen et al., 2008). *E. coli* pJ13 and pAT451 were used as positive controls for *tet(M)* and *tet(S)* genes respectively (Bryan et al., 2004).

3. Examination of mutations of the QRDRs in the *gyrA* and *parC* genes

The *Vibrio* isolates resistant to ciprofloxacin and enrofloxacin were examined for mutation(s) in the QRDRs of *gyrA* and *parC* using PCR and DNA sequencing. Amplifications of the QRDRs of the *gyrA* were performed using a primer set, VGYR-2 and VGYR-4 as previously described (Okuda et al., 1999). For the *parC* gene, the QRDR was PCR amplified using primers VGYR-3 and VGYR-4. PCR thermocycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 45 second, 55°C for 45 second, and 72°C for 30 second, with a final step at 72°C for 5 minutes. All PCR products were gel purified and submitted for nucleotide sequencing. Nucleotide sequencing analysis was performed using the NCBI Blast search. The resulting nucleotide sequence of both strands were compared with the published DNA sequence of *Vibrio* spp. (GenBank accession numbers AB023569 and AB0235570 for *gyrA* and *parC*, respectively) using the Chromas ver.1.45 and Seqman (DNA-STAR) program. Two *Vibrio* isolates susceptible to both ciprofloxacin and enrofloxacin were used as negative controls.

Phase III Determination of the presence of the plasmid DNA

Originally, the experimental design proposed was to determine if class 1, 2 and 3 integrons with antibiotic resistance gene cassettes could be transferred to other bacteria. However, no class 1 integrons with resistance gene cassettes was found and none of the *Vibrio* isolates carried class 2 and 3 integrons (see results on page 28). Therefore, this part of the proposed experiment was not performed.

The experiment in this phase was determination of the presence of plasmid DNA as described below.

All *Vibrio* isolates were determined for the presence of plasmid DNA. Plasmid DNA were extracted with the conventional-plasmid minipreparation method (Kraft et al., 1988). Bacteria were grown on LB broth supplemented with 1% NaCl and incubated at 37°C for 24 h. The 1.5-ml portion of the culture was transferred into an eppendorf tube and centrifuged at 14,000 rpm for 2 minutes. The supernatant was removed and the bacterial pellet was washed with 1 ml phosphate buffered saline (PBS, Diagxotics®, Wilton, USA). A hundred-µl of 10 mg/ml Lysozyme (BioBasic Inc®, Canada) was added and the pellet was re-suspended by vortexing. After incubation at room temperature for 5-10 minutes, 200-µl of freshly-prepared lysis solution consisting of 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS, Amresco®, Ohio, USA) was added and the mixture was mixed by inversion. At this step, the sample will become viscous. After incubation on ice for 5 minutes, 150-µl of cold potassium acetate pH 4.8 was added. The mixture was mixed by vortexing, incubated for 5 minutes on ice and centrifuged at 14,000 rpm for 5 minutes. The supernatant approximately 400 µl in volume was transferred to a new eppendorf tube. Two-µl of 10 mg/ml Ribonuclease A (RNase, Fermentas®) and was added and the mixture was incubated at 37°C for 30 minutes. The equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 ratio) mixture, which was approximately 400 µl was added. The sample was again mixed by vortexing and centrifuged at 14,000 rpm for 2 minutes. The aqueous phase was transferred to a new eppendorf tube and mixed 2.5 volume of cold absolute ethanol. The mixture was mixed by inversion,

incubated at -80°C for 30 minutes and centrifuged at 14,000 rpm for 5 minutes. The clear supernatant was removed. The DNA pellets were rinsed with 1 ml of cold 70% ethanol and centrifuged for 1 minute. The supernatant was discarded. The DNA pellet was air-dried at room temperature for 10 minutes. The DNA pellets were dissolved in 15 µl sterile water and stored at -20°C. The plasmid DNA obtained was separated on agarose gel electrophoresis.

Instruments and chemical substances

1. PCR assay
    1.1 Master Mix (Fermentas®, Mainz, Germany)
    1.2 DNA marker (Fermentas®, Mainz, Germany)
    1.3 Loading dye (Fermentas®, Mainz, Germany)
    1.4 Agarose gel (Molecular grade)
    1.5 Gel electrophoresis buffer (TAE)
    1.6 Ethidium Bromide 10 mg/ml (Sigma Aldrich Inc®, USA)
2. Thermocycler (Thermo electron corporation®, Cambridge, UK)
3. Gel electrophoresis system (OWL Scientific Inc®, USA)
4. Gel document system (Vilber Lourmat®, Marne La Valle, France)
5. PCR tubes and Microcentrifuge tube 1.5 ml
6. Centrifuge and Microcentrifuge
7. Micropipette and Micropipette tips
8. A -20°C refrigerator
9. A -80°C refrigerator
10. Experimental glasswares

CHAPTER IV

RESULTS

1. Susceptibilities to antimicrobials

The MICs range, MIC_{50} and MIC_{90} of the *Vibrio* isolates is shown in Table 4. The MIC_{50} and MIC_{90} were defined as the minimum concentration of antimicrobials required to inhibit 50% and 90% of the isolates tested.

Table 4: MICs range of *Vibrio* isolates to antimicrobials susceptibilities

Antimicrobials	MIC (μg/ml)		
	MIC_{50}	MIC_{90}	MIC range
ampicillin	32	128	1- >128
chloramphenicol	1	2	0.5-4
ciprofloxacin	0.5	4	0.016-16
enrofloxacin	0.5	2	0.031-16
erythromycin	4	16	2-32
kanamycin	4	16	0.25-32
streptomycin	8	16	2-32
sulfamethoxazole	19	76	2.38-152
tetracycline	2	32	0.25-64
trimethoprim	1	8	0.125-32

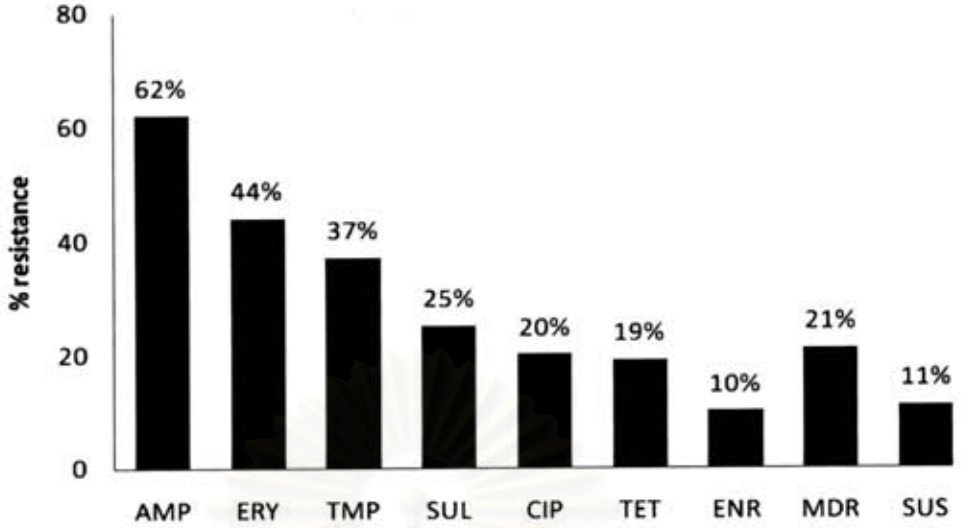


Figure 5: Distribution of antimicrobial resistance in *Vibrio* isolates. Abbreviations: AMP, ampicillin; ERY, erythromycin; TMP, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; TET, tetracycline; ENR, enrofloxacin; MDR, multiple drug resistant; SUS, susceptible to all antibiotics tested

Distribution of antimicrobial resistance of the *Vibrio* isolates is shown in Figure 5. In this study, 9 isolates (11%) were susceptible to all antimicrobials tested and 74 isolates (89%) were resistant to at least one antimicrobial. Most of the isolates were resistant to ampicillin (62%) and erythromycin (44%). None of the isolates was resistant to chloramphenicol, kanamycin and streptomycin. Seventeen isolates (21%) were resistant to at least 3 different antimicrobial classes and considered multidrug resistance (MDR).

Antimicrobial resistance patterns are also analyzed and shown in Table 6. All *Vibrio* strains could be grouped into 27 resistance patterns. The most common resistance pattern was AMP (20.48%) and the most common MDR phenotypes were the AMP-SMX-TMP (4.82%).

Table 5: Antimicrobial resistance patterns of *Vibrio* isolates (*n*=83)

Antimicrobial resistance pattern	No. of isolates (%)
AMP	17(20.48)
ERY	9 (10.84)
SMX	2 (2.41)
TET	3 (3.61)
AMP-CIP	4 (4.82)
AMP-ERY	7 (8.43)
AMP-TET	3 (3.61)
CIP-ERY	2 (2.41)
ERY-TET	1 (1.20)
SMX-ERY	1 (1.20)
SMX-TMP	2 (2.41)
AMP-CIP-ENR	1 (1.20)
AMP-ERY-SMX	1 (1.20)
AMP-ERY-TET	2 (2.41)
AMP-SMX-TET	1 (1.20)
AMP-SMX-TMP	4 (4.82)
CIP-ERY-ENR	1 (1.20)
CIP-ENR-TMP	1 (1.20)
AMP-CIP-ERY-ENR	1 (1.20)
AMP-CIP-ENR-TET	1 (1.20)
AMP-ERY-SMX-TMP	2 (2.41)
AMP-TET-TMP-SMX	1 (1.20)
AMP-CIP-ERY-SMX-TMP	1 (1.20)
AMP-ERY-SMX-TET-TMP	1 (1.20)
CIP-ERY-SMX-TET-TMP	1 (1.20)
AMP-CIP-ERY-ENR-SMX-TMP	2 (2.41)
AMP-CIP-ERY-ENR-SMX-TMP-TET	2 (2.41)
Susceptible to all antibiotics	9 (10.84)
Total	83

2. Determination of genetics of antibiotic resistance in *Vibrio* spp.

2.1 Molecular characterization of class 1, 2 and 3 integrons

Five isolates (6%) yielded a 497 bp amplicon when they were amplified with the primers specific for the *intI1* gene. Nucleotide analysis confirmed that the sequence of the amplicon was identical to the published sequence of class 1 integrase gene. The PCR amplicons of *intI1* are shown in Figure 8. None of strains tested were positive to *intI2* and *intI3* genes.

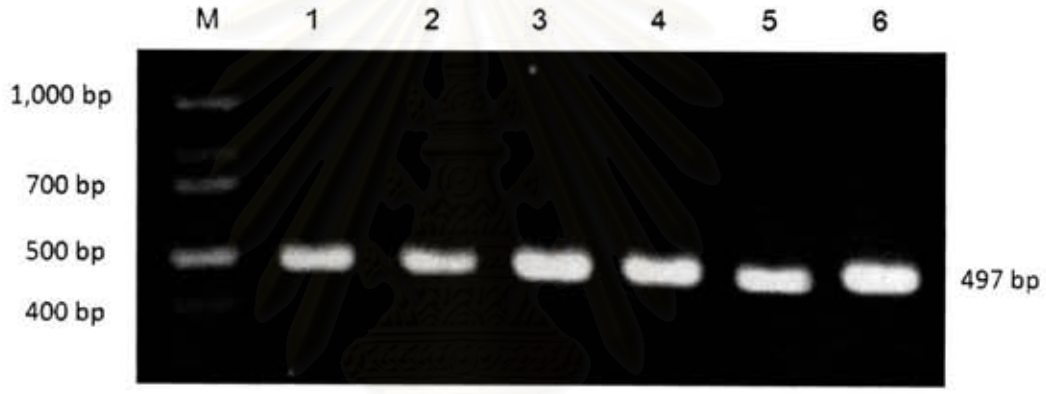


Figure 6: PCR amplification of *intI1* in the *Vibrio* isolates; Lane M, 100-bp marker; Lane 1-5, the *intI1*-containing *Vibrio* strains; Lane 6, *Pseudomonas aeruginosa* P90 was used positive control.

The *Vibrio* isolates carrying *intI1* gene were examined for the presence of the typical 3'-conseved regions. Since none of the *intI1*-positive strains carried the *qacEΔ1-sul1* fused structure, all of them lacked the typical 3'CS.

The *Vibrio* isolates carrying *intI1* gene were examined for the presence of gene cassettes inserted in variable regions. One of the *intI1*-positive strains contained gene cassettes in variable region with 428 bp in size. Nucleotide sequencing analysis revealed that the PCR amplicon of the variable regions were the incomplete *rumA* gene.

2.2 The presence of the *tet* genes

All *Vibrio* isolates were determined for the presence of the *tet(K)*, *tet(L)*, *tet(M)* *tet(O)* and *tet(S)*. None of them were positive the *tet* genes tested. The PCR amplicons of the positive controls of *tet* genes were 401, 1,723 and 573 bp in size when primers sets for *tet(M)*, *tet(O)* and *tet(S)* were used respectively. These confirmed that the appropriate primers and PCR reactions were used in this study.

2.3 Mutation(s) in QRDRs of *gyrA* and *parC*

Seventeen *Vibrio* isolates resistant to ciprofloxacin and enrofloxacin were examined for mutations within the QRDRs of *gyrA* and *parC*. Ten *Vibrio* spp. isolates (59%) resistant to ciprofloxacin harbored a mutation in the *gyrA* gene, which was replacement of G at position of 248 with T leading to a Ser-to-Ile change at amino acid residue position 83 in GyrA. Nine *Vibrio* isolates (53%) carried silent mutations that were replacement of C at position of 363 with T in *gyrA*. The positions of mutations in the gene are shown in Figure 7. No mutations were found in the QRDRs of *parC* in any strains. There were no mutations in the QRDRs of the quinolone susceptible *Vibrio* spp. control strains as well.

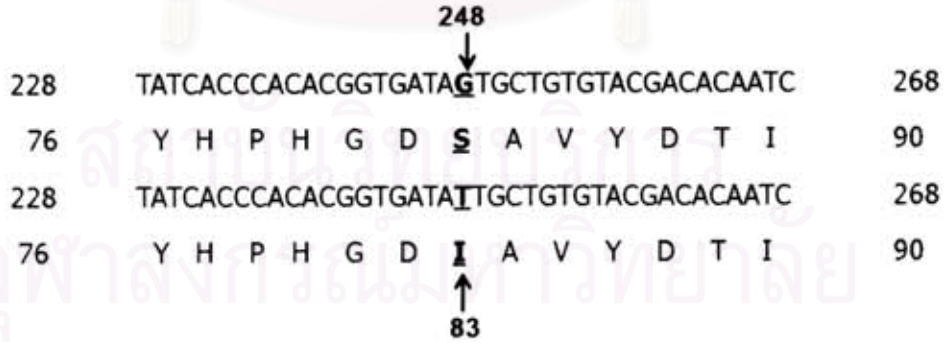


Figure 7: A point mutation observed in the *gyrA* gene sequences of the quinolone-resistant *Vibrio* isolate. The mutation found was a G248T replacement resulting in a Ser-83-Ile substitution in GyrA.

3. Determination of the presence of the plasmid DNA

All *Vibrio* isolates were determined for the presence of plasmid DNA. Seventeen isolates (20.5%) were found to carry plasmid DNA. Plasmid profile could be defined into 4 groups according to number and size of plasmids (Figure 8).

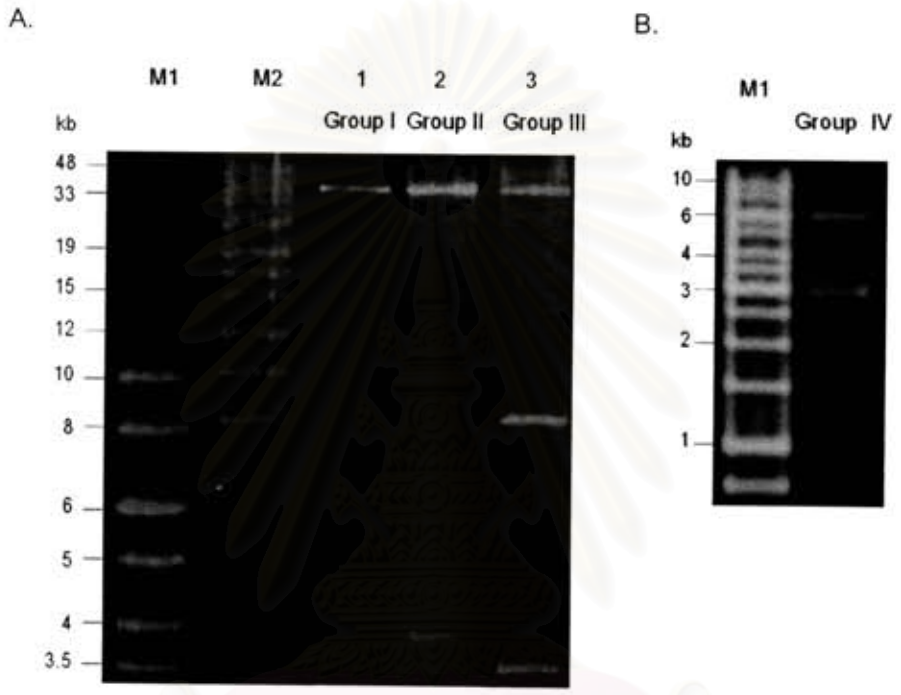


Figure 8: Plasmid profile of the *Vibrio* isolates. Lane M1, 1 Kbp marker; Lane M2, Lamda mix marker; (A) Lane1, Group I; Lane 2, Group II and Lane 3, Group III and (B) Group IV

Twelve isolates (70.5%) that harbored one plasmid of approximately 35,000 bp in size were classified in Group I. Group II consisted of one isolate that carried 4,000-bp and 35,000 bp plasmid. In group 3, there were 2 *Vibrio* isolates containing up to 3 plasmids with the size of 3,500 bp, 8,000 bp and 35,000 bp. In the last group, there were 2 *Vibrio* isolates that carried 2 plasmids with the size of 3,000 bp and 6,000 bp. The characteristics of plasmid profile in each group are summarized and shown in Table 6.

Table 6: Plasmid profile of the *Vibrio* isolates in this study

Group	Number of plasmids	Plasmid profile (size: bp)	Number of strains (%)
I	1	35,000	12 (70.5)
II	2	35,000, 4,000	1 (5.9)
III	3	35,000, 8,000, 3,500	2 (11.8)
IV	2	6,000, 3,000	2 (11.8)

The MIC value for antibiotics of all the *Vibrio* isolates carrying plasmid is shown in Table 7. Most of the isolates containing plasmid(s) (76.5%) were resistant to at least one antibiotic and 23.5% were multidrug resistant. 23.5 percent of the *Vibrio* strains were susceptible to all antimicrobials tested.

Table 7: MIC value of the *Vibrio* strains containing plasmid(s)

Strain	MIC (μg/ml)[a]						
	AMP	CIP	ENR	ERY	SMX	TET	TMP
V. cholera 33	4	0.02	0.03	2	9.5	2	0.13
V. cholera 45	2	8	4	2	16	8	16
V. cholera 76	128	2	2	4	0.25	2	0.25
V. cholera 128	2	0.5	1	0.5	38	8	1
V. cholera 140	2	0.13	0.25	1	38	8	1
V. cholera 180	1	0.03	0.06	2	9.5	1	0.13
V. parahaemolyticus 59	32	0.13	0.25	0.5	76	2	1
V. parahaemolyticus 64	64	0.25	0.5	4	2.38	0.5	0.5
V. parahaemolyticus 235	16	0.25	0.5	2	152	1	>16
V. parahaemolyticus 270	1	0.25	0.25	8	19	0.5	8
V. parahaemolyticus 282	64	8	4	16	76	0.5	16
V. vulnificus 5	64	0.25	0.5	16	152	0.5	8
V. vulnificus 25	32	8	16	4	38	1	2
V. vulnificus 34	16	0.5	0.5	8	38	1	2
V. vulnificus 61	32	1	1	4	19	1	0.5
V. fluvialis 7	64	0.5	1	8	4.75	0.5	0.5
V. fluvialis 49	32	1	2	1	9.5	1	0.13

[a] Number underlined indicates resistance to antibiotics

CHAPTER V

DISCUSSION

As farmed-marine shrimp is one of the most beneficial exporting goods, *Vibrio* spp. a commonly-found pathogen in shrimps has been a major cause of zoonotic disease (so called vibriosis). Antibiotics have been used for treatment and prevention of bacterial infections in cultured shrimps for a long period of time. It has been known that improper antibiotic use in animal production including shrimp farmings could result in development of drug-resistant bacteria, transfer of drug resistance features and reduced efficacy of antibiotic treatment (OIE/FAO/WHO, 2004; 2006). To date, incidence of antibiotic-resistant *Vibrio* spp. has been increasing worldwide. Therefore, concerns of development and dissemination of multidrug-resistant *Vibrio* strains have been raised and particularly, those from farmed-marine shrimps (Falbo et al., 1999; Amita et al., 2003; Song et al., 2006a).

One of the main findings of this study is the widespread of multidrug resistance among *Vibrio* species from farmed-marine shrimps. In Thailand, the commonly used antibiotics in aquaculture include tetracycline, quinolones and sulfonamide (Holmstrom et al., 2003; Sapkota et al., 2008). This could be the explanation for high resistance rates to these antibiotics in the *Vibrio* isolates in the present study.

Most of the isolates tested in this study were resistant to ampicillin (62%) and erythromycin (44%), which is in agreement with the results of previous studies in Mexico (Roque et al., 2001) showing that 68% of the isolates were resistant to ampicillin and in India (Vaseeharan et al., 2005) reporting that 100% and 47% of the isolates were resistant to ampicillin and erythromycin, respectively.

Nineteen percent of the *Vibrio* isolates were resistant to tetracycline, which is less than the results of previous reports in Philippines (53.7%), Thailand (49%), Mexico (43%) and India (43%) (Pearson et al., 1995; Tendencia et al., 2001; Roque et al., 2001;

Vaseeharan et al., 2005). In contrast, the isolates in this study showed higher resistance rates to enrofloxacin (10%) than those in previous reports (Barton et al., 2006; Roque et al., 2001). These previous studies showed that all the *Vibrio* isolates from shrimps and water in Australia (Barton et al., 2005) and Mexico (Roque et al., 2001) were susceptible to fluoroquinolones. Differences of resistance phenotypes observed are likely due to differences of antimicrobial use in different geographical areas.

Antimicrobial resistance genes in bacteria could be acquired and transmitted through several mechanisms, including the acquisition of genes through mobile genetic elements and dissemination through horizontal transfer (Mazel, 1998; Fluit et al., 2004). As observed in many Gram-negative bacteria, antimicrobial resistance genes in *Vibrio* spp. are often associated with mobile genetic elements especially integrons (Dalsgaard et al., 2000; Falbo et al., 1999). In this study, 6 % of the isolates were found to contain class 1 integrons, which is comparable to the results of a previous study in Thailand. The latter showed that 3% of the *Vibrio* isolates from environmental water and shrimp products contained class 1 integron (Dalsgaard et al., 2000). To date, there are very limited data on class 1 integrons in the *Vibrio* isolates from shrimps. Therefore, the findings in this study are compared with those from different hosts previously observed. The incidence of class 1 integrons is much less than that of a former study in Italy reporting that 26.3 % of the environmental isolates from waste water carried the integrons (Columbo et al., 2007). It is also much less than those previously reported in clinical isolates from humans in Thailand (30%) (Dalgaard et al., 2000), in Africa (36.2%) (Mwansa et al., 2007) and in Laos (48%) (Toma et al., 2004). A study of human isolates in India (Yamasaki et al., 2006) showed that 10.5% of were found to contain class 1 integrons, which is similar to the results of this study. Taken together, these data demonstrate that class 1 integrons are widespread in *Vibrio* isolates worldwide.

In previous studies, class 1 integrons containing antibiotic resistance gene cassettes have been indentified in *V. cholera*, *V. parahemolyticus* and *V. fluvialis* (Dalsgaard et al., 2000; Columbo et al., 2006a; Srinivasan et al., 2006). Most of them were found to contain resistance gene cassettes including *aadA1*, *aadA2* and *aadB*

encoding resistance to streptomycin and spectinomycin, *blaP1* encoding resistance to β-lactams, *dfrA1* and *dfrA15* encoding resistance to trimetroprim (Dalsgaard et al., 2000; Toma et al., 2004; Columbo et al., 2007). In this study, only one *intI1*-positive strain carried gene cassettes in variable region, which was the incomplete *rumA* gene. The *rumA* gene is located on the *rumAB* operon and encodes 23sRNA uracil-5-methyltransferase enzyme that is involved in UV mutagenic DNA repair (Woodgate et al., 1996). In *Vibrio* spp., the *rumAB* operon usually resides in SXT elements. It is unclear how this partial gene has been inserted into the variable regions. This insertion may happen through an aberrant recombination event during integron transfer (Waldor et al., 2001; 2002; Toma et al., 2004). However, this partial *rumA* gene does not provide any benefits to the *Vibrio* host strain.

Class 1 integrons without inserted gene cassettes were also identified. These empty integrons may be a result of the incomplete PCR amplification because the size of gene cassettes may be too large to be amplified. Another explanation could be that these integrons have lost the resistance gene cassettes in the environment where selective pressure is absent or that they have exchanged the resistance gene cassettes with integrons in other bacteria. These empty variable regions are, therefore, available for accepting new coming gene cassettes (Roy and Bissonneitte, 1992).

Since all the class 1 integrons lacked the *qacEΔ1-sul1* fused structure, none of the *intI1*-positive strains in the present study carried the typical 3'-conserved regions. Therefore, they were not the *sul1*-associated class 1 integrons. Class 1 integrons that carried atypical 3'-conserved regions were previously described in *E. coli* (Antunes et al., 2007) and *Salmonella* (Antunes et al., 2005) and those with the *qacH-sul3* fused structure has been reported in *E. coli* (Antunes et al., 2007) and *Salmonella* (Chuanchuen et al., 2008b). However, the *Vibrio* isolates in this study were negative to *sul3*. Therefore, further study is required to elucidate the structure of these unusual 3'conserved regions.

In the present study, none of the strains tested were positive to class 2 and 3 integrons. Class 2 integrons was previously identified in only two isolates of *V. cholera*. One was a clinical isolate from human cases in India and the other one was an environmental isolate from waste water in Bangladesh (Ahmed et al., 2006). Class 3 integrons have never been found in *Vibrio* spp.. To date, they have been only identified in *Serratia marcescens*, *Klebsiella pneumonia* and *Delftia* spp. (Duarte et al., 2003; Xu et al., 2007).

All *Vibrio* isolates in this study were determined for the presence of the *tet(K)* and *tet(L)* genes encoding for efflux pumps and the *tet(M)*, *tet(O)* and *tet(S)* genes encoding for ribosomal protection proteins. None of them were positive the *tet* genes tested. To date, 39 different tetracycline-resistance encoding genes have been characterized in bacteria (Robert et al., 2001; Suzuki et al., 2002; Jeong et al., 2007). Among these, *tet(A-E)* and *tet(G)* were widespread in Gram-negative bacteria isolated from marine animals (Dang et al., 2006; Song et al., 2006). The *tet(H-M)*, *tet(O)* and *tet(S)* genes have been present in less frequency (Robert et al., 2001; Suzuki et al., 2002; Barton et al.,2007).

Occurrence of *tet(M)* gene in *Vibrio* spp. have been usually associated with high-level tetracycline MICs that is equal to or greater than 250 µg/ml (Suzuki et al., 2004). However, tetracycline MICs of the *Vibrio* isolates in the present study range 16-64 µg/ml and *tet(M)* was absent in all of them.

The *tet* genes that were previously identified in *Vibrio* spp. include *tet(A-E)*, *tet(G)* and *tet(35)* that encode efflux protein (Olsvik et al., 1995; Song et al., 2006a; 2006b; Barton et al.,2007; Jeong et al., 2007) and *tet(34)* that encodes xanthine-guanine phosphoribosyltransferase enzyme (Suzuki et al., 2002; Jeong et al., 2007). However, these genes were not tested in the current study. Future studies to examine the involvement of other *tet* genes in tetracycline resistance are suggested.

Fluoroquinolones resistance in *Vibrio* spp. has been shown to be mediated by mutations within the QRDRs of *gyrA* gene. The major mutation associated with high-level resistance to fluoroquinolones is a single point mutation G248T in *gyrA* gene, which results in serine 83 being substituted with isoleucine (Ser-83-Ile) in GyrA. Such mutation has been commonly identified in *V. cholera* (Kundu et al., 2002), *V. parahaemolyticus* (Okuda et al., 1999), *V. fluvialis* (Srinivisan et al., 2006) and *V. anguillarum* (Colquhoun et al., 2007; Aoki et al., 2008). This is in agreement with our findings that a point mutation G248T in *gyrA* gene was most common (59%) in fluoroquinolone-resistant isolates.

Serine is an amino acid that has a short group ending with a hydroxyl group and it is very hydrophilic. Isoleucine is a hydrophobic amino acid that has large aliphatic hydrophobic side chains (Nelson et al., 2000). The point mutation G248T is located within the DNA-binding domain on the surface of gyrase and topoisomerase protein. Changing from hydrophilic serine to hydrophobic isoleucine may affect the interaction between enzymes and floroquinolones resulting in the reduced susceptibility of bacteria to the drugs (Ruiz, 2003; Vila, 2005). In addition, 9 *Vibrio* isolates (53%) harbored silent mutations, which was the replacement of C at position of 363 with T in *gyrA*. These silent mutations do not change amino acid, arginine, and thus have no affects in fluoroquinolones susceptibility.

As single Ser-83-Ile has been the most commonly found mutation mediated fluoroquinolones in *Vibrio* spp., a double mutation Ser-83-Ile and Asp-100-Tyr was also previously determined at very less frequency. MIC values of the strains with single or double mutations were not different and no correlation of the second mutation with increased resistance level was apparent (Kundu et al., 2002).

In this study, no mutations were identified in ParC. This enzyme has been shown to be a secondary target for fluoroquinolones action (Ruiz, 2003; Vila, 2005). The *Vibrio* strains resistant to fluoroquinolones were previously shown to contain a single base pair change in the *parC* sequence resulting in a serine to leucine change at residue

position 85 (Ser-85-Leu). This mutation was also found in *V. cholera* (Kundu et al., 2002), *V. parahaemolyticus* (Okuda et al., 1999), and *V. anguillarum* (Colquhoun et al., 2007; Aoki et al., 2008). Previous studies showed that *Vibrio* spp. carrying mutations in both *gyrA* and *parC* usually had high fluroquinolones MICs. For example, *V. parahaemolyticus* with Ser-83-Ile in GyrA and Ser-85-Leu in ParC had fluoroquinolones MIC above 50 µg/ml (Okuda et al., 1999; Colquhoun et al., 2007). However, in this study, the ciprofloxacin and enrofloxacin MICs in the *Vibrio* isolates ranged 4-16 µg/ml.

Up to 41% of fluoroquinolones resistant strains did not have mutations in *gyrA* and *parC*, indicating that other unidentified resistance mechanisms do exist. Fluoroquinolones resistance in Gram-negative bacteria including *Vibrio* spp. has been attributed to plasmid-borne genetic elements e.g. *qnr*-like elements (Nordmann et al., 2005; Fonseca et al., 2008), transmembrane efflux pump e.g. ABC multidrug transporters (Tsuchiya et al., 2003) and other mutations in topoisomerase genes e.g. *gyrB* and *parE* (Ruiz, 2003; Vila, 2005). These possibilities need to be examined in future studies.

Plasmid is one of the most important contributors to resistance gene transfer and has been contributed to the widespread dissemination of antibiotic resistance genes in the environment with selective pressure (Osborn et al., 2002). A previous study has reported occurrence of plasmids among the *Vibrio* isolates from shrimps was 80% and also showed that a 21.2 kb plasmid carried gene conferring resistance to cephalothin (Bruno et al., 2002). The involvement of plasmids in antimicrobial resistance has been previously demonstrated in the *Vibrio* isolates (Bruno et al., 2002; Wang et al., 2006). In this study, 20.5% of the isolates were found to carry plasmid(s) and a plasmid that is 3.5 kb in size was commonly found. Among the plasmid-containing strains, 13 isolates were resistant to at least one antimicrobial. However, the associations of the resistance phenotypes and the presence of plasmid were not examined. These could be a subject of interest for a future study.

Conclusion and suggestions

From the findings of this study, the conclusions could be made as follows:

1. Multidrug resistance is widely distributed among the *Vibrio* isolated from farmed marine shrimp.
2. Among the strains tested, class 1, 2 and 3 integrons did not play an important role in dissemination of antibiotic resistance.
3. The *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)* and *tet(S)* genes do not contribute tetracycline resistance in this strain collection.
4. A single base pair mutation G248T in the QRDR of *gyrA* is the major mechanism of resistance to fluoroquinolone among the resistance isolates.

Data obtained in this study could be beneficially used as follows:

1. To be part of antimicrobial resistance monitoring. At present, most antimicrobial resistance surveillance in Thailand relies on results of antimicrobial susceptibility test. Epidemiological data of antimicrobial resistance is still limited. Little is known about molecular mechanism of pathogens from aquatic animals

2. To be applied in risk analysis of the distribution and development of antimicrobial resistance. As risk analysis of antimicrobial resistance is mandatory for new drugs that will be used in aquaculture. The genetic data of antimicrobial resistance among bacteria of aquaculture origins in Thailand is limited. The data from other countries cannot be always applied to situations in Thailand due to differences in antimicrobial use in different countries. Therefore, epidemiological situations of antibiotic resistance need routine monitoring in Thailand.

3. To prove link of antimicrobial resistance along the food chain. This could be accomplished when combined with data from the isolates from related-food and

humans. However, data collection from a huge number of strains from different sources along the food chain is needed for the effective comparison.

From the results of this study, the suggestion for further studies could be as follows:

1. Since there were many resistant *Vibrio* isolates that did not harbor the resistance determinants tested in the study, other mechanism(s) underlying such resistance must exist: Studies of other resistance mechanisms may be performed e.g. another classes of integrons, active efflux pumps, integrative and conjugative elements (ICEs) etc.

2. Data on genetics of antimicrobial resistance of *Vibrio* spp. along the food chain will help to create the project to reduce antimicrobial resistance. However, such data are still insufficient especially in developing countries. Therefore, studies of genetics of antimicrobial resistances in *Vibrio* isolates from other sources e.g. humans, aquatic animal/products and environment are recommended.

3. Study of genetic relatedness of *Vibrio* isolates from various sources should be performed. This will prove the link of antimicrobials resistance among *Vibrio* spp. along the food chain.

4. Study of resistance gene transfer in antimicrobial-resistant *Vibrio* isolates harboring plasmid(s) could be achieved. In addition, characterization of these plasmids should be performed. This will prove the route of dissemination of antibiotic resistance among *Vibrio* species and also other bacteria.

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APPENDICES

APPENDIX A

Bacterial Growth Media

1. Muller Hinton Agar (MHA) (Difco™, MD, USA)

- Beef Extract Powder	2.0	g
- Acid Digest of Casien	17.5	g
- Starch	1.5	g
- Agar	17.0	g

2. Luria-Bertani Agar (LB) (Difco™, MD, USA)

- Trptone	10.0	g
- Yeast Extract	5.0	g
- Sodium chloride	5.0	g
- Agar	15.0	g

3. Tryptic Soy Agar (TSA) (Difco™, MD, USA)

- Pancreatic digest of casein	17.0	g
- Enzymatic Digest of Soybran Meal	3.0	g
- Dextrose	25.0	g
- Sodium chloride	5.0	g
- Dipotassium phosphate	2.5	g
- Agar	15.0	g

4. Thiosulfate Citrate Bile Salts Agar (TCBS) (Eiken, Tokyo, Japan)

- Yeast extract	5.0	g
- Peptone	10.0	g
- Sodium Citrate	10.0	g
- Sodium thiosulfate	7.0	g
- Oxgall	5.0	g

- Sodium cholate	3.0	g
- Sachharose	20.0	g
- Sodium chloride	10.0	g
- Ferric Citrate	1.0	g
- Bromthymol Blue	0.04	g
- Thymol blue	0.04	g
- Agar	15.0	g

APPENDIX B

Reagents

Reagents for PCR reaction

1. The Fermentas MasterMix (2X) (Fermentas®, Mainz, Germany) contains

- *Taq* DNA Polymerase (recombinant) in reaction buffer	0.05	units/µl
- $MgCl_2$	4	mM
- dNTPs (dATP, dCTP, dGTP, dTTP)	0.4	mM of each

Reagents for agarose gel electrophoresis

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

- Tris base	242.0	g
- Glacial acetic acid	57.1	ml
- 0.5 M EDTA pH 8.0	100.0	ml
- Distilled deionized water	1,000	ml

2. 10 mg/ml Ethidium bromide

- Ethidium bromide	1	g
- Distilled deionized water	1,000	ml

3. 0.5 M EDTA, pH 8.0 1000 ml contains

- Disodium ethylene diamine tetraacetate. $2H_2O$	186.1	g
- Distilled deionized water	800.0	ml
- Adjust pH to 8.0		
- Adjust Volume to 1,000 ml		

4. 1 M Tris HCl, pH 8.0 1000 ml contains

- Tris (ultrapure)	121.1	g
- Distilled deionized water	800.0	ml
- Adjust pH to 8.0 by adding conc. HCl	42.0	ml

APPENDIX C

Summary of characteristics of antimicrobial resistance in the *Vibrio* isolates

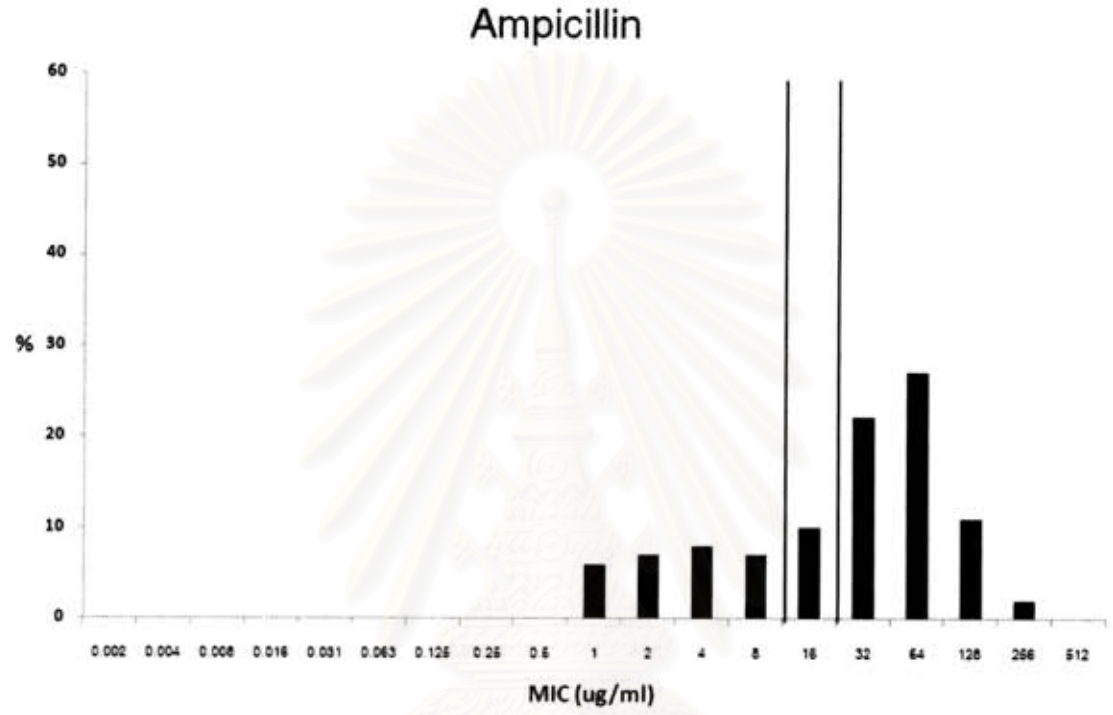
Strains	Antibiotic resistance patterns	*IntI1*-positive	Mutation in *gyrA* gene	Plasmids	
				Group	Size (bp)
V. cholera 16	AMP	-	-	-	-
V. cholera 32	AMP-SMX-TMP	-	-	-	-
V. cholera 33	-	+	-	1	35,000
V. cholera 45	CIP-ENR-TMP	-	-	1	35,000
V. cholera 76	AMP	-	-	4	6,000, 3,000
V. cholera 77	AMP-CIP-ERY-ENR	-	+	-	-
V. cholera 78	ERY	-	-	-	-
V. cholera 106	AMP-ERY-SMX-TMP	-	-	-	-
V. cholera 125	AMP-SUL-TET	-	-	-	-
V. cholera 127	AMP-ERY-SMX	-	-	-	-
V. cholera 128	-	-	-	1	35,000
V. cholera 140	-	-	-	1	35,000
V. cholera 143	SMX-TMP	-	-	-	-
V. cholera 157	AMP-CIP	-	-	-	-
V. cholera 179	AMP	-	-	-	-
V. cholera 180	-	-	-	3	35,000, 8,000, 3,500
V. cholera 193	-	-	-	-	-
V. cholera 197	ERY-SMX	-	-	-	-
V. parahaemolyticus 14	AMP	-	-	-	-
V. parahaemolyticus 19	AMP-ERY-TET	-	-	-	-
V. parahaemolyticus 20	-	-	-	-	-
V. parahaemolyticus 58	AMP-ERY-SMX-TMP	-	-	-	-
V. parahaemolyticus 59	AMP	-	-	4	6,000, 3,000
V. parahaemolyticus 62	AMP-ERY	-	-	-	-
V. parahaemolyticus 64	AMP	-	-	2	35,000, 4,000
V. parahaemolyticus 83	AMP-TET	-	-	-	-
V. parahaemolyticus 94	AMP-ERY	-	-	-	-
V. parahaemolyticus 103	ERY	-	-	-	-
V. parahaemolyticus 104	AMP	-	-	-	-
V. parahaemolyticus 177	AMP	-	-	-	-
V. parahaemolyticus 178	AMP-CIP	-	+	-	-
V. parahaemolyticus 184	AMP-TET	-	-	-	-
V. parahaemolyticus 191	ERY	-	-	-	-

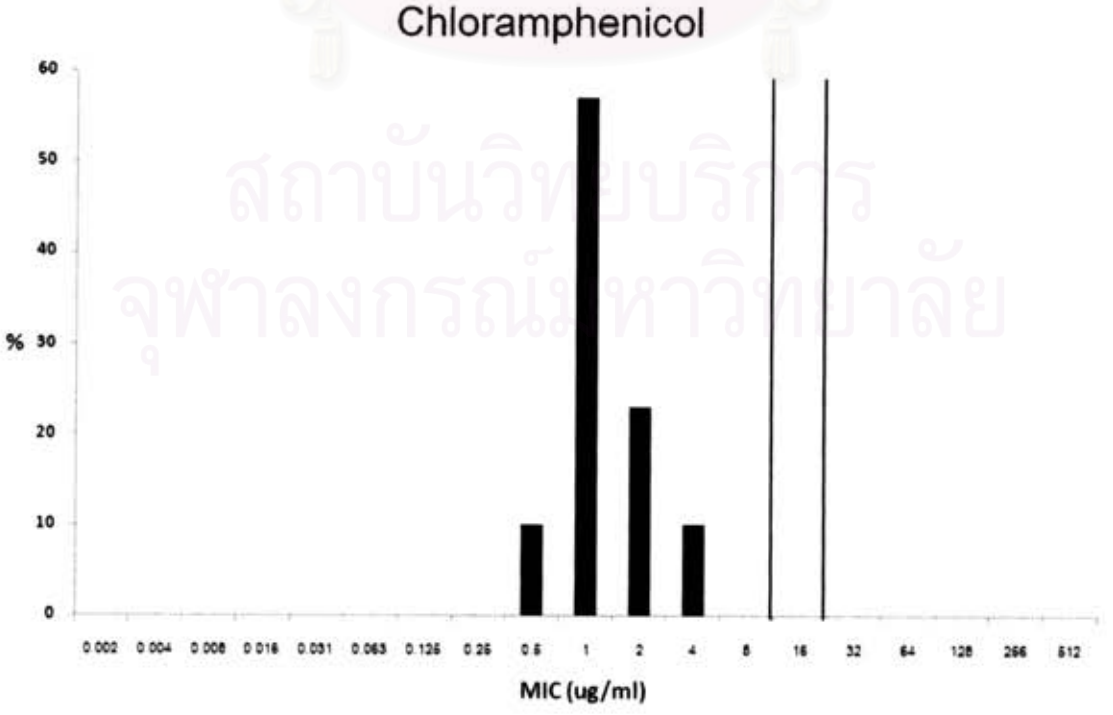
Strains	Antibiotic resistance patterns	*IntI1*-positive	Mutation in *gyrA* gene	Plasmids	
				Group	Size (bp)
V. parahaemolyticus 234	ERY	-	-	-	-
V. parahaemolyticus 235	SMX	-	-	1	35,000
V. parahaemolyticus 237	ERY	-	-	-	-
V. parahaemolyticus 245	AMP –SMX- TET-TMP	-	-	-	-
V. parahaemolyticus 250	SMX	-	-	-	-
V. parahaemolyticus 251	CIP-ERY	-	+	-	-
V. parahaemolyticus 265	AMP	-	-	-	-
V. parahaemolyticus 270	ERY	-	-	3	35,000, 8,000, 3,500
V. parahaemolyticus 279	AMP-CIP	-	-	-	-
V. parahaemolyticus 281	AMP-CIP	-	+	-	-
V. parahaemolyticus 282	AMP-CIP-ERY-ENR-SMX-TMP	-	+	1	35,000
V. vulnificus 5	AMP-ERY-SMX-TMP	-	-	1	35,000
V. vulnificus 17	AMP-CIP-ERY-ENR-SMX-TMP	-	+	-	-
V. vulnificus 25	AMP-CIP-ENR	-	+	1	35,000
V. vulnificus 31	-	-	-	-	-
V. vulnificus 34	ERY	-	-	1	35,000
V. vulnificus 43	ERY-TET	-	-	-	-
V. vulnificus 44	AMP-CIP-ENR-TET	-	-	-	-
V. vulnificus 46	AMP	-	-	-	-
V. vulnificus 61	AMP	-	-	1	35,000
V. vulnificus 67	AMP-CIP-ERY-SMX-TMP	-	+	-	-
V. vulnificus 109	AMP-ERY	-	-	-	-
V. vulnificus 183	TET	-	-	-	-
V. vulnificus 217	TET	-	-	-	-
V. vulnificus 223	SMX-TMP	-	-	-	-
V. vulnificus 267	AMP-ERY	-	-	-	-
V. vulnificus 268	AMP	-	-	-	-
V. fluvialis 1	AMP-CIP-ENR-ERY-SMX-TET-TMP	-	-	-	-
V. fluvialis 3	AMP-CIP-ENR-ERY-SMX-TET-TMP	-	-	-	-
V. fluvialis 6	AMP- ERY-SMX-TET-TMP	-	-	-	-
V. fluvialis 7	AMP-ERY	-	-	1	35,000
V. fluvialis 11	AMP	-	-	-	-

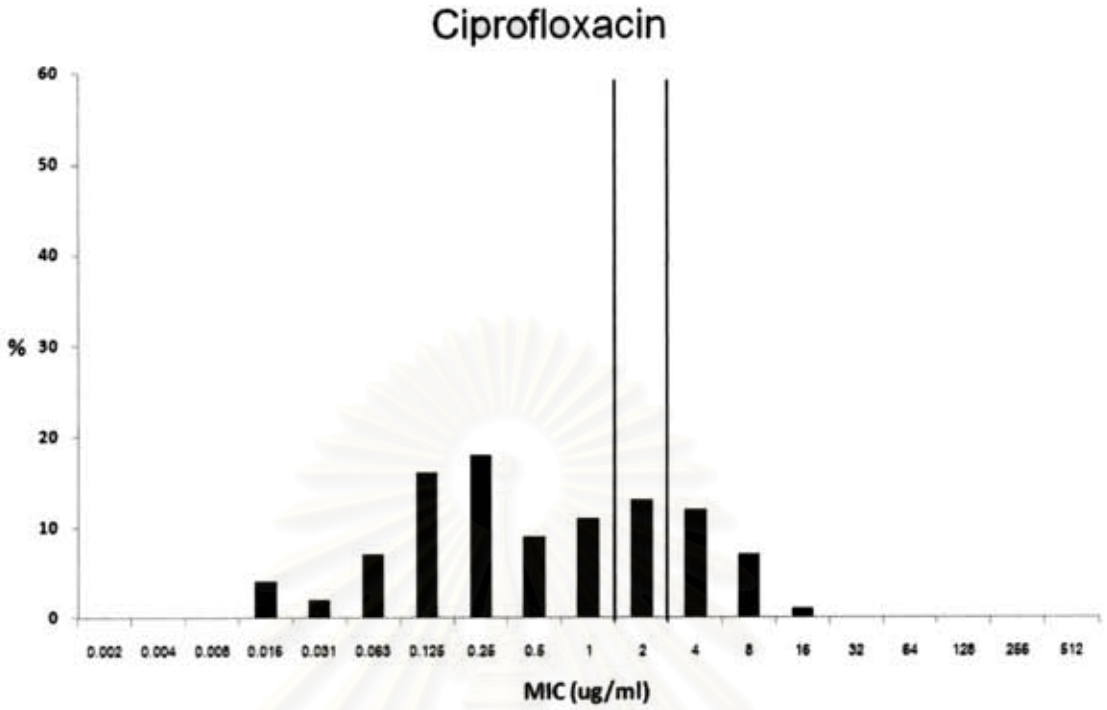
Strains	Antibiotic resistance patterns	*Intl1*-positive	Mutation in *gyrA* gene	Plasmids	
				Group	Size (bp)
V. fluvialis 13	ERY	-	-	-	-
V. fluvialis 21	AMP-SMX-TMP	-	-	-	-
V. fluvialis 28	-	-	-	-	-
V. fluvialis 49	AMP	+	-	1	35,000
V. fluvialis 57	AMP-ERY	-	-	-	-
V. fluvialis 63	AMP-SNX-TMP	-	-	-	-
V. fluvialis 69	-	-	-	-	-
V. fluvialis 72	AMP-ERY-TET	+	-	-	-
V. fluvialis 73	AMP	+	-	-	-
V. fluvialis 75	AMP-ERY	-	-	-	-
V. fluvialis 81	AMP-TET	-	-	-	-
V. fluvialis 132	CIP-ERY-TET-SMX-TMP	-	-	-	-
V. fluvialis 153	AMP	-	-	-	-
V. fluvialis 159	CIP-ENR-ERY	-	+	-	-
V. fluvialis 164	CIP-ERY	-	+	-	-
V. fluvialis 186	TET	-	-	-	-
V. fluvialis 192	AMP	-	-	-	-
V. fluvialis 286	ERY	-	-	-	-

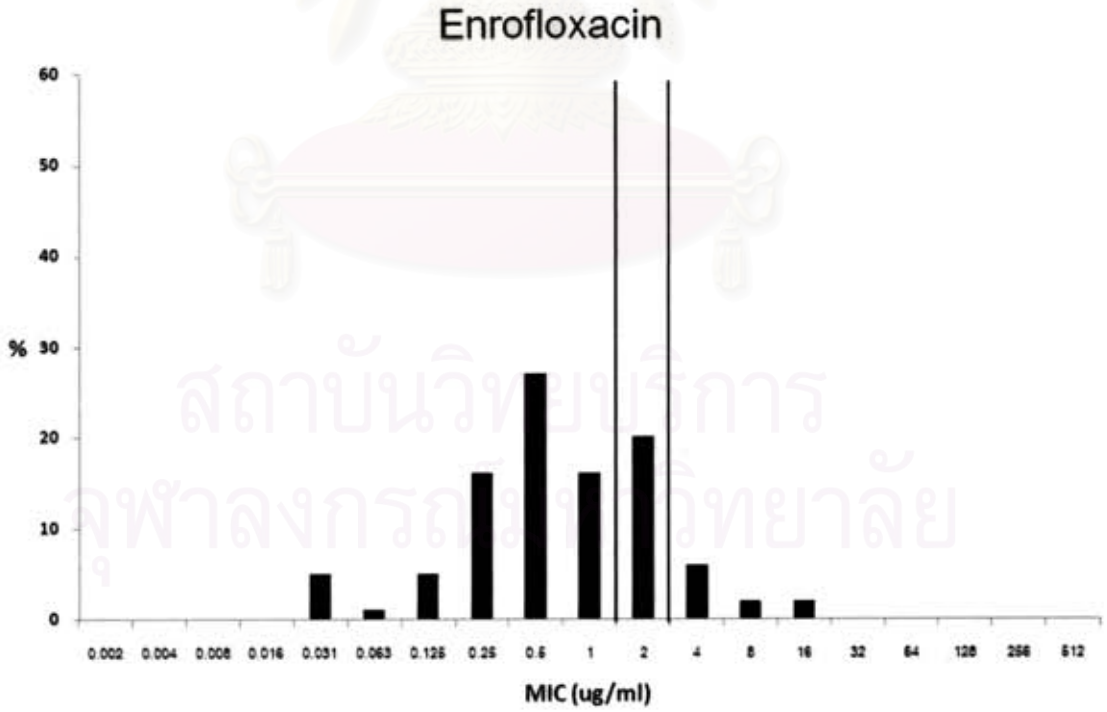
APPENDIX D

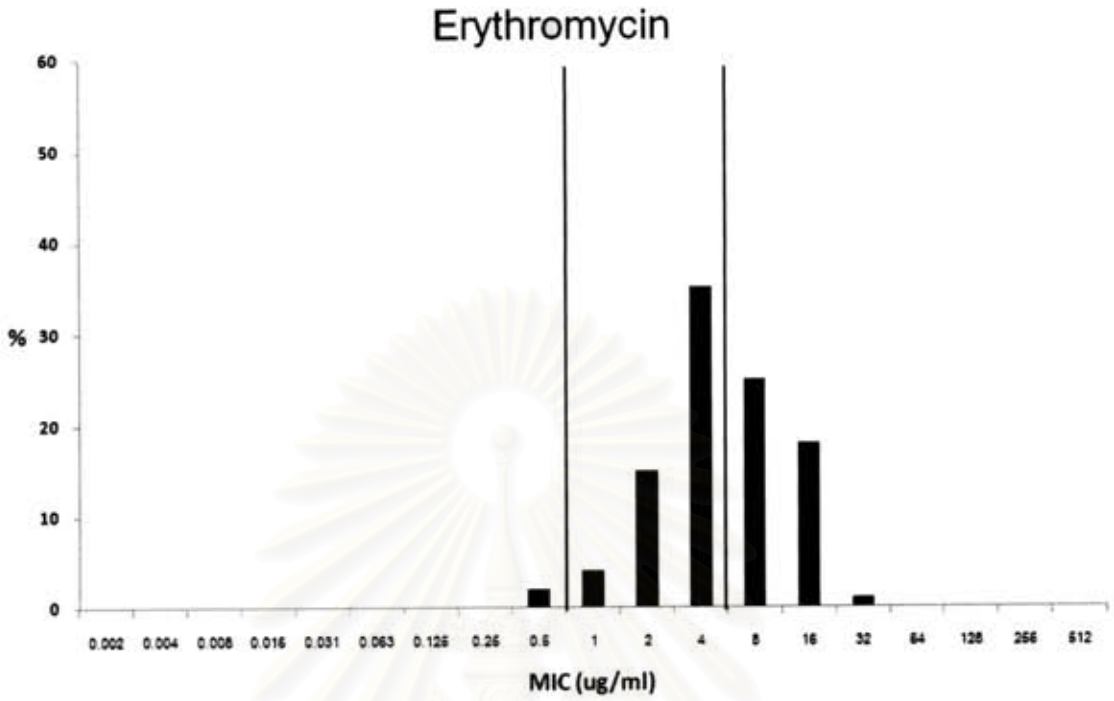
Distribution of the MIC values of *Vibrio* spp.

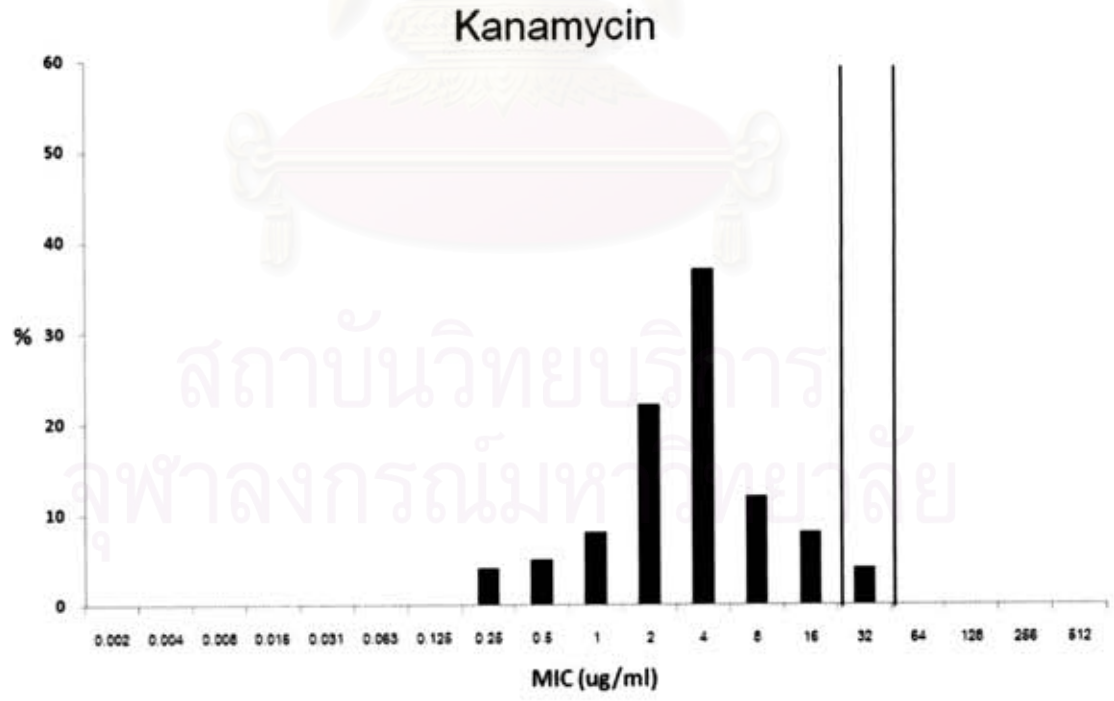


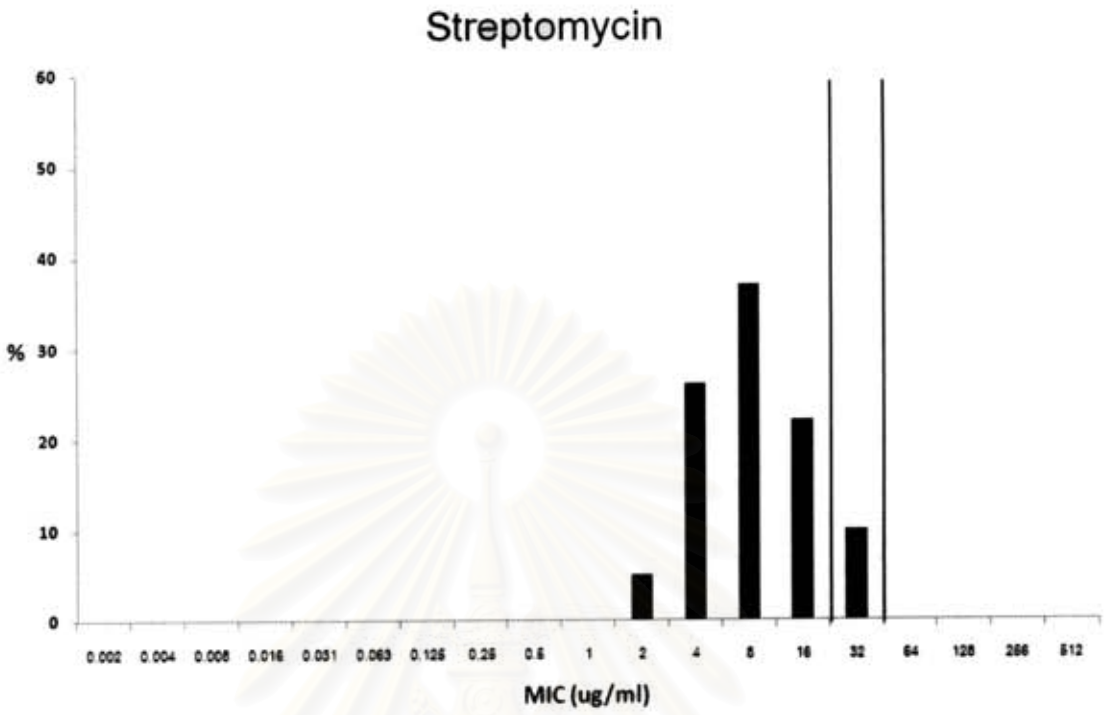


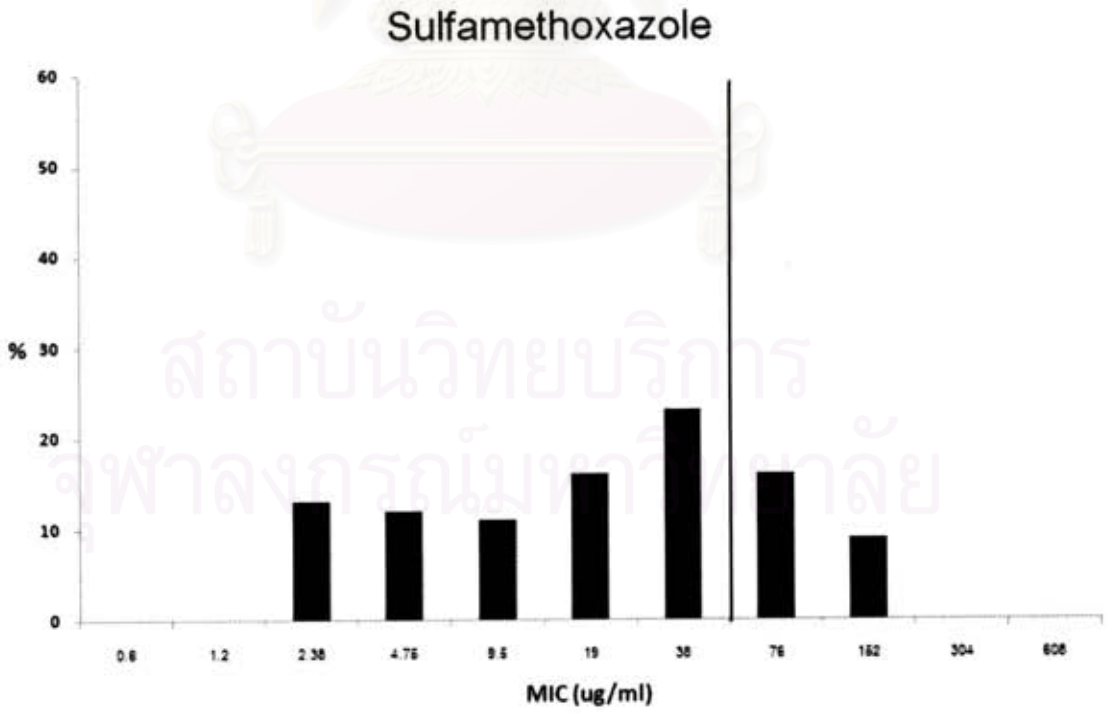


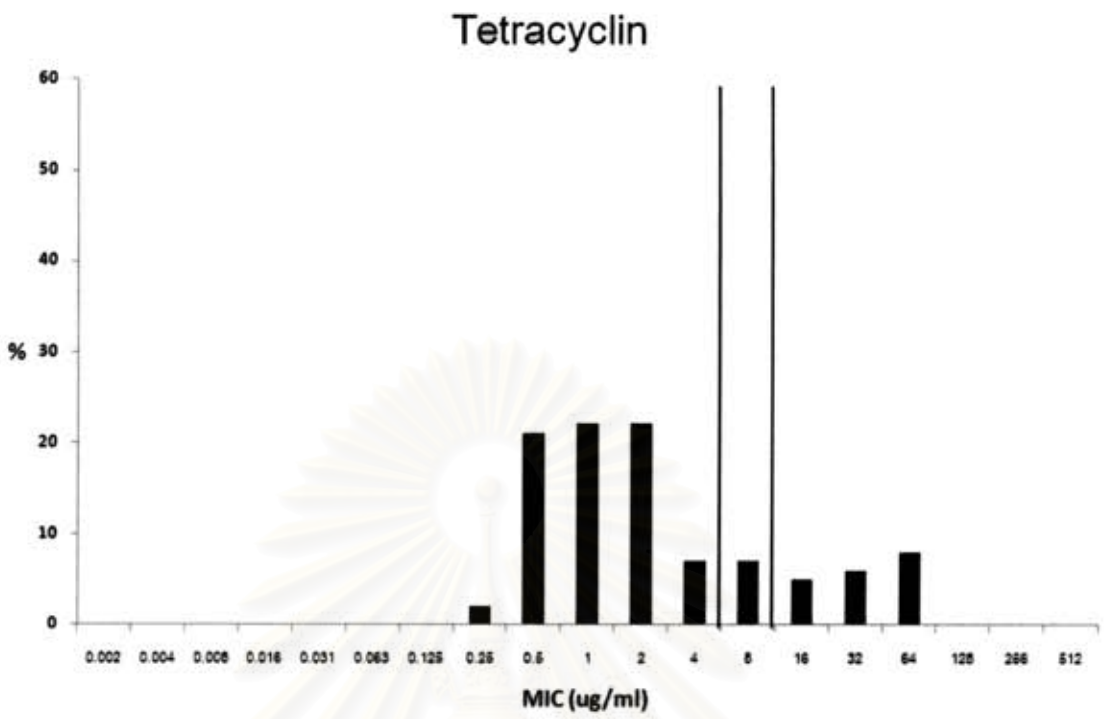


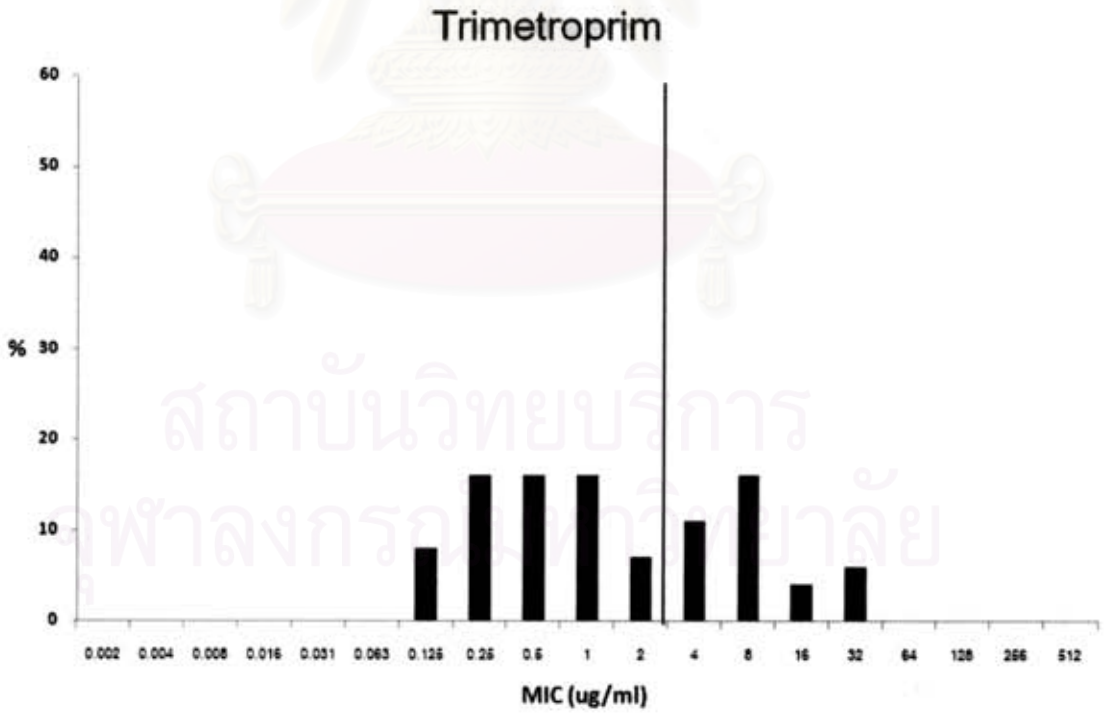












BIOGRAPHY

Miss Sirikorn Kitiyodom was born on June 24, 1982 in Bangkok, Thailand. She got the degree of Doctor of Veterinary Medicine (2nd Class Honours) from the Faculty of Veterinary Medicine, Chulalongkorn University, Thailand in 2005. After that she has worked at the Aquaculture technical service and development office, Charoen Pokpahand food PLC. In 2007, she enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since 2008.