Class 1 integrons และ AheABC efflux system ในเชื้อ *Aeromonas hydrophila* ที่แยกได้จากปลานิลเพาะเลี้ยงในประเทศไทย

นางสาวมินตรา ลักขณา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

CLASS 1 INTEGRONS AND THE AheABC EFFLUX SYSTEM IN *AEROMONAS HYDROPHILA* ISOLATED FROM FARMED NILE TILAPIA IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Public Health

Department of Veterinary Public Health

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2010

Thesis Title	CLASS 1 INTEGRONS AND THE AheABC EFFLUX SYSTEM IN *AEROMONAS HYDROPHILA* ISOLATED FROM FARMED NILE TILAPIA IN THAILAND
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มินตรา ลักขณา : Class 1 integrons และ AheABC efflux system ในเชื้อ *Aeromonas hydrophila* ที่แยกได้จากปลานิลเพาะเลี้ยงในประเทศไทย (CLASS 1 INTEGRONS AND THE AheABC EFFLUX SYSTEM IN *AEROMONAS HYDROPHILA* ISOLATED FROM FARMED NILE TILAPIA IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.สพ.ญ.ดร. รุ่งทิพย์ ขวนชื่น, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.สพ.ญ.ดร. เจนนุช ว่องธวัชชัย, 75 หน้า.

ศึกษาเชื้อ *Aeromonas hydrophila* จำนวน 50 เชื้อที่แยกได้จากปลานิล (*Oreochromis nilotica*) เพาะเลี้ยงในประเทศไทย โดยทดสอบค่าความไวของเชื้อต่อยาปฏิชีวนะจำนวน 16 ชนิด ศึกษาลักษณะทางพันธุกรรมของ class 1 integrons รวมทั้งศึกษาการแสดงออกของ AheABC efflux system และตรวจการกลายพันธุ์ในส่วน Quinolone Resistance-Determining Regions (QRDRs) ของยีน *gyrA* และ *parC* ในเชื้อที่ดื้อต่อยา enrofloxacin และ/หรือ ciprofloxacin จำนวน 13 เชื้อ ผลการวิจัยพบว่า 94% ของเชื้อดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน โดยดื้อต่อยา streptomycin มากที่สุด (92%) รองลงมาคือยา trimethoprim (88%) และ amoxicillin (86%) รูปแบบการดื้อยาที่พบมากที่สุด คือ AMO-CAR-TMP-STR (20%) การศึกษา class 1 integrons พบเชื้อที่มีการปรากฏของยีน *intI1* จำนวน 23 เชื้อ และพบเชื้อที่มี gene cassettes ใน variable regions จำนวน 14 เชื้อ ลักษณะของ gene cassettes ที่พบ คือ *aadA2*, *dfrA1-orfC*, *dfrA12-aadA2* และ incomplete *aadA2* เชื้อจำนวน 3 เชื้อ สามารถถ่ายทอด class 1 integrons ไปยัง *E. coli* ได้ ศึกษาการแสดงออกของ AheABC efflux system พบการแสดงออกของยีน *aheB* ในเชื้อจำนวน 39 เชื้อ วัดระดับการแสดงออกของยีน *aheB* ในเชื้อที่สุ่มมาจำนวน 10 เชื้อ พบว่าทุกเชื้อมีการแสดงออกของยีน *aheB* สูงกว่าเชื้อ *A. hydrophila* ATCC 35654 โดยระดับการแสดงออกไม่ได้สัมพันธ์กับค่าความไวของเชื้อต่อยาปฏิชีวนะ ศึกษาการกลายพันธุ์ในส่วน QRDRs ของยีน *gyrA* และยีน *parC* ทุกเชื้อที่ทดสอบพบการกลายพันธุ์ในยีน *gyrA* ที่ตำแหน่ง 83 และยีน *parC* ที่ตำแหน่ง 80 ซึ่งทำให้กรดอะมิโน serine เปลี่ยนเป็น isoleucine ในทั้งสองตำแหน่ง ผลการศึกษานี้แสดงให้เห็นว่าเชื้อ *A. hydrophila* ที่แยกได้จากปลานิลเพาะเลี้ยงในประเทศไทยดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน และ class 1 integrons และ AheABC efflux system ที่ศึกษามีส่วนเกี่ยวข้องกับการดื้อยาและอาจทำให้เกิดการแพร่กระจายของเชื้อดื้อยาได้ ผลการวิจัยชี้ให้เห็นถึงความจำเป็นในการใช้สารฆ่าจุลชีพในสัตว์น้ำอย่างเหมาะสมและสุขุมรอบคอบ

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต มินตรา ลักขณา

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม

##5175577731 : MAJOR VETERINARY PUBLIC HEALTH

KEYWORDS : *Aeromonas hydrophila* / Integrons / Multidrug efflux systems / Multidrug resistance / Tilapia/ AheABC

MINTRA LUKKANA: CLASS 1 INTEGRONS AND THE AheABC EFFLUX SYSTEM IN *AEROMONAS HYDROPHILA* ISOLATED FROM FARMED NILE TILAPIA IN THAILAND. THESIS ADVISOR: ASST. PROF. RUNGTIP CHUANCHUEN, THESIS CO-ADVISOR: ASSOC. PROF. JANENUJ WONGTAVATCHAI, 75 pp.

A total of 50 *Aeromonas hydrophila* strains isolated from farmed Nile tilapia (*Oreochromis nilotica*) in Thailand were used in this study. All strains were examined for susceptibilities to 16 antimicrobials, characterized for class 1 integrons and determined for the AheABC efflux system. Thirteen strains resistant to enrofloxacin and/or ciprofloxacin were examined for the mutations in the Quinolone Resistance-Determining Regions (QRDRs) of *gyrA* and *parC*. Up to 94% of the isolates were considered multidrug-resistant. Most isolates were resistant to streptomycin (92%), trimethoprim (88%) and amoxicillin (86%) and common resistance pattern was AMO-CAR-TMP-STR (20%). Class 1 integrons were detected in 23 isolates. Fourteen isolates harbored inserted gene cassettes i.e. *aadA2*, *dfrA1-orfC*, *dfrA12-aadA2* and incomplete *aadA2*. Three strains could horizontally transfer class 1 integrons to the *E. coli* recipient. All strains were screened for the expression of the AheABC efflux system. Transcriptions of the *aheB* gene were detected in 39 isolates. Ten isolates were randomly selected for determination of the transcription level of *aheB* gene. The *aheB* transcription level in all isolates was higher than that of *A. hydrophila* ATCC 35654. The expression level was not correlated with multidrug resistance phenotype. All the strains tested were found to contain mutations in both *gyrA* and *parC* gene. The substitutions in *gyrA* and *parC* gene were Ser-83-Ile and Ser-80-Ile, respectively. The results of this study show that the multidrug-resistant *A. hydrophila* are widely distributed in farmed Nile tilapia in Thailand. Class 1 integrons and the AheABC efflux pump play an important role in multidrug-resistance and dissemination of antibiotic resistance. The data indicate the requirement of proper and prudent use of antimicrobial agents in aquaculture.

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Field of Study: Veterinary Public Health Advisor's Signature Rungtip Chuanchuen

Academic Year: 2010 Co-Advisor's Signature

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my advisor, Assistant Professor Dr. Rungtip Chuanchuen, for her guidance, patience and useful suggestions, which encouraged and helped me in completing the project work in time. Without her guidance and persistent help this thesis would not have been possible.

I wish to express my deep sense of gratitude to my co-advisor, Associate Professor Dr. Janenuj Wongtavatchai, who has the innovative ideas. I am sincerely thankful for her advice throughout the period of this study.

I would like to thank my committee chair, Associate Professor Dr. Alongkorn Amonsin, for his questions and support throughout the completion of the project.

I would like to thank my committee members, Dr. Taradon Luangtongkum and Assistant Professor Dr. Chanwit Tribuddharat for their constructive criticisms and valuable suggestions.

I would like to thank the staff of the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University and my friends from RC lab, AA lab and JW lab for their help and love to empower me that never fail all the time.

I would like to thank my colleagues from the National Bureau of Agricultural Commodity and Food Standards (ACFS), Ministry of Agriculture and Cooperative for their help and wishes for the successful completion of this project.

I would like to express my heartfelt thanks to my beloved parents for their unlimited love. I am grateful to be a part of this wonderful family. Thank you for staying with me and cheering me up.

Finally and importantly, I would like to thank The Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumbol Adulyadej for financial support. I also thank The Thailand Research Fund (TRF-MAG windowII: MRG-WII525S030) cofunding by the 90th Anniversary of Chulalongkorn University Fund Grant (Ratchadaphiseksomphot Endowment Fund) for research funding.

CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DW	distilled water
e.g.	exampla gratia, for example
et al.	et alii, and others
g	gram (s)
g	gravity
h	hour (s)
i.e.	id est, that is
M	molar
min	minutes (s)
mg	milligram (s)
ml	milliliter (s)
mM	millimolar
µg	microgram (s)
µl	micro liter (s)
µM	micro molar
NSS	normal saline solution
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
Real-time qRT-PCR	Real-time Quantitative Reverse Transcription PCR
RT-PCR	Reverse Transcription Polymerase Chain Reaction
sec	sec (s)
TAE	Tris-Acetate-EDTA
U	unit

CHAPTER I

INTRODUCTION

Freshwater aquaculture has been practiced in Thailand over eight decades. Currently, there are more than 50 kinds of cultured-aquatic species. Among these, tilapia (*Oreochromis nilotic*a) has gained increasing popularity and become one of the top five main cultured species in Thailand. Department of Fisheries are undergoing to increase the production yield of tilapia, upgrade Good Aquaculture Practice (GAP) for tilapia farm management and promote the tilapia to a new export item. At the end of 2007, the export value of tilapia production was 538.72 million bahts (11,177.23 tons) and has been continually increasing to 751.71 million bahts (16,170.44 tons) in 2008 (TFFA, 2010).

In the past, the tilapia cultivation was mainly performed in ponds, rice field, ditches and cages to serve home consumptions. To date, most farming systems have expanded to supply both exportations and domestic consumptions. The fast-growing massive production has led to inappropriate farm management systems e.g., increasing stocking densities, fluctuating temperature, poor water exchange and aeration. These factors are the predisposing causes of aquatic infections that provoke stress and increase susceptibility to infections. As a result, farmed tilapia has faced disease outbreaks, including streptococcosis, edwardsiellosis, columnaris, monogenetic trematodes and motile aeromonas septicaemia (El-Sayed, 2006; FAO, 2010).

A Gram-negative bacterium, *Aeromonas hydrophila*, is one of the important opportunistic pathogens that cause diseases in tilapia when they are under stress from either management or environment. The prevalence of infections in cultured tilapia is higher than that of wild fish and the mortality rate is very high up to 80-100% in summer season (Faisal et al., 1989). These could result in economic loss due to their high share in national income. *A. hydrophila* Infected tilapia show the clinical signs, which are similar to those of other bacterial infections e.g., loss of scales, skin ulcers, tail rot,

fin rot, exophthalmia, abdominal distention and hemorrhagic septicemia (Farmer et al., 2006). The outbreaks of *A. hydrophila* infections in tilapia have been reported in many countries; for example, Indonesia, Malaysia, Papua New Guinea (Tonguthai, 1985), Thailand (Menasveta, 1985), India (Jhingran and Das, 1990), Western Australia (Callinan, 1994) and the Philippines (Yambot, 1998). Particular concern is *A. hydrophila* can cause infections not only in tilapia, but also in humans. The organism is considered a foodborne pathogen because people can get infection by ingesting contaminated food and water e.g., seafood, raw meat, raw vegetable and raw milk. This bacterium can grow at refrigerator temperature and this ability are important in foodborne infection (Daskalov, 2006). *A. hydrophila* is also found in drinking water; therefore, listed as the microbiological contaminant on the Environmental Protection Agency's Drinking Water Contaminant Candidate List (USEPA, 2005). Various clinical sings have been observed in infected patients, for example gastroenteritis, septicemia, vomiting and nausea (Daskalov, 2006; Palu et al., 2006). Moreover, several studies have revealed that the organism can cause the extraintestinal infections such as cellulitis, colitis, meningitis (Ellison and Mostow, 1984), traumatic and wound infections (Farmer et al., 2006; Subashkumar et al., 2006). The clinical cases of *A. hydrophila* infection in humans have been reported in many countries e.g., USA (Joseph, 1979), England, Wales, Ireland (Ellison and Mostow, 1984; Seetha, 2004) and India (Seetha, 2004). In Thailand, the *Aeromonas* species were most frequently isolated pathogens from patients who had traumatic wound and exposed to contaminated water after the 2004-tsunami wave (Hiransuthikul et al., 2005).

Antibiotics have been used for treatment of *Aeromonas* infections in both tilapia and humans. In aquaculture, antibiotics have been mixed with feed for oral administration to treat bacterial infections for a long time. Drugs that are approved for such purposes include oxytetracycline, florfenicol and sulfadimethoxine/ormetoprim (USFDA, 2009). Antimicrobial agents in the same classes have been also used for medicated treatment in human; for example, ampicillin, ciprofloxacin, chloramphenicol, carbenicillin, cephalothin, fluoroquinolone, gentamicin, thiamphenicol and sulfamethoxazole/trimethoprim (Joseph, 1979; David and Ronald, 1981; Chompoonuch

et al., 2009). Improper and over-use of antibiotics can lead to development of antibiotic resistance in bacteria. Particular public concern is the pathogens may become resistant to multiple drugs and can transfer resistance determinants to other bacteria intra- and inter-species (Son et al., 1997; Chang et al., 2007). Resistant bacteria could be passed to humans resulting in resistant infections (Fluit and Schmitz, 2004). An infection of these bacteria restricts the antimicrobial use for treatment of illness, increases the severity of infection and increases hospitalization (Molbak, 2004). The incidence of antibiotic-resistant *A. hydrophila* has been increasing reported worldwide. For example, the *Aeromonas* strains isolated from food samples in Brazil were resistant to ampicillin/sulbactam, cefoxitin and tetracycline (Palu et al., 2006). The environmental strains in the Netherland during 2008 were shown to be resistant to oxytetracycline and sulfamethoxazole/trimethoprim (Penders and Stobberingh, 2008). In India, *A. hydrophila* isolates from fish were resistant to ampicillin and colistin (Kaskhedikar and Chhabra, 2010).

Antimicrobial-resistance mechanisms have been extensively studied in bacteria; for example, acquisition of resistance determinants, and expression of efflux pumps and mutations of the target enzymes. Class 1 integrons have been shown to play an important role in the acquired resistance among Gram-negative bacteria (Fluit and Schmitz, 2004). They are mobile genetic elements that are capable to harboring several resistance gene cassettes and confer multidrug-resistance phenotype (Perez-Valdespino et al., 2009). Class 1 integrons could be horizontally transferred among bacteria inter- and intra-species and have been previously identified in *A. hydrophila*. Resistance gene cassettes that have been found to be associated with class 1 integrons include *aadA1*, *aadA2, dfrA12, catB3, cmlA4, dfrA15, dfrB4* and *oxa2* (Chang et al., 2007; Jacobs and Chenia, 2007). Multidrug efflux systems play a major role in multidrug-resistance among bacteria (Poole, 2004). To date, only the AheABC efflux pump system has been demonstrated in *A. hydrophila*. The system has been shown to be involved the multidrug-resistance phenotype of the pathogen and its antimicrobial substrates include cefoperazone, cefuroxime, erythromycin, lincomycin, minocycline, trimethoprim and rifampin (Hernould et al., 2008).

The study of antimicrobial resistance mechanisms is essential for better understanding of resistance mechanisms and distribution of resistant bacteria. Such data will facilitate development of action plan to prevent the emergence and reduce the spread of resistant bacteria. To date, the studies of molecular mechanisms of antibiotic resistance in *A. hydrophila* have been conducted in many countries e.g., Norway (L'Abee-Lund and Sorum, 2001), South Africa (Jacobs and Chenia, 2007), Taiwan (Chang et al., 2007; Lee et al., 2008) and France (Hernould et al., 2008). However, genetics of antibiotic resistance in *A. hydrophila* isolated from freshwater fish have never been reported in Thailand. Therefore, the purposes of this study were to characterize class 1 integrons and the AheABC efflux pump system in *A. hydrophila* isolated from farmed Nile tilapia in Thailand.

The study focused on characterization of class 1 integrons and test for their transferability, determination of the expression of the AheABC efflux system and its involvement in multidrug-resistance phenotypes. The results obtained can be used to explain distribution and transmission of antibiotic resistance genes from farmed tilapia to humans when combined with the antibiotic resistance data in *A. hydrophila* from infected humans. The outcomes of this study can be a part of antibiotic resistance monitoring program and risk analysis especially antibiotic resistance originated from aquatic animals. The *A. hydrophila* strains containing class 1 integrons and the AheABC efflux system could be used for future studies such as the genetics characterization of other integrons, the relationship between class 1 integrons, the role of multidrug efflux systems in bacteria from aquatic animals.

CHAPTER II

REVIEW LITERATURES

1. General characteristics of *A. hydrophila*

A. hydrophila is Gram-negative rod with 0.3-1.0 μm width and 1.0-3.5 μm length, non-spore forming and facultative anaerobe bacteria classified in the family *Aeromonadaceae* (Farmer et al., 2006). *A. hydrophila* grows in general laboratory media i.e., Luria-Bertani agar (LBA), tryptic soy agar (TSA) and forms smooth brown-pigmented colonies (Figure 1). The pathogen has an optimal growth temperature of about 28°C but can grow at refrigerator temperature (Daskalov, 2006). In addition, it is motile by a single polar flagellum. *A. hydrophila* is generally catalase positive, oxidase positive, and glucose fermentative (Farmer et al., 2006).

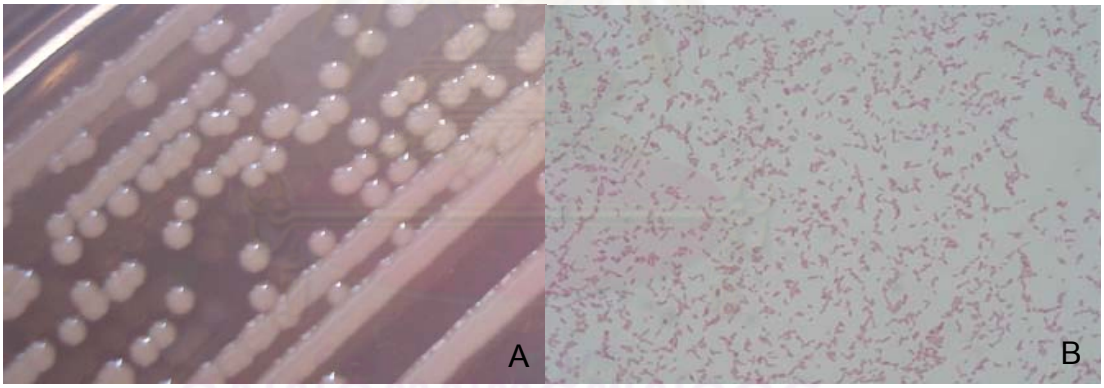

Figure 1: *A. hydrophila*

A. Appearance of colonies on Luria-Bertani agar (Photo by Mintra Lukkana)

B. Gram-stained smear (Photo by Assoc. Prof. Janenuj Wongtavatchai)

A. hydrophila is widely distributed in the environment especially aquatic environment such as freshwater lake, river and waste water system (Farmer et al., 2006). The bacterium is considered an important opportunistic pathogen of humans and animals. It can infect both cold-blooded (e.g., fish, amphibians, reptiles) and warm-blooded animals (e.g., mammal and birds) (Daskalov, 2006).

2. Pathogenesis of *A. hydrophila*

A. hydrophila has emerged as a pathogen among warm water fish (Farmer et al., 2006). The pathogen is ubiquitous and could be isolated from the healthy fish, aquatic plants and pound mud (Abulhamd, 2009). Therefore, it appears impossible to eliminate this bacterium from aquaculture environment (Camus et al., 1998). The infected fish has nonspecific clinical signs such as raised scales, loss of scales, skin ulcerative, fin rot, abdominal distention and exophthalmia, which are similar to clinical signs of other bacterial infections (Noga, 1996). For farmed tilapia, *Aeromonas* infections can cause high mortality rates up to 100% especially in summer resulting in the high economic loss (Kaskhedikar and Chhabra, 2010). There have been several reports of *Aeromonas* infections among other animals, for example, red leg syndrome in frogs, hemorrhagic bacteremia in snakes, diarrhea in piglets and birds and septicemia in dogs (Farmer et al., 2006).

A. hydrophila also causes infections in humans. The organism can infect both healthy and immunocompromised patients (Figueras et al., 2007). The reported incidences of *A. hydrophila* in human have been found in many countries e.g., USA, England, Wales, Ireland and India (Joseph, 1979; Ellison and Mostow, 1984; Seetha, 2004). *A. hydrophila* has also emerged as a human foodborne pathogen (Daskalov, 2006). Foodborne illness associated with *A. hydrophila* has been frequently reported in many countries e.g., Western Australia (Burke et al., 1983), India (Vasaikar et al., 2002) and Spain (Vila et al., 2003). The organism is able to produce toxins, including exotoxins and endotoxins that are the virulence factors associated with gastroenteritis (Farmer et al., 2006). Human could get infected via ingestion of contaminated food and water e.g., seafood, raw meat, raw milk, raw vegetable and drinking water (Daskalov, 2006; Palu et al., 2006). Moreover, several studies have revealed that the organism can cause the extraintestinal infections and able to penetration through abrasion, puncture and wound on the skin. People who handle or work with aquatic animals can expose to the pathogen via direct contact with the mucus and tissue from infected animals. The clinical signs include cellulitis, myonecrosis around the wound, soft-tissue inflammation

(Joseph, 1979; David and Ronald, 1981; Gold and Salit, 1993; Lehane and Rawlin, 2000; Lowry and Smith, 2007; Chompoonuch et al., 2009).

3. Antimicrobial resistance in *A. hydrophila*

At present, tilapia farming has been expanded rapidly and led to many poor conditions in farm environments, for example, increasing stocking densities, poor water quality and aeration. These situations increase the disease outbreak and demand the use of veterinary drugs. The antimicrobials have been mixed with feed for oral administration. The antimicrobial agents are leached and diffused into the aquaculture system and eventually remain in the aquatic environment (Cabello, 2006; Smith, 2008). The use of antimicrobials has led to development of multidrug resistance in fish pathogens including *A. hydrophila*. Previous studies reported that *A. hydrophila* resistant to antimicrobials has been increasing around the aquaculture areas (Hatha et al., 2005; Jacobs and Chenia, 2007; Smith, 2008). Clinical isolates of *A. hydrophila* was shown to be resistant to many groups of antimicrobials, for example, ampicillin, amoxicillin, oxytetracycline, tetracycline and streptomycin (Hatha et al., 2005; Jacobs and Chenia, 2007; Penders and Stobberingh, 2008). Furthermore, *A. hydrophila* isolated from food products and natural water exhibited multidrug resistance to methicillin, rifampicin, bacitracin, novobiocin, aminoglycosides, cephalosporins, nitrofurantoin, fluroquinolones, chloramphenicol, sulphonamides, tetracyclines, penicillin and polymixin (Vivekanandhan et al., 2002; Palu et al., 2006; Chang et al., 2007). It was also evident that *A. hydrophila* can transfer the resistance determinants to other pathogens (Son et al., 1997; Bruun et al., 2003) and these resistant bacteria could be transferred from animals to human through the food chain (Teale, 2002; Walsh and Fanning, 2008). As a result, multiple drug-resistant *A. hydrophila* can become problems in animals and public health including poor growth performance, increase morbidity and mortality rate, high cost and long term of treatment.

4. Genetics of antimicrobial resistance in *A. hydrophila*

4.1 Class 1 Integrons in *A. hydrophila*

Bacteria develop antimicrobial resistance through several mechanisms, for example, acquisition of resistance determinants, mutations of the target enzyme, and expression of efflux pumps. As seen in other bacteria, resistance mechanisms of *A. hydrophila* could be associated with chromosome or plasmid. Previous studies showed that antimicrobial resistance genes in *A. hydrophila* can be transmitted inter- and intra-species via plasmid (Son et al., 1997). The spread of antimicrobial resistance genes are facilitated by mobile genetic elements, such as integrons. Class 1 integrons plays an important role in the carriage and spread of antimicrobial resistance genes among gram-negative bacteria including *A. hydrophila* (L'Abee-Lund and Sorum, 2001). These are mobile genetic elements that usually contain 2 conserved regions (CS), 5´CS and 3´CS (Figure 2). The 5´CS contains the *intI1* gene, which encodes the class 1 integrase enzyme. The *attI* site is responsible for integration of gene cassettes that encode for the resistance phenotypes in bacteria (Chang et al., 2007). The 3´CS contains *qacEΔ1-sul1* gene that encodes resistance to quaternary ammonium compounds and sulfonamide, respectively (Jacobs and Chenia, 2007). The variable region contains different inserted one or more gene cassettes which can be present in class 1 integrons. A variety of gene cassettes in class 1 integrons have contributed to multiple antimicrobial resistance phenotypes (Perez-Valdespino et al., 2009). A study in Taiwan showed that *Aeromonas* spp. isolated from food products, aquatic animals and poultry carried the gene cassettes i.e., *aadA1* and *aadA2*, bla_{OXA2a}, *catB3* and *catB8*, *dfr12* and *dfr2d* which encode resistance to aminoglycosides, β-lactam, chloramphenicol and trimethoprim, respectively (Chang et al., 2007; Jacobs and Chenia, 2007). Early study in South Africa indicated that inserted gene cassettes are *ant(3'')Ia* and *aac(6')Ia, dhfr1* that encode resistance to aminoglycosides and trimethoprim, respectively. Recent study in United Kingdom has revealed that class 1 integrons of *Aeromonas* spp. isolated from ornamental fish harbored variety of dihydrofolate reductase, aminoglycoside transferase coding regions and also carried

the *floR*, bla_{TEM-1}, *tet*(A), *tet*(D), *tet*(E) genes (Verner-Jeffreys et al., 2009). Different genetics of antimicrobial resistance in class 1 integrons were characterized in *Aeromonas* spp. from different countries owing to different antimicrobials use in various geographical areas.

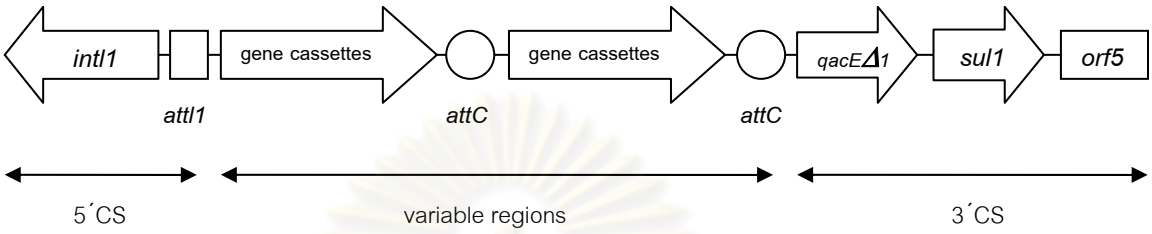

Figure 2: The typical model of class 1 integrons. The 5´ conserved sequence (5´CS) contains the *intI1* gene, which encodes the type 1 integrase enzyme. The 3´CS contains *qacEΔ1-sul1* gene that encodes resistance to quaternary ammonium compounds and sulfonamide. Variable regions contain gene cassettes encoding antibiotic resistance.

4.2 Drug efflux pump systems in *A. hydrophila*

Drug efflux pump has been known to play an important role in the antimicrobial resistance especially in Gram-negative bacteria (Poole, 2004). An active efflux mechanism extrudes antibacterial agents from inside of the cells leading to the reduction of intracellular. This could prevent the approach of the drugs to their target sites (Tenover, 2006). Multidrug efflux systems are currently classified into 5 families including the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (Poole, 2004).

At present, only one multidrug efflux pump system has been reported in *Aeromonas* species, the AheABC system. This pump is closely related to the AcrAB-TolC Multidrug Efflux Pump of *E. coli* classified in the RND family. Hernould et al. (2008) demonstrated that this RND efflux pump is involved in the intrinsic resistance of

A. hydrophila ATCC 7966 and inactivation of the *aheR* gene inhibited the pump function. The *aheABC* operon consists of the *aheA, aheB*, and *aheC* genes that are arranged in the same direction and encode for AheA, AheB, and AheC, respectively (Figure 3) (Hernould et al., 2008). The *aheR* gene encodes for the AheR regulatory protein responsible for suppression of the *aheABC* transcription. If there is a mutation in *aheR* or the *aheR-aheA* intergenic region, AheR cannot suppress the expression of AheABC and the transcription can constitutively occur. When the antimicrobial substrates get into the bacterial cells, they will be recognized and pumped out of the cells. Therefore, the substrate concentration will be reduced and not sufficient to hit the targets. The bacteria will become resistant (Chuanchuen et al., 2008a).

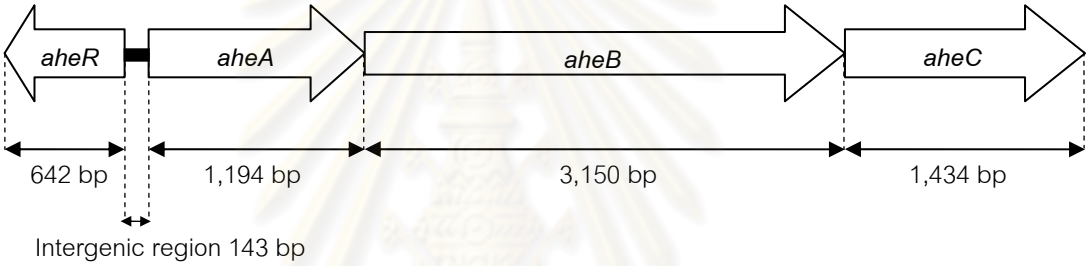

Figure 3: The model of the *aheRABC* gene cluster (Hernould et al., 2008)

4.3 Fluoroquinolones resistance in *A. hydrophila*

The broad spectrum antibacterial agents, fluoroquinolones, e.g. norfloxacin, enrofloxacin and ciprofloxacin are one of the antibiotic groups for treatment of gastroenteritis in human. Fluoroquinolones inhibit DNA gyrase or the topoisomerase IV enzyme, which are essential for bacterial DNA synthesis. The 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. DNA gyrase plays a major role in the relaxation of negative supercoils and it is essential for initiation and prolongation of the DNA replication fork. Topoisomerase IV is responsible for the decatenation of daughter chromosomes after replication (Hawkey, 2003).

Resistance to fluoroquinolones has been developed in *A. hydrophila* by mutation(s) in the Quinolone Resistance-Determining Regions (QRDRs) which are located within the DNA gyrase or the topoisomerase IV genes. The mutation(s) of the DNA gyrase and/or topoisomerase IV enzymes result in reduced sensitivity to fluoroquinolones. Previous studies of QRDRs in *Aeromonas* spp. have shown that mutations in *gyrA* gene is the primary target of quinolone resistance in *Aeromonas* spp., while mutations in both *gyrA* and *parC* genes can also contribute to high level quinolone resistance. Mutations in *gyrB* and *parE* genes have never been reported in *Aeromonas* spp. The mutations have been reported at position 83 in the QRDR region of the GyrA subunit which resulted in serine substituted with isoluecine or arginine (Ser-83-Ile or Arg) (Alcaide et al., 2010). For ParC subunit, the mutation at position 80 responsible for a serine to isoluecine change (Goni-Urriza et al., 2002).

CHAPTER III

MATERIALS AND METHODS

This study was divided into 3 phases as follows: phase 1, genetic confirmation of *A. hydrophila* by multiplex PCR; phase 2, test of antimicrobial susceptibility, and phase 3, characterization of antibiotic resistance in *A. hydrophila*. The experimental outline is shown in Figure 4.

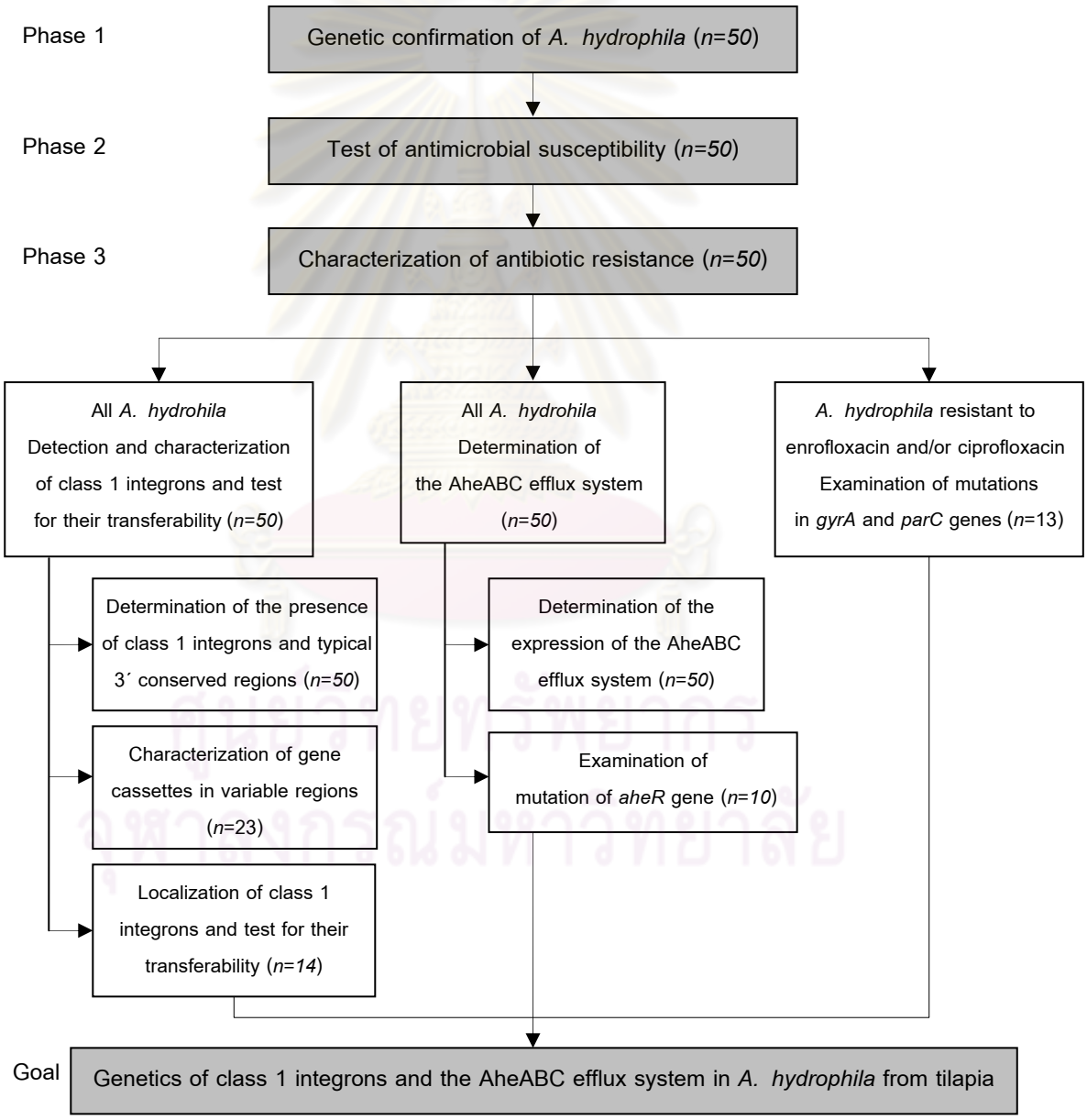

Figure 4: The research plan of study.

A. hydrophila isolates

A total of 50 *A. hydrophila* isolates were included in this study. All of the isolates were originated from the strain collection of Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. They were isolated from clinical cases of in farmed tilapia in Thailand during 2007 and 2009 (Appendix A). The bacteria strains were isolated using the methods described in Fish Disease: Diagnosis and Treatment (Noga, 1996). The biochemical characteristics of *A. hydrophila* were confirmed by using API® 20 E or API® 20 NE (Biomerieux®, France). The *A. hydrophila* isolates were sent to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for further investigations. All the isolates were stored in 20% glycerol at -80°C.

Phase 1 Genetic confirmation of *A. hydrophila*

All the bacterial isolates were genetically confirmed to be *A. hydrophila* using multiplex polymerase chain reaction (PCR) technique with genus-specific primers (Aero1 and Aero2) and species-specific primers (16S rDNA1 and 16S rDNA2) as previously described (Chu and Lu, 2005). The primers used are listed in Table 1.

Template DNA was prepared by the whole cell boiled lysate procedure (Levesque et al., 1995). Bacteria were grown on Luria-Bertani (LB) agar (Difco, MD, USA) at 37°C for 24 hours. A single colony was selected and resuspended in 50 µl sterile distilled water and heated in a boiling water bath for 10 minutes. The suspension was centrifuged at 12,000 x *g* for 5 minutes. The supernatant was transferred to a new eppendorf tube and stored at -20°C.

Table 1: Primers used in phase 1

Primer	Sequence (5´-3´)	Gene	Amplicon size (bp)	Reference
Aero1	CTC AGT CCG TGC GAC CGA CT	AHCYTOENT	462	Chu and Lu, 2005
Aero2	GAT CTC CAG CCT CAG GCC TT		462	
16S rDNA1	GAA AGG TTG ATG CCT AAT ACG TA	*16S rDNA*	685	Chu and Lu, 2005
16S rDNA2	CGT GCT GGC AAC AAA GGA CAG		685	

All PCR assays were carried out using PCR MasterMix (Fermentas, Burlington, Canada) according to the manufacturer's instructions. The multiplex PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of Fermentas MasterMix, 3.5 µl of sterile-distilled water, 1 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). PCR thermal cycling conditions included an initial denaturation at 94°C for 5 minutes, and 30 cycles of denaturation for 2 minutes at 94°C, and primer annealing for 2 minutes at 56°C, and DNA extension for 2 minutes at 72°C and a final extension at 72°C for 5 minutes. PCR products obtained were separated on 1-1.2% agarose gel electrophoresis (Sigma-Aldrish®, St Louis, MO, USA) in 1X Tris-acetate/EDTA buffer and applying 100 V for 30-40 minutes. The gels were stained in ethidium bromide solution (Sigma-Aldrish®) and visualized using the Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, Ventura, CA, USA). The PCR amplicons were gel purified using Nucleospin Gel Extraction kit (Nucleospin®, Gutenburg, France). The DNA representatives were submitted for sequencing by Dideoxy Chain Termination at Molecular Informatic Lab, N.T., Hongkong. Nucleotide sequence analysis was performed using the Blast algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). *A. hydrophila* ATCC 35654 was used as control for confirmation of genus and species.

Phase 2 Antimicrobial susceptibility test

Antimicrobial susceptibility tests were conducted to determine the Minimum Inhibitory Concentrations (MICs) of antimicrobials, including amoxicillin (AMO), carbenicillin (CAR), cefoperazone (CEF), chloramphenicol (CHP), ciprofloxacin (CIP), enrofloxacin (ENR), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), minocycline (MIN), oxytetracycline (OTC), rifampin (RIF), sulphamethoxazole (SMX), streptomycin (STR), tetracycline (TET) and trimethoprim (TMP). All antimicrobials agents were purchased from Sigma-Aldrich. The two-fold agar dilution technique was performed as described by Clinical and Laboratory Standards Institute (CLSI, 2006). The *A. hydrophila* isolates were grown overnight at 37°C in Tryptic Soy agar (TSA; Difco, MD USA) for 18-24 h. Single colonies were transferred to 0.85% NaCl solution (NSS) and the cell density was adjusted to 0.5 McFarland (~10^8 CFU/ml). Then, the suspension was ten fold diluted to 10^7 CFU/ml in NSS. The suspension was inoculated using multi-point inoculators onto the Muller-Hinton agar (MHA; Difco) containing suitable concentrations of antibiotics. For antibiotic preparation, all were dissolved in appropriate diluents and filter-sterilized. The diluents and antimicrobial concentrations used are shown in Table 2. After 18-24 hr incubation, the MIC defined as the lowest concentration of an antimicrobial agent yielding no visible growth of bacteria was recorded. Breakpoints, the antimicrobial concentrations used for discriminating the isolates susceptible or resistant, are as the interpretation guidelines established by CLSI. Multidrug resistance (MDR) was defined as resistance to at least 3 or more different classes of antimicrobials. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *A. hydrophila* ATCC 35654 were used as quality control organisms.

Table 2: Solvents, concentrations and breakpoint of antimicrobial agents used in this study

Antimicrobial	Solvent	Concentration range (µg/ml)	Breakpoint (µg/ml)
amoxicillin	0.1N NaOH	0, 0.125-128	32
carbenicillin	sterile distilled water	0, 0.125-128	32
cefoperazone	sterile distilled water	0, 0.03-16	32
chloramphenicol	95% ethanol	0, 0.5-128	32
ciprofloxacin	0.1N NaOH	0, 0.06- 64	4
enrofloxacin	0.1N NaOH	0, 0.06-16	4
erythromycin	95% ethanol	0, 0.06-128	32
gentamicin	sterile distilled water	0, 0.5- 512	16
kanamycin	sterile distilled water	0, 0.125-256	16
minocycline	sterile distilled water	0, 0.03-16	16
oxytetracycline	0.1N NaOH	0, 0.06-64	16
rifampin	dimethylsulfoxide	0, 0.06-128	NA
streptomycin	sterile distilled water	0, 0.125-256	16
sulphamethoxazole	0.1N NaOH	0, 1-1,024	76
tetracycline	70% ethanol	0, 0.25-256	16
trimethoprim	dimethylacetamide	0, 0.125-1,240	4

NA: Not available

Phase 3 Characterization of antibiotic resistance

The experiment included 3 steps as follows: the first step was to characterize class 1 integrons and test for their transferability. The sec step was to determine expression of the AheABC efflux system. The third step was to examine the mutation(s) of the Quinolone Resistance Determination Regions (QRDRs) in *gyrA* and *parC* genes. For all steps, DNA template was prepared by the whole cell boiled lysate procedure as described above. Preparation of RNA is described in the section where the RNA template was used.

All primers used in this phase are listed in Table 3. Ten primers were designed in the present study i.e., Ahy_BF, Ahy_BR, Ahy_BUp, Ahy_BDown, Ahy_rpsLUp, Ahy_rpsLDown, Ahy_RAF, Ahy_RAR, Ahy_ParCF and Ahy_ParCR using the Primer3 program available at www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.

Table 3: Primers used in phase 3

Primer	Sequence (5´-3´)	Gene	Amplicon size (bp)	Reference
Class 1 integrons				
Int1F	CCT GCA CGG TTC GAA TG	*IntI1*	497	Chuanchuen et al., 2007
Int1R	TCG TTT GTT CGC CCA GC		497	
5´CS	GGC ATC CAA GCA GCA AG	Variable	Variable	Levesque et al., 1995
3´CS	AAG CAG ACT TGA CCT GA	regions	Variable	
qacEF	TAA GCC GTA CAC AAA TTG GGA GAT AT	*qacEΔ1*	363	Chuanchuen et al., 2007
qacEΔ1R	GCC TCC GCA GCG ACT TCC ACG		363	
sul1F	CGG ACG CGA GGC CTG TAT C	*sul1*	591	Chuanchuen et al., 2007
sul1R	GGG TGC GGA CGT AGT CAG G		591	
qacEF	TAA GCC CTA CAC AAA TTG GGA GAT AT	*qacEΔ1-sul1*	1,198	Chuanchuen et al., 2007
sul1R	GGG TGC GGA CTA GTC AGC		1,198	
AheABC efflux system				
Ahy_BF	GAG GAG TTC AAC GAC ATC CTG	*aheB*	252	This study
Ahy_BR	GTA CGG GTA GAC AAT CTC CAT GTT		252	
Ahy_BUp	TAT CGG TCA CCC TCA CCT TC	*aheB*	142	This study
Ahy_BDown	CTG CTG GAG GTC TTC TGG AC		142	
Ahy_rpsLUp	CCA CGG ATC AGA ACA ACA GA	*rpsL*	142	This study
Ahy_rpsLDown	TAT ACA CCA CCA CCC CGA AG		142	
Ahy_RAF	GCT GGG TAC TGC CCT GTT C	*aheR*	906	This study
Ahy_RAR	ATA AGG GCC GCC AGT GTT		906	
Mutation(s) in the QRDRs				
AsalgyrAF	TCC TAT CTT GAT TAC GCC ATG	*gyrA*	441	Goni-Urriza et al., 2002
AsalgyrAR	CAT GCC ATA CCT ACC GCG AT		441	
Ahy_ParCF	GTC CAC TCC ACC GTA CCC	*parC*	363	This study
Ahy_ParCR	GTA CGT CAT CAT GGA TCG TG		363	

1. Characterization of class 1 integrons and test for horizontal transfer

1.1 Determination of the presence of class 1 integrons and typical 3´conserved regions

Location of primers used in this step and the size of corresponding PCR-amplicons is shown in Figure 5.

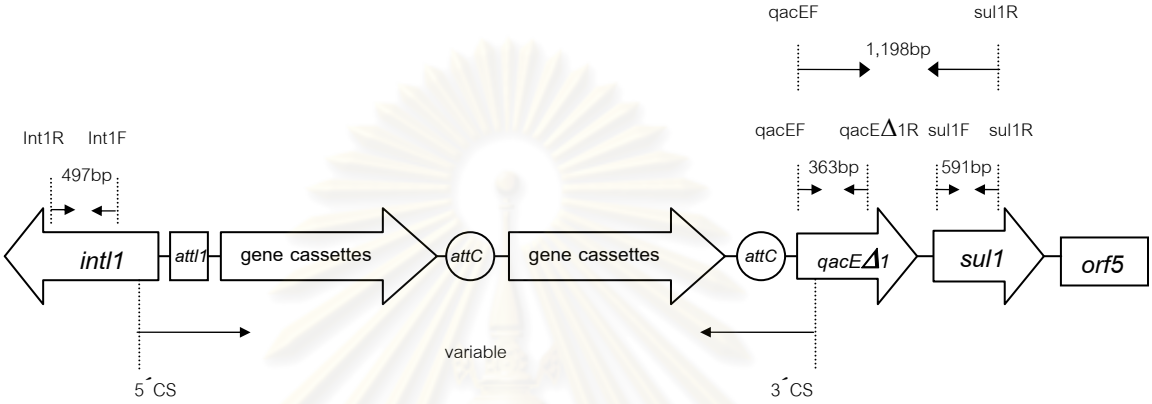

Figure 5: Localization of primers used in characterization of class 1 integrons and the size of expected PCR products. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers. The map is not drawn to scale.

All of the *A. hydrophila* strains were assayed for the presence of the class 1 integrase gene, *intI1*, by PCR using Int1R and Int1F primers. The PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of Fermentas MasterMix, 5.5 µl of sterile-distilled water, 1 µl of each primer at 10 µM and 5 µl of DNA template. PCR thermal cycling conditions consisted of an initial denaturation cycle at 94°C for 5 minutes, followed by 30 cycles of 45 sec at 94°C, 45 sec at 50°C, and 1 minutes at 72°C and a final extension of 72°C for 5 minutes.

All the *intI1*-positive strains were examined for the presence of the typical 3´conserved regions using primers specific for *qacEΔ1* (qacEF and qacEΔ1R), *sul1* (sul1F and sul1R) and *qacEΔ1-sul1* genes (qacEF and sul1R). The *qacEΔ1-sul1* amplicon was performed to confirm that *qacEΔ1* is located at 5´ end of *sul1.* Thermal cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, and

30 cycles of denaturation for 45 sec at 94°C, primer annealing for 45 sec at 65°C for *qacEΔ1*, 45 sec at 60°C for *sul1*, 45 sec at 67°C for *qacEΔ1-sul1* and extension for 1 minute at 72°C and a final extension at 72°C for 5 minutes.

1.2 Characterization of gene cassettes in variable regions

All the *intI1*-positive strains were further characterized for the presence of resistance gene cassettes in variable regions using CS-PCR technique with the specific primer pairs 5´CS and 3´CS (Levesque et al., 1995). Thermal cycling conditions consisted of a predenaturation cycle at 94°C for 5 minutes, and 30 cycles of 45 sec at 94°C, 1 minute at 54°C, 3 minutes at 72°C and a final step of 72°C for 5 minutes. The PCR amplicons were visualized on agarose gel and purified. Then, the representatives of purified PCR amplicons were submitted for sequencing. Nucleotide sequence was compared with the sequences deposited in GenBank.

The PCR amplicons of the same size were digested with selected restriction endonuclease according to the sequence results. The size of common PCR amplicon approximately 2,000 bp that was expected to be *dfrA12-aadA2* was digested with *EcoRI* (Fermentas) and separated in a 1.2% agarose gel. The amplicons with the same restriction pattern were considered identical.

1.3 Localization of class 1 integrons and test for their transferability

Determination of the presence of plasmid DNA

The *A. hydrophila* strains containing class 1 integrons with the resistance gene cassettes were screened for their plasmid using the alkaline lysis method (Liou et al., 1999). Bacteria were grown in LB broth and incubated at 37°C for 24 hours. The 1.5 ml of the culture was transferred into a new eppendorf tube and centrifuged at 16,000 *x g* for 5 minutes. The supernatant was removed. Then, the cell pellet was washed with 1 ml of phosphate buffer saline (PBS, Diagxotics®, Wilton, USA). A hundred-µl of 10mg/ml lysozyme (Biobasic Inc®, Canada) was added and the bacterial pellet was

resuspended by pipetting. The cell suspension was incubated at room temperature for 5-10 minutes. Then, two hundred-µl of lysis solution containing 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS, Amresco®, Ohio, USA) was added and the mixture was mixed by inversion 6-8 times. After incubation on ice for 5 minutes, one hundred fifty-µl of cold potassium acetate pH 4.8 was added. The mixture was mixed by vortexing, incubated on ice for 5 minutes and centrifuged at 16,000 x *g* for 5 minutes. The supernatant was transferred to a new eppendorf tube. Two-µl of 10mg/ml Ribonuclease A (RNase, Fermetas®) was added and the mixture was incubated at 37°C for 30 minutes. Four hundred-µl of phenol: chloroform: isoamyl alcohol (25:24:1 ratio) was added. The mixture was mixed by vortexing, and centrifuged at 16,000 x *g* for 2 minutes. The aqueous phase was transferred to a new eppendorf tube and 1 ml of cold absolute ethanol was added. The mixture was incubated at -20°C for 24 hours and centrifuged at 16,000 *x g* for 5 minutes. The supernatant was discarded. The pellet was washed with 1 ml cold 70% ethanol. The DNA pellet was air-dried at room temperature for 10-15 minutes and dissolved in 15 µl sterile distilled water. The DNA preparation was stored at -20°C. The plasmid DNA obtained was visualized on agarose gel electrophoresis.

Conjugation experiment

The test for transferability of class 1 integrons was performed using conjugation technique as previously described (Schmidt et al., 2001). The *A. hydrophila* strains containing class 1 integrons with the resistance gene cassettes were used as donors and the spontaneous rifampicin-resistant derivatives of *E. coli* MG1655 rif^r were used as recipients (Khemtong and Chuanchuen, 2008). The donors and recipients were grown in 4 ml LB broth and 4 ml LB broth containing rifampicin (32 µg/ml), respectively, at 37°C for 18-24 hours in a shaking incubator. Eighty-µl of aliquots of each overnight culture of the donors and recipients were added into 4 ml of fresh LB broth and incubated at 37°C for 4 hours in a shaking incubator to log phase of growth. Seven-hundred µl portions from each donor and recipient were gently mixed in fresh eppendrof tube and centrifuged at 5,000 x rpm for 1 minute. The supernatant was removed and the pellet was resuspended in 30 µl LB broth warmed at 37°C. The cell mixture was

gently dropped on a sterile membrane filter (0.45 µm pore size, Millipore, Massachusetts, USA) that was placed on a LB agar plate without antibiotic and incubated overnight at 37°C. The filter was transferred to 1 ml of 0.85% NaCl solution in a new eppendrof tube. The tube was vortexed to dislodge the cells and then the empty filter paper was discarded. The suspension was centrifuged at 13,000 *x g* for 1 minute and the supernatant was removed. A hundred-µl of fresh LB broth was added into the bacterial pellets. The conjugation mixture was spread on Eosin Methylene Blue (EMB) agar (Difco) supplemented with rifampicin (32 µg/ml) and streptomycin (10 µg/ml) and incubated overnight at 37°C. Transconjugants that were *E. coli* MG1655 rif^r with resistance plasmid, showed metallic sheen colonies on EMB agar. Plasmid DNA was extracted from each transconjugant using alkaline lysis method as described above and tested for the possession of class 1 integrons with gene cassettes corresponding to those in the donors.

2. Determination of the AheABC efflux system

2.1 Determination of the expression of the AheABC efflux system

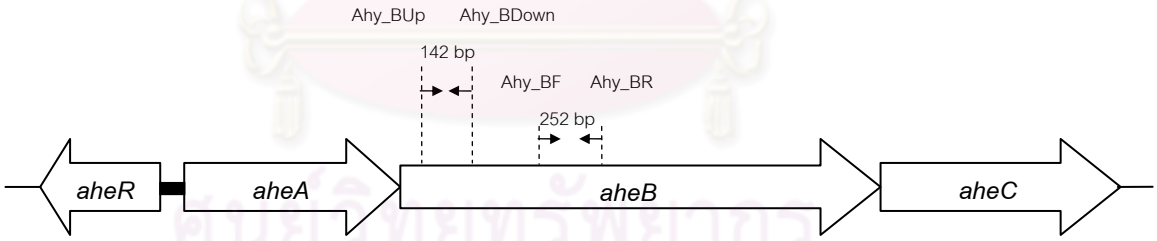

Figure 6: Localization of primers used in amplification of the *aheB* gene and the size of expected PCR products. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers.

Screening for the transcription of the aheB gene

All the *A. hydrophila* strains were screened for the expression of the AheABC efflux system by detecting the transcription of the *aheB* gene. The transcription of the *aheB* gene was examined using reverse transcription (RT)-PCR. All *A. hydrophila* isolates were grown in LB broth and incubated at 37°C for 24 hours. Total RNA was extracted using QIAGEN RNeasy mini kit (Qiagen® Hilden, Germany) as described by the manufacturer and treated by DNaseI (Fermentas) according to the manufacturer's protocol.

In the two step RT-PCR reaction, 1 µg of DNaseI treated RNA sample was used to prepare cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. For the cDNA synthesis, the reactions were performed in a final volume of 20 µl consisting of 1 µl of 2 pmol Ahy_BR primer, 1 µl of 10 mM dNTP Mix and 0.5 µg of total RNA. The mixture was incubated at 65°C for 5 minutes and then, on ice for 1 minute. Then, 4 µl of 5X First-Strand Buffer, 1 µl 0.1 M DTT and 1 µl of SuperScript™ III Reverse Transcriptase were added. The cDNA synthesis conditions were as follows: 25°C for 5 minutes, followed by 55°C for 45 minutes, and inactivation of the reaction at 70°C for 15 minutes. The cDNA obtained was stored at -20°C and used as DNA template for PCR. Amplification of the *aheB* gene was performed using primers Ahy_BF and Ahy_BR. PCR thermocycling conditions included an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 2 minutes, with a final extension step at 72°C for 5 minutes. Two representatives of purified PCR products were submitted for sequencing to confirm the specificity of primers used.

The reaction without the reverse transcriptase enzyme served as the negative control for cDNA synthesis to confirm the absence of DNA contamination. *A. hydrophila* ATCC 35654 with the constitutive expression of the AheABC efflux system was used as positive control.

Determination of the expression level of aheB

A. hydrophila strains with the expression of *aheB* were randomly selected (*n*=10) for measurement of the transcription level of *aheB* using real-time quantitative reverse transcription (qRT) PCR. The transcription levels of *aheB* in these selected strains were compared with that of *A. hydrophila* ATCC 35654, of which *aheB* is constitutively expressed. A new primer set, Ahy_BUp and Ahy_BDown, was designed for more specificity and efficiency of amplification. The housekeeping gene, *rpsL*, was chosen as internal control and Ahy_rpslUp and Ahy_rpslDown primers were used for detection of the transcription level of *rpsL*.

Total RNA was extracted as described above and treated with DNaseI. Then, cDNA was synthesized using Ahy_BDown primer for *aheB* and Ahy_rpslDown for *rpsL* gene. The cDNA obtained were stored at -20°C until use.

The real-time qRT-PCR was performed using Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A., Madrid, Spain) according to the manufacturer's instructions. The cDNA templates were 1:100 diluted in sterile-distilled water before analysis. The reactions were performed in a final volume of 25 µl consisting of 10 µl of QUANTISYG 2X, 8 µl of sterile-distilled water, 1 µl of each primer at 10 µM and 5 µl of cDNA template. The PCR amplification was carried out in triplicate in a Rotor-Gene™ 3000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia). The PCR thermal cycling conditions were an initial denaturation at 95°C for 5 minutes, and 45 cycles of denaturation for 10 sec at 96°C, and primer annealing for 15 sec at 57°C, and DNA extension for 20 sec at 72°C and a final extension at 72°C for 45 sec. The sterile distilled water was included for negative control in each run. Samples were considered positive when the negative control yielded no signal and the melt-curve analysis showed a single primer-specific melting temperature (Appendix D).

The threshold cycle or the Ct value is the cycle number where the fluorescence signal of the reaction crosses the baseline. The Ct value used to calculate the average *aheB* copy number. The copy number of each sample was normalized using the average *rpsL* copy number of the same sample. The results exhibit the relative level of *aheB* expression in the tested strains compared to the reference strain. The standard

curve (r^2>0.999) was plotted from the Ct values against the log of given concentrations data of serial 10-fold diluted *A. hydrophila* ATCC 35654. The cDNA of tested strains were diluted and expression levels of each sample fell within the limits of the standard curve.

2.2 Examination of mutations of *aheR* and the *aheR-aheA* intergenic region

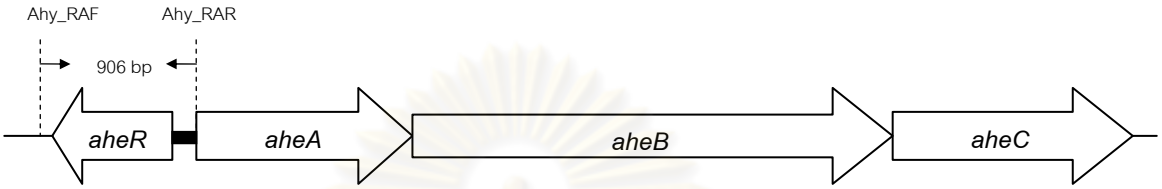

Figure 7: Localization of primers used in amplification of *aheR* and the *aheR-aheA* intergenic region and the size of expected PCR products. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers.

Ten *A. hydrophila* isolates that were investigated for the *aheB* transcription level were examined for mutation(s) in the *aheR* gene and the *aheR-aheA* intergenic region using PCR and DNA sequencing. Chromosomal DNA was prepared using IsoQuick® Nucleic acid extraction kit (IsoQuick®, WA, USA). Amplification of the target region was performed using a primer pair, AheRA_F and AheRA_R. PCR thermocycling condition was as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 sec, 52°C for 1 minute, and 72°C for 1 minute 30 sec, with a final extension step at 72°C for 10 minutes. After gel purification, all PCR products were submitted for nucleotide sequencing. Nucleotide sequences were compared with the *A. hydrophila* ATCC 35654 using the Chromas ver. 1.45 and Seqman (DNA-STAR) program.

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

The *A. hydrophila* strains exhibiting resistance to enrofloxacin and/or ciprofloxacin were tested for mutations in the QRDRs of *gyrA* and *parC* using PCR and DNA sequencing. Amplification of the QRDRs of *gyrA* was performed using a primer pair, AsalgyrAF and AsalgyrAR as previously described (Goñi-Urriza et al., 2002). For

the *parC* gene, the QRDRs were PCR amplified using primers Ahy_ParCF and Ahy_ParCR. PCR thermocycling conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 minute, with a final extension step at 72°C for 10 minutes. PCR products were purified and submitted for nucleotide sequencing. Nucleotide sequences were analyzed by comparison to the published *A. hydrophila* sequence (GenBank accession numbers AY027901 and AF435419.1 for *gyrA* and *parC,* respectively). Two *A. hydrophila* isolates susceptible to both enrofloxacin and ciprofloxacin were used as negative controls.

Instruments, bacterial growth media and chemical substances

1. Bacterial growth media
    1.1 Luria-Bertani (Difco)
    1.2 Tryptic Soy Agar (Difco)
    1.3 Muller-Hinton agar (Difco)
2. PCR assay
    2.1 MasterMix (Fermentas)
    2.2 DNA marker (Fermentas)
    2.3 Loading Dye (Fermentas)
    2.4 Agarose gel (Sigma-Aldrish®)
    2.5 Gel electrophoresis buffer (TAE)
    2.6 Ethidium Bromide 10mg/ml (Sigma-Aldrish®)
    2.7 PCR Tpersonal combi model® (Biometra®)
3. Reverse transcription (RT)-PCR assay
    3.1 QIAGEN RNeasy mini kit (Qiagen®)
    3.2 DNaseI (Fermentas)
    3.3 SuperScript™III Reverse Transcriptase (Invitrogen)
4. Real-time quantitative reverse transcription (qRT)-PCR assay
    4.1 Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A.)
    4.2 Rotor-Gene™ 3000 Real Time Thermal Cycler (Corbett Research)

CHAPTER IV

RESULTS

1. Genetic confirmation of *A. hydrophila*

A total of 50 *A. hydrophila* isolates used in this study were genetically confirmed using multiplex PCR. The size of PCR amplicon obtained from primers specific genus *Aeromonas* and species *A. hydrophila* was 462 bp and 685 bp, respectively (Figure 8). Nucleotide sequencing of these 2 amplicons confirmed the specificity of the primers.

Figure 8. Genetic confirmation of *A. hydrophila* using multiplex PCR. Chromosomal DNA was PCR amplified using primers specific for genus (Aero1 and Aero2) and species (16S rDNA1 and 16S rDNA2). The PCR products were 462 bp and 685 bp in size, respectively.

2. Antimicrobial susceptibility test

The MIC_{50} and MIC_{90} were defined as the minimum inhibitory concentration of antimicrobial agent required to inhibit 50% and 90% of the tested strains. Sulphamethoxazole had the highest MIC_{50} and MIC_{90} values. Cefoperazone and chloramphenicol had the lowest MIC_{90} values. The MIC range, MIC_{50} and MIC_{90} of the *A. hydrophila* strains are shown in Table 4.

Table 4. MICs range of *A. hydrophila* isolates to antimicrobial susceptibilities

Antimicrobial	MIC (μg/ml)		
	MIC_{50}	MIC_{90}	MIC range
amoxicillin	128	128	2-128
carbenicillin	128	128	2-128
cefoperazone	0.125	0.5	0.03-1
chloramphenicol	0.5	0.5	0.5-8
ciprofloxacin	1	4	0.06-16
enrofloxacin	0.25	8	0.06-8
erythromycin	4	128	0.06-128
gentamicin	1	4	0.5-4
kanamycin	4	16	2-256
minocycline	0.5	2	0.03-16
oxytetracycline	0.25	16	0.06-64
rifampin	1	4	0.5-8
sulphamethoxazole	512	1,024	1-1,024
streptomycin	16	128	8-256
tetracycline	1	16	0.25-128
trimethoprim	16	128	0.5-128

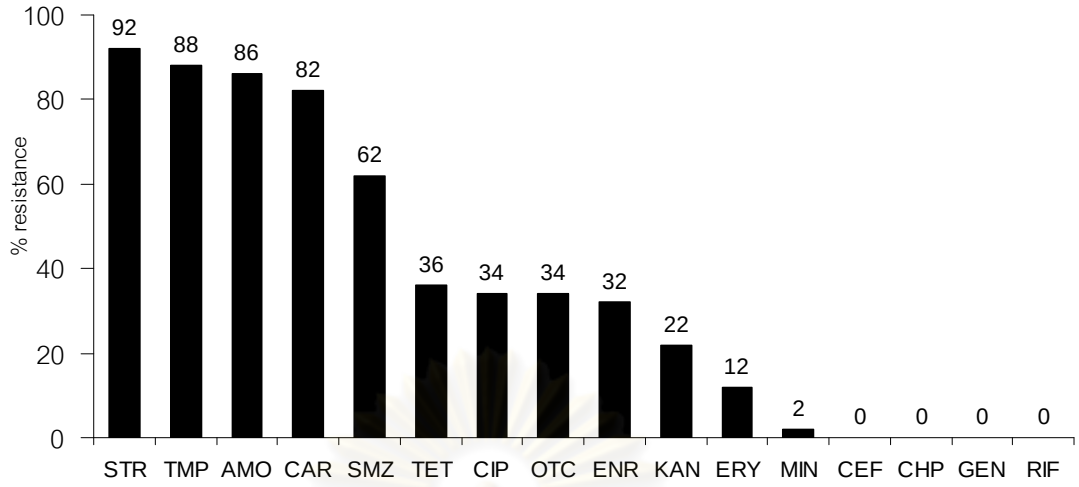

Figure 9. Distribution of antimicrobial resistance in *A. hydrophila* (*n*=50). Abbreviation: amoxicillin, AMO; carbenicillin, CAR; cefoperazone, CEF; chloramphenicol, CHP; ciprofloxacin, CIP; enrofloxacin, ENR; erythromycin, ERY; gentamicin, GEN; kanamycin, KAN; minocycline, MIN; oxytetracycline, OTC; rifampin, RIF; sulphamethoxazole, SMX; streptomycin, STR; tetracycline,TET; trimethoprim,TMP

Distribution of antimicrobial resistance is shown in figure 9. All isolates were resistant to at least one antibiotic, while most isolates were resistant to streptomycin (46/50, 92%), trimethoprim (44/50, 88%), amoxicillin (43/50, 86%), carbenicillin (42/50, 82%) and sulphamethoxazole (31/50, 62%). Resistance to gentamicin, chloramphenicol, rifampin and cefoperazone was not detected.

Antibiotic resistance phenotypes could be arranged into 29 patterns (Table 5). The most common resistance pattern was AMO-CAR-STR-TMP (10/50, 20%). Ninety four percent of the isolates were resistant to at least 3 different classes of antimicrobial agents and considered multidrug-resistant (MDR).

Table 5. Antibiotic resistance patterns of *A. hydrophila* isolates (*n*=50)

Antibiotic resistance pattern	No. of isolate (%)
STR	1 (2)
SMZ	2 (4)
CAR-SMZ-STR	1 (2)
AMO-CAR-STR-TMP	10 (20)
AMO-CAR-ERY-TMP	2 (4)
AMO-CAR-TMP-SMZ	1 (2)
CAR-CIP-SMZ-STR-TMP	1 (2)
CAR-KAN-SMZ-STR-TMP	1 (2)
AMO-ENR-SMZ-STR-TMP	1 (2)
AMO-CAR-ERY-SMZ-STR-TMP	1 (2)
AMO-CAR-KAN-SMZ-STR-TMP	5 (10)
AMO-CAR-OTC-STR-TET-TMP	1 (2)
AMO-CAR-SMZ-STR-TET-TMP	1 (2)
AMO-ENR-KAN-SMZ-STR-TMP	1 (2)
AMO-OTC-SMZ-STR-TET-TMP	1 (2)
AMO-CAR-CIP-ENR-OTC-STR-TMP	1 (2)
AMO-CAR-CIP-ENR-OTC-STR-TET	2 (4)
AMO-CAR-CIP-ENR-SMZ-STR-TMP	1 (2)
AMO-CAR-ERY-SMZ-STR-TET-TMP	2 (4)
AMO-CAR-OTC-SMZ-STR-TET-TMP	1 (2)
AMO-CIP-ERY-KAN-SMZ-STR-TMP	1 (2)
CIP-ENR-OTC-SMZ-STR-TET-TMP	1 (2)
AMO-CAR-CIP-ENR-KAN-SMZ-STR-TMP	1 (2)
AMO-CAR-CIP-ENR-OTC-STR-TET-TMP	1 (2)
AMO-CAR-CIP-OTC-SMZ-STR-TET-TMP	1 (2)
AMO-CAR-KAN-OTC-SMZ-STR-TET-TMP	1 (2)
AMO-CAR-CIP-ENR-MIN-OTC-STR-TET-TMP	1 (2)
AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP	5 (10)
AMO-CIP-ENR-KAN-OTC-SMZ-STR-TET-TMP	1 (2)
Total	**50**

Abbreviation: amoxicillin, AMO; carbenicillin, CAR; cefoperazone, CEF; chloramphenicol, CHP; ciprofloxacin, CIP; enrofloxacin, ENR; erythromycin, ERY; gentamicin, GEN; kanamycin, KAN; minocycline, MIN; oxytetracycline, OTC; rifampin, RIF; sulphamethoxazole, SMX; streptomycin, STR; tetracycline,TET; trimethoprim,TMP

3. Characterization of antibiotic resistance

3.1 The presence of class 1 integrons and typical 3´conserved regions

The *intI1* gene was detected in 23 isolates (46%). The representative PCR product was sequenced and confirmed the specific amplification. The PCR amplicons of *intI1* are shown in Figure 10.

Figure 10. The PCR amplicons of *intI1* in the *A. hydrophila* isolates. PCR amplifications were performed using primers int1F and int1R and the corresponding amplicon size was 497 bp. M, 100 bp marker; lane 1-5, the *intI1*-positive *A. hydrophila* strains.

All the *intI1*-positive strains were screened for the presence of typical 3´ conserved regions. Among these *intI1*-positive strains, sixteen (69.6%) isolates had typical 3´ conserved regions. The PCR amplicons of *qacEΔ1, sul1* and *qacEΔ1-sul1* genes are shown in Figure 11.

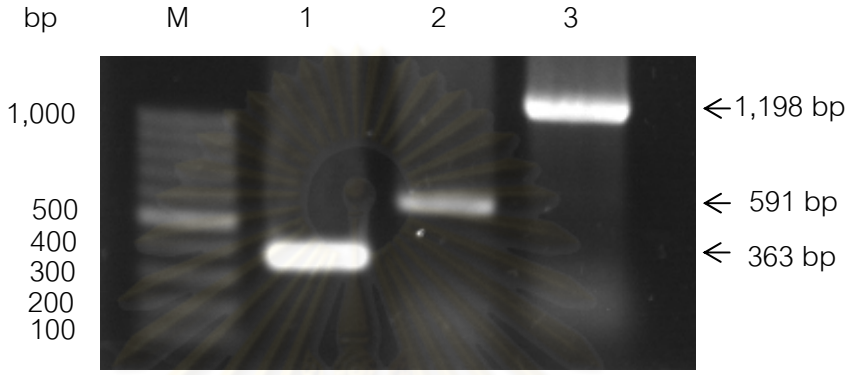

Figure 11. Typical 3´ conserved regions. The size of *qacEΔ1, sul1*, *qacEΔ1-sul1* was 363 bp, 591 bp and 1,198 bp, respectively. M, 100 bp marker; lane 1, *qacEΔ1*; lane 2, *sul1*; lane 3, *qacEΔ1-sul1.*

3.2 Characterization of gene cassettes in variable regions

Among the 23 isolates of the *intI1*-positive strains, fourteen isolates (60.8%) harbored antibiotic resistance gene cassettes inserted in variable regions. Thirteen isolates with gene cassettes in variable regions (56.5%) contained typical 3´ conserved regions. Only one isolate (4.3%) harbored the gene cassettes but lacked typical 3´ CS. Nine *intI1*-positive strains (39.1%) yielded a 150 bp amplicon with out inserted gene cassette. Among these strains, 3 isolates contained typical 3´ CS.

The size of gene cassette varied from 700 to 2,000 bp. Four gene cassette arrays were identified, including *aadA2, dfrA1-orfC*, *dfrA12-aadA2* and partial *aadA2* (Figure 12). The gene cassette array most commonly found was *dfrA12-aadA2* (26.09%). Eleven strains carried a single class 1 integrons, of which 1 isolate had a partial gene inserted in variable region. Three isolates contained 2 distinct class 1 integrons consisting, *aadA2* and *dfrA1-orfC* gene array. All class 1 integrons positive strains were resistant to at least 5 antimicrobial agents. The *intI1*-positive strains and their antimicrobial resistance pattern are shown in table 6.

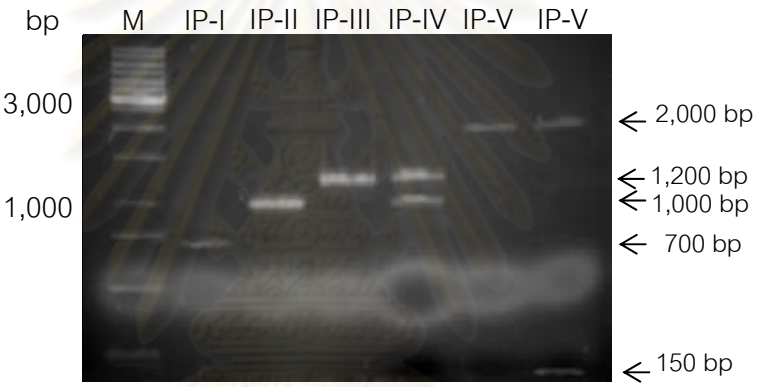

Figure 12. Integron profiles (IP). IP-I partial *aadA2*; IP-II *aadA2*; IP-III *dfrA1-orfC*; IP-IV *aadA2* (lower) and *dfrA1-orfC* (upper); IP-V *dfrA12-aadA2*; IP-VI 150 bp amplicon and *dfrA12-aadA2*. M, 1 kb marker

Table 6. Antimicrobial resistance patterns of the *intI1*-positive *A. hydrophila* strains (*n* =23)

Integron profile	Isolate no.	*IntI-1*	3'CS	Variable region size (bp)	Gene cassette[a]	Antimicrobial resistance pattern[b]	No. of isolate (%)
I	AH147[c]	+	+	700	Incomplete *aadA2*	CAR-CIP-SMZ-STR-TMP	1 (4.3%)
II	AH171[c]	+	+	1,000	*aadA2*	AMO-CAR-CIP-ENR-OTC-STR-TET	4 (17.4%)
	AH172	+	+	1,000	*aadA2*	AMO-CAR-CIP-ENR-OTC-STR-TET	
	AH175	+	+	1,000	*aadA2*	AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP	
	AH176	+	+	1,000	*aadA2*	AMO-CAR-CIP-ENR-OTC-STR-TET	
III	AH146	+	+	1,200	*dfrA1-orfC*	AMO-CAR-CIP-ENR-SMZ-STR-TMP	2 (8.7%)
	AH199	+	+	1,200	*dfrA1-orfC*	AMO-STR-OTC-SMZ-TET-TMP	
IV	AH173	+	+	1,000, 1,200	*aadA2*, *dfrA1-orfC*	AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP	1 (4.3%)
V	AH195	+	+	2,000	*dfrA12-aadA2*	AMO-KAN-ENR-SMZ-STR-TMP	4 (17.4%)
	AH196	+	+	2,000	*dfrA12-aadA2*	AMO-ENR-SMZ-STR-TMP	
	AH197	+	+	2,000	*dfrA12-aadA2*	AMO-CAR-CIP-ENR-OTC-SMZ-STR-TET-TMP	
	AH108	+	-	2,000	*dfrA12-aadA2*	AMO-CAR-ERY-SMZ-STR-TET-TMP	
VI	AH198	+	+	150, 2,000	*dfrA12-aadA2*	AMO-CAR-OTC-SMZ-STR-TET-TMP	2 (8.7%)
	AH200[d]	+	+	150, 2,000	*dfrA12-aadA2*	AMO-CIP-ERY-KAN-SMZ-STR-TMP	
No gene cassette	AH182	+	+	150	-	AMO-CIP-ENR-KAN-OTC-SMZ-STR-TET-TMP	9 (39.1%)
	AH191	+	+	150	-	AMO-CAR-CIP-OTC-SMZ-STR-TET-TMP	
	AH192	+	+	150	-	AMO-CAR-KAN-OTC-SMZ-STR-TET-TMP	
	AH184	+	-	150	-	AMO-CAR-KAN-SMZ-STR-TMP	
	AH185	+	-	150	-	CAR-KAN-SMZ-STR-TMP	
	AH187	+	-	150	-	AMO-CAR-KAN-SMZ-STR-TMP	
	AH188	+	-	150	-	AMO-CAR-KAN-SMZ-STR-TMP	
	AH189	+	-	150	-	AMO-CAR-KAN-SMZ-STR-TMP	
	AH190	+	-	150	-	AMO-CAR-KAN-SMZ-STR-TMP	

[a] *aadA2* encodes aminoglycoside adenyltransferases; *dfrA1* and *dfrA12* encode trimethoprim resistance dihydrofolate reductase.

[b] amoxicillin, AMO; carbenicillin, CAR; cefoperazone, CEF; chloramphenicol, CHP; ciprofloxacin, CIP; enrofloxacin, ENR; erythromycin, ERY; gentamicin, GEN; kanamycin, KAN; minocycline, MIN; oxytetracycline, OTC; rifampin, RIF; sulphamethoxazole, SMX; streptomycin, STR; tetracycline,TET; trimethoprim,TMP

[c] Transfer of class 1 integrons was detected.

[d] Only empty integrons was transferred.

According to the sequencing data, the 2,000 bp amplicons from 5 isolates were expected to be *dfrA12-aadA2*; therefore they were digested with *Eco*RI. The restriction yielded three fragments of 940 bp, 490 bp and 420 bp (Figure 13).

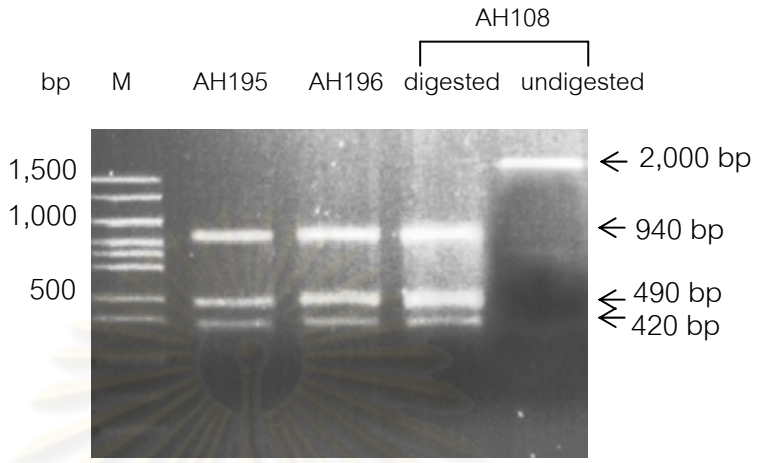

Figure 13. The *Eco*RI restriction patterns of *dfrA12-aadA2* array. The 2,000 bp from AH195, AH196 and AH108 were digested with *Eco*RI expected amplicon size. The undigested gene array was on the last lane. M, 100 bp marker

3.3 Localization of class 1 integrons and their transferability

All fourteen isolates of *intI1*-positive strains harbored antibiotic resistance gene cassettes inserted in variable regions were investigated for the presence of plasmid DNA. Plasmid DNA sizes ranging from 2-8 kb was found in 13 isolates and categorized into 7 groups according to the size and number of plasmids (Figure 14). All 13 isolates carried at least 2 plasmids. The most commonly found plasmid profile was group 6. The plasmid profile is shown in Table 7.

The conjugation experiments indicated that 3 *A. hydrophila* i.e. AH171, Ah147 and AH200 could horizontally transfer class 1 integrons to the *E. coli* recipient. It is noted that AH171 carry incomplete *aadA2* gene. However, AH200 carried 2 class 1 integrons and only empty class 1 integrons carrying plasmid was found to transfer.

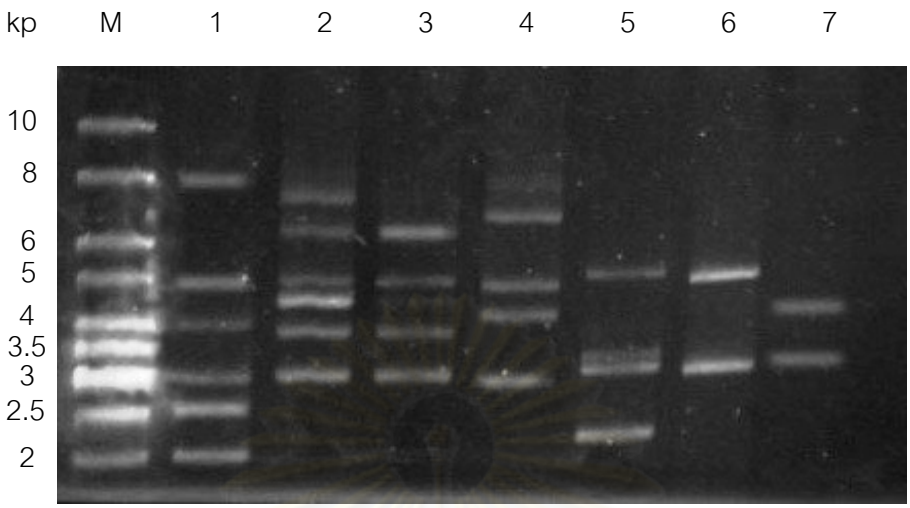

Figure 14. Plasmid profile of the *intI1*-positive *A. hydrohila* strains (*n*=13).

M, 1 kb marker; lane 1-7, group 1-7, respectively.

Table 7. Plasmid profile of the *intI1*-positive *A. hydrohila* strains in this study (*n*=13).

Group	Plasmid profile (kb)	No. of isolates (%)
1	2, 2.5, 3, 4, 5, 8	1(7.7)
2	3, 3.9, 4.7, 5, 6.5, 7.5	2(15.4)
3	3, 3.9, 4.7, 5, 6.5	1(7.7)
4	3, 4.5, 5, 7, 8	1(7.7)
5	2.4, 3.3, 3.5, 5.2	1(7.7)
6	3.3, 5.2	4(30.7)
7	3.3, 4.5	3(23)

Thirteen isolates containing plasmids were resistant to at least 5 antibiotics. All the isolates were resistant to streptomycin and amoxicillin, while resistance rates to, trimethoprim and sulphamethoxazole were 75% and 65%, respectively. Resistance to cefoperazone, chloramphenicol, erythromycin, gentamicin, minocycline and rifampin was not detected. The MIC values for antibiotics of all the *A. hydrophila* isolates containing plasmid is shown in table 8.

Table 8. MIC values of the *A. hydrophila* isolates containing plasmid (*n*=13).

Group	Isolate no.	MIC (μg/ml)*															
		AMO	CAR	CEF	CHP	CIP	ENR	ERY	GEN	KAN	MIN	OTC	RIF	SMZ	STR	TET	TMP
1	200	128	4	0.06	0.5	4	0.25	128	2	256	0.25	0.25	2	512	256	1	16
2	173	≥128	≥128	0.5	0.5	8	8	8	0.5	8	2	16	1	1024	128	32	≥128
	175	≥128	128	0.5	0.5	4	8	8	0.5	4	2	16	1	1024	128	32	≥128
3	171	≥128	≥128	0.5	0.5	4	8	0.06	0.5	8	2	16	1	64	64	16	2
4	196	≥128	4	0.06	0.5	2	4	8	4	8	0.5	0.25	4	512	256	1	16
5	108	≥128	64	1	0.5	1	0.5	128	0.5	4	1	0.25	2	1024	64	16	≥128
6	146	≥128	≥128	0.06	2	4	4	4	0.5	4	0.5	8	2	1024	16	4	16
	172	≥128	≥128	0.5	0.5	4	8	0.06	0.5	8	2	16	1	64	64	16	4
	195	64	4	0.5	0.5	2	4	8	2	16	0.5	0.25	4	512	128	1	16
	197	128	256	0.125	0.5	4	4	8	4	8	1	16	4	512	128	16	16
7	147	16	32	0.5	8	8	<0.06	4	1	4	0.125	8	0.5	1024	128	4	≥128
	198	≥128	256	0.125	2	0.25	0.25	8	2	8	0.5	16	2	512	256	16	16
	199	≥128	4	0.06	0.5	0.125	0.25	8	2	8	1	16	2	512	16	16	16

*The MIC values above the breakpoints underlined

Abbreviation: amoxicillin, AMO; carbenicillin, CAR; cefoperazone, CEF; chloramphenicol, CHP; ciprofloxacin, CIP; enrofloxacin, ENR; erythromycin, ERY; gentamicin, GEN; kanamycin, KAN; minocycline, MIN; oxytetracycline, OTC; rifampin, RIF; sulphamethoxazole, SMX; streptomycin, STR; tetracycline,TET; trimethoprim,TMP

4. Determination of the AheABC efflux system

4.1 The expression of the AheABC efflux system

All the *A. hydrophila* were screened for the expression of the AheABC efflux system. Transcription of the *aheB* gene was detected in 39 isolates (78%). The representative PCR product was sequenced and confirmed to be the *aheB* nucleotide sequence. The *aheB* amplicons generated from RT-PCR are shown in Figure 15.

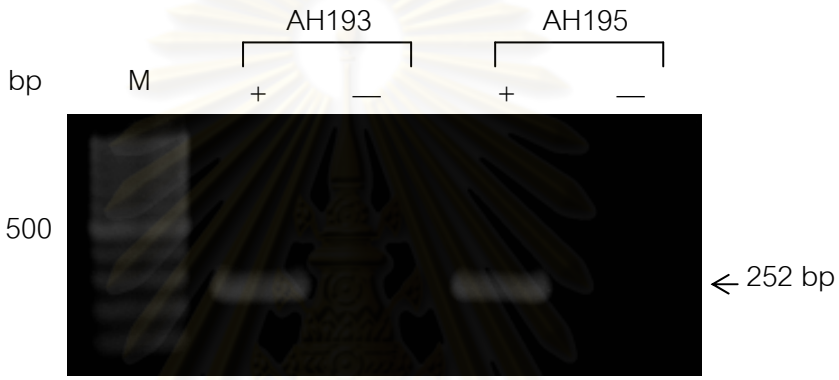

Figure 15. Detection of transcription of *aheB* using RT-PCR. The expected PCR amplicon size was 252 bp. (+) and (-) indicated RT-PCR with or without reverse transcriptase. M, 100 bp marker

Ten *A. hydrophila* isolates (26%) were randomly selected for determination of the transcription level of *aheB* gene by real-time qRT-PCR. The *aheB* transcription level in all isolates was 1.7 to 221.3 fold higher than that of *A. hydrophila* ATCC 35654. The maximum expression level (221.3 fold) was detected in AH194 and the minimum expression level (1.7 fold) was detected in AH173. AH194 was resistant only to a single antibiotic sulphamethoxazole, while AH173 was resistant up to 9 antimicrobials i.e., amoxicillin, carbenicillin, ciprofloxacin, enrofloxacin, oxytetracycline, streptomycin, sulphamethoxazole, tetracycline and trimethoprim. The expression level of *aheB* and antibiotic susceptibilities of the selected *A. hydrophila* isolates are shown in table 9.

Table 9. The expression levels of *aheB* and antibiotic susceptibilities in *A. hydrophila* (*n*=10)

Isolate	Expression	MIC (μg/ml)*															
no.	level of *aheB*	AMO	CAR	CEF	CHP	CIP	ENR	ERY	GEN	KAN	MIN	OTC	RIF	STR	SMZ	TET	TMP
AH194	221.3	2	2	0.06	0.5	0.06	<0.06	2	2	4	0.06	0.06	0.5	8	256	0.5	1
AH200	213.6	128	4	0.06	0.5	4	0.25	128	2	256	0.25	0.25	2	256	512	1	16
AH198	112.8	≥128	256	0.13	2	0.25	0.25	8	2	8	0.5	16	2	256	512	16	16
AH179	71.5	≥128	64	0.13	0.5	0.25	0.125	0.06	0.5	4	0.5	0.25	1	16	512	1	16
AH195	51.8	64	4	0.5	0.5	2	4	8	2	16	0.5	0.25	4	128	512	1	16
AH106	38.3	≥128	128	1	0.5	0.125	0.125	128	0.5	2	1	0.25	2	8	8	0.25	16
AH172	21	≥128	≥128	0.5	0.5	4	8	0.06	0.5	8	2	16	1	64	64	16	4
AH193	4.9	2	2	0.06	0.5	0.06	<0.06	2	2	4	0.06	0.06	0.5	8	256	0.5	0.5
AH160	4.8	128	≥128	0.03	0.5	1	<0.06	4	1	4	0.03	0.25	1	16	8	0.5	16
AH173	1.7	≥128	≥128	0.5	0.5	8	8	8	0.5	8	2	16	1	128	1024	32	≥128

*The MIC values above the breakpoints underlined

Abbreviation: amoxicillin, AMO; carbenicillin, CAR; cefoperazone, CEF; chloramphenicol, CHP; ciprofloxacin, CIP; enrofloxacin, ENR; erythromycin, ERY; gentamicin, GEN; kanamycin, KAN; minocycline, MIN; oxytetracycline, OTC; rifampin, RIF; sulphamethoxazole, SMX; streptomycin, STR; tetracycline,TET; trimethoprim,TMP

4.2 Mutations of *aheR* gene and *aheR-aheA* intergenic region

Ten *A. hydrophila* isolates were examined for mutations within the *aheR* gene and *aheR-aheA* intergenic region. However, PCR amplification was successful in only, 1 isolate i.e. AH106. This strain contained 7 point mutations in *aheR* gene. These were G-284-A leading to a Glu-95-Gly, C-289-A, G-291-C leading to a Leu-97-Ile, G-292-A leading to a Val-98-Ile, T-301-A leading to a Cys-101-Ser and A-307-G, C-309-G leading to a Asn-103-Glu. Thirteen silent mutations were found in *aheR* gene of AH106 (Appendix E). No mutation was observed in the *aheR-aheA* intergenic region.

5. Mutations of the QRDRs in *gyrA* and *parC* genes

In this study, nineteen (38%) *A. hydrophila* isolates were resistant to enrofloxacin and/or ciprofloxacin. Among these strains, thirteen (68.4%) strains were randomly selected for examination of mutations in the QRDRs of *gyrA* and *parC*. All the strains tested were found to contain mutations in both *gyrA* and *parC* gene. The point mutations identified in *gyrA* include G-248-T and T-249-T, C leading to a Ser-83-Ile. Only a C to A transition at position 80 was identified in *parC* and this resulting in Ser-80-Ile substitution. Eighteen silent mutations were found in *gyrA*, while 21 silent mutations were found in *parC* (Appendix F). No mutations were identified in QRDRs of the quinolone susceptible *A. hydrophila* strains.

CHAPTER V

DISCUSSIONS

Tilapia is one of the most important agriculture products in Thailand. Production of tilapia production has been continually increasing to supply both national consumptions and international exportations. The intensive farming and improper farming practices have led to severe disease outbreaks, including *A. hydrophila* infection among aquatic animals. In fish farming, antimicrobials have been widely used for prevention and treatment of bacterial infections. It is evident that improper uses of antibiotics are a major cause of antibiotic resistance in bacteria (Angulo et al., 2004). Currently, the emergence of multidrug-resistant *A. hydrophila* has been increasing worldwide (Palu et al., 2006; Penders and Stobberingh, 2008; Kaskhedikar and Chhabra, 2010). Previous studies have shown that multi-resistance in bacteria is usually associated with integrons and multidrug efflux system (L'Abee-Lund and Sorum, 2001; Chang et al., 2007). Class 1 integrons have been reported in *A. hydrophila* (L'Abee-Lund and Sorum, 2001; Fluit and Schmitz, 2004) and the AheABC pump is the only efflux system that has been reported in the pathogen so far (Hernould et al., 2008). Genetics of resistance have never been characterized in *A. hydrophila* isolated from tilapia, in spite of the public health importance of *A. hydrophila* and the high economic value of tilapia. Therefore, class 1 integrons and the AheABC efflux pump system were characterized in *A. hydrophila* isolated from farmed Nile tilapia in the present study. Since data regarding genetics of antimicrobial resistance in *A. hydrophila* from fish is very limited, the results obtained in this study will be compared with those from different species and/or hosts in certain context.

In this study, most of the *A. hydrophila* isolates were multidrug-resistant (MDR) and the most common resistance phenotype found was AMO-CAR-STR-TMP. This is in agreement with the earlier work showing that most of isolated from fish in Malaysia, India (Son et al., 1997; Hatha et al., 2005), from catfish and eel in The Netherlands (Penders and Stobberingh, 2008) and from food in India, Abu Dhabi (Vivekanandhan et al., 2002;

Awan et al., 2009) were multidrug-resistant (MDR). Taken together, these data confirm the widespread of multidrug-resistance among *A. hydrophila*.

High resistance level of streptomycin, trimethoprim, amoxicillin, carbenicillin and sulphamethoxazole was found in this study. Up to ninety two percent of *A. hydrophila* isolates were resistant to streptomycin, which is much higher than the results of early studies in Malaysia (57%) (Son et al., 1997) and United Kingdom (49%) (Verner-Jeffreys et al., 2009). In contrast, Vivekanandhan et al. (2002) reported that 9.3% *A. hydrophila* isolated from marketed fish and prawns were resistant to this antibiotic and Hatha et al. (2005) found that none of the isolates from fish was resistant to streptomycin. However, streptomycin has been rarely applied in aquaculture. The explanations for such high resistance may include cross resistance between this drug and other antimicrobials and co-selection of resistance genes located on the same elements. Another reason may be the expression of unidentified multidrug systems, of which the expression is turned on by other antimicrobial agents and streptomycin is one of its substrates. This hypothesis worth further investigations.

A. hydrophila demonstrated high resistance rates to sulphamethoxazole (62%), trimethoprim (88%), tetracycline (36%) and oxytetracycline (34%). These results were consistent with previous studies (Ko et al., 1996; Vivekanandhan et al., 2002; Jun et al., 2010). This is likely to be due to the extensive use of these antibiotics in farmed fish (Sapkota et al., 2008).

High resistance to amoxicillin and carbenicillin was observed, consistent with previous studies in *A. hydrophila* isolated from fish and eel (Son et al., 1997; Penders and Stobberingh, 2008; Verner-Jeffreys et al., 2009). This phenomenon may not be surprising because *A. hydrophila* is intrinsically resistant to β-lactam antibiotics. Such intrinsic resistance of this pathogen is due to its outer membrane permeability (Zemelman et al., 1984; Fosse et al., 2004; Tayler et al., 2010).

None of the isolates tested were resistant to cefoperazone, chloramphenicol, gentamicin and rifampin in agreement with the reports of Jacobs and Chenia (2007) and Awan et al. (2009). Cefoperazone and rifampin have never been used in farmed fish, while chloramphenicol has been banned for using in food producing animals since 1997 (FDA, 1997). This could support the low incidence of resistance to those antibiotics identified.

Up to 46% of the *A. hydrophila* isolates had the *intI1* gene and 60.8% carried resistance gene cassettes. The results were similar to those previously observed among *Aeromonas* spp. in southern Taiwan (Lee et al., 2008), Taiwan (Chang et al., 2007), South Africa (Jacobs and Chenia, 2007) and United Kingdom (Verner-Jeffreys et al., 2009). This indicated that class 1 integrons are widespread among *Aeromonas* spp. worldwide. However, the class 1 integrons without inserted gene cassettes or the empty integrons was identified in this study. The empty integrons were previously found in *A. salmonicida* isolated from fish in Denmark, Scotland, Canada, USA and Norway (Schmidt et al., 2001) and *A. encheleia, A. sobria* and *A. hydrophila* isolated from an aquatic environment in South Africa (Jacobs and Chenia, 2007). It is still unclear how these empty integrons have emerged. They could be the integrons that lose their gene cassettes in the environment where absence of selective pressure or have exchanged the gene cassettes with other integrons (Partridge et al., 2002). Regardless of the reasons, these empty integrons are available to receive new gene cassettes and contribute to resistance dissemination among the pathogens (Bissonnette and Roy, 1992).

In the present study, 3 gene cassette arrays were identified in *A. hydrophila* strains, including *aadA2, dfrA1-orfC* and *dfrA12-aadA2*. All of these cassettes have been reported previously in *A. hydrophila* (Lee et al., 2008), *A. veronii* and *A. caviae* (Chang et al., 2007) and *A. salmonicida* (L'Abee-Lund and Sorum, 2001). The *dfrA12-aadA2* was most commonly found in our collection. The identical gene array was identified in *A. veronii* and *A. caviae* isolated from foodborne outbreak-suspected samples and human in Taiwan (Chang et al., 2007; Lee et al., 2008). Moreover, this

gene array was previously reported in other bacteria from different geographical locations, for example, *Salmonella enterica* from poultry and swine in Thailand (Khemtong and Chuanchuen, 2008), *E. coli* from poultry in Korea (Kang et al., 2005) and *Vibrio cholerae* from human and environmental samples in India (Thungapathra et al., 2002). The *aadA2* was also commonly found in this study. The gene cassette *aadA2* was previously described in *Aeromonas* spp. from humans in Mexico (Pérez-Valdespino et al., 2009), *V. cholerae* from human and environmental samples in Thailand (Dalsgaard et al., 2000) and *S. enterica* from food in Portugal (Antunes et al., 2005). The *dfrA1-orfC* gene array was detected in only 3 *A. hydrophila* strains. This gene array was previously found in *V. cholerae* from human in India (Thungapathra et al., 2002; Shi et al., 2006), *S. enterica* from humans and animals in Australia (Levings et al., 2005) and *S.* Oslo and *S.* Baerily from imported seafood in USA (Khan et al., 2006). The presence of the identical class 1 integrons in different bacterial species from different locations suggests the horizontal transfer of the elements and confirms their important role in dissemination of antibiotic resistance among bacteria (Severino and Magalhaes, 2002).

All of the class 1 integrons-positive strains in this study were multidrug-resistant. This is consistent with the results of several studies, which suggested that multidrug-resistance among gram negative bacteria, including *A. hydrophila*, is usually related to the existence of gene cassettes in class 1 integrons (Fluit and Schmitz, 2004; Chang et al., 2007; Jacobs and Chenia, 2007; Lee et al., 2008). This also confirms that the extensive use of antibiotics plays an important role in distribution of antibiotic resistance in bacteria.

Several *A. hydrophila* isolates without class 1 integrons were also multidrug-resistant isolates. Similarly, the presence of resistant gene cassettes in class 1 integrons did not support total resistance phenotypes. This indicated that antibiotic resistance in these strains was a result of none class 1 integron-borne resistance mechanisms such as chromosomal mutation, the existence of antibiotic resistance

genes not located on class 1 integrons and other resistance integron types (Chang et al., 2007).

Among 23 *intI1*-positive strains, 16 isolates (69.6%) carried the typical 3´CS *qacΔE1-sul1* fused structure. Class 1 integrons without typical 3´ CS were previously identified (Schmidt et al., 2001). This confirmed that the typical 3´CS is not always typical like its name (Chuanchuen et al., 2007; Chuanchuen et al., 2008b). The *qacEF-sul3* associated region was previously found in class 1 integrons from *S. enterica* isolated from chicken in Thailand (Chuanchuen et al., 2008b) and *P. aeruginosa* isolated from aquatic environment in United Kingdom (Rosser and Young, 1999) but was not detected in this study. The results suggest the existence of atypical 3´CS that has never been characterized. This also suggests that the presence of 3´CS cannot be used as an absolute marker for class 1 integrons detection.

In conjugation experiments, only 3 *A. hydrophila* isolates could horizontally transfer class 1 integrons-carrying plasmid to *E. coli* recipient. Among these, only a class 1 integrons with resistant gene cassette i.e. AH171 was transmissible. The other two transferable integrons included class 1 integrons-carrying partial *aadA2* that was no longer functional and class 1 integrons with no inserted gene cassette. The explanation could be that horizontal transfer of these two class 1 integrons was mediated by streptomycin resistance-encoding gene(s) located elsewhere on the same plasmids. Even though low transfer rate of class 1 integrons was detected in this study, it suggested that horizontal transfer could happen between *A. hydrophila* and other bacteria, at least in *in vitro* situation.

Of 14 class 1 integrons-carrying isolates, 13 isolates harbored plasmids with size ranging from 2-8 kb and at least 2 plasmids were identified in each isolate. The plasmid profile were different from those previously reported in *A. hydrophila* from patients in Australia (Chang and Bolton, 1987) and from tilapia in Malaysia (Son et al., 1997). The plasmid-borne resistance to antibiotics has been reported previously in *A. hydrophila* from fish in Japan (Aoki and Takahashi, 1987), *A. salmonicida* from brown

trout in Scotland (Adams et al., 1998) and *A. allosaccharophila* isolated from water samples in Switzerland (Picao et al., 2008). However, the relationship of the resistance phenotypes and the existence of plasmid were not determined in the present study. This could be the subject of interest for a future study.

In this study, transcription of the *aheB* gene was detected in 39 isolates (78%), indicating that the *aheABC* efflux is not constitutively expressed in all clinical isolates. The expression level of *aheB* in all 10 strains selected was higher than that of *A. hydrophila* ATCC 35654. The isolate with the highest *aheB* expression (i.e. AH194, 221.3 folds), was resistant to a single antibiotic. In contrast, the strain with *aheB* expression 1.7 folds higher than that of *A. hydrophila* ATCC 35654 (i.e. AH173) was resistant up to 9 antimicrobials. This suggested that the *aheABC* expression level was not correlated with multidrug resistance phenotype in these clinical isolates.

When consider individual antibiotics, the AheABC expression was not correlated with the susceptibility level of most substances. The evidences included AH194 with 221.3 fold expressions had low MICs to amoxicillin, carbenicillin, streptomycin, tetracycline and trimethoprim but AH173 with 1.7 fold expressions exhibited much higher MIC level of these antibiotics. In generally, there are several mechanisms involved in resistance to only a certain drug, for example, target mutation, enzymatic deactivation and also the presence of other efflux systems. Therefore, it cannot rule out that the AheABC is the sole mechanism responsible for resistance phenotypes among *A. hydrophila* strains tested. Further investigations are required to determine contribution of this system, in particular using gene inactivation strategy. However, such experiment was not performed in the present study.

Despite the expression of the AheABC efflux, all isolates were susceptible to cefoperazone, chloramphenicol, gentamicin, minocycline and rifampin, indicating that these four antibiotics are not the substrates for the AheABC efflux system. In contrast, a previous study showed that the AheABC efflux system was involved in the multidrug-resistance phenotypes of *A. hydrophila* and its antimicrobial substrates included

cefoperazone, cefuroxime, erythromycin, lincomycin, minocycline, trimethoprim and rifampin (Hernould et al., 2008). The reason(s) for the difference is still unknown. This may be due to variations between laboratory and clinical strains. Further investigations are suggested.

Determination of role of mutation in the *aheR* repressor and the *aheR-aheA* intergenic region in the *aheABC* expression level was one of the efforts in this study. However, we had failed to amplify *aheR* gene and *aheR-aheA* intergenic region in *A. hydrophila* ATCC 35654 and some clinical isolates. Several attempts have been done to overcome the problem, including adjustment of PCR mixture and amplification conditions and new primer design. However, it was not successful. Therefore, the sequencing results obtained was compared with the *A. hydrophila* ATCC 7966 (GenBank accession number EF613320.1). Among ten isolates tested amplification of *aheR* and *aheR-aheA* intergenic region was successful in only one isolates i.e. AH106. AH106 contained 5 amino acid changes in AheR including Glu-66-Gly, Leu-68-Ile, Val-69-Ile, Cys-72-Ser and Asn-74-Glu. No mutations were identified in *aheR-aheA* intergenic region. However, this data is not sufficient for thorough investigation of the involvement of mutations in *aheR* and the *aheR-aheA* intergenic region in *aheB* expression.

In *A. hydrophila*, the fluoroquinolone resistance was shown to be associated with mutations within the QRDRs of *gyrA, gyrB*, *parC* and *parE* genes (Goni-Urriza et al., 2002). Only a single mutation Ser-83-Ile substitution in GyrA was identified in this study. This amino acid mutation was previously found in GyrA of the quinolone resistant *A. hydrophila* (Goni-Urriza et al., 2002; Alcaide et al., 2010). Several amino acid changes at position 83 were previously demonstrated e.g. Ser-83-Arg and Ser-83-Val. The Ser-83-Ile amino acid substitution was also formerly found to co-exist with Asp-87-Asn in *A. caviae* isolated from diarrhea patients in India (Sinha et al., 2004). However, this double mutation was not correlated to higher resistance level. In addition to a point mutation in GryA, all ciprofloxacin and enrofloxacin resistant isolates carried a C to A transition in *parC* resulting in Ser-80-Ile substitution. This Ser-80-Ile amino acid

substitution was previously observed in ParC of *A. hydrophila* (Goni-Urriza et al., 2002; Sinha et al., 2004; Alcaide et al., 2010). It was previously shown in other bacteria that the bacterial strains simultaneously carrying mutations in both GyrA and ParC exhibited MIC level higher than that in the strain with mutation(s) in only one gene. In this study, all strains tested had mutation in both GyrA and ParC and MIC level varied (4-16 μg/ml). This suggested that the co-existence of mutations in both GyrA and ParC did not affect the resistance level of the *A. hydrophila* strains in this collection.

In aquaculture practice, the antimicrobials have been mixed with feed and spreaded into the aquaculture area. Their residues are deposited in the aquatic environment and contribute to the antibiotic resistance among aquatic bacteria such improper uses of antibiotics can induce occurrence of multiple drug resistance in bacteria. This also coselects the resistance even of some agents that have not been used in fish farming. Multiple drug-resistant pathogens can become problems in animals and public health including high cost and long term of treatment, poor growth performance and increasing morbidity and mortality rate (Molbak, 2004). The results from this study highlight the widespread of multidrug resistance and resistance-encoding determinants among aquatic pathogens. Therefore, appropriate and prudent use of antimicrobials in aquaculture is required. Moreover, the study of antimicrobial resistance mechanisms is essential for better understanding of the resistance mechanisms among aquatic pathogens. The antimicrobial resistance data are necessary to build up the action plan to reduce and prevent development and spread of resistant bacteria.

Conclusion and suggestions

From the findings of this study, we can conclude that this is the widespread of multidrug-resistance among *A. hydrophila* isolated from farmed Nile tilapia. Class 1 integrons and the *aheABC* pump play an important role in multidrug-resistance and dissemination of antibiotic resistance. The *aheABC* efflux is not constitutively expressed in these clinical isolates and its expression level was not correlated with multidrug resistance phenotype in clinical isolates. The data obtained could be beneficially used as follows:

1. Currently, most antibiotic resistance surveillance in Thailand results based on antimicrobial susceptibility test. The data on antimicrobial resistance of pathogens from aquatic animals is still limited. Therefore, the results could be a part of antimicrobial resistance monitoring program especially among aquatic animals.

2. The data can be applied in risk analysis of antimicrobial resistance. Risk analysis of antimicrobial resistance is required for the drug registration including those for aquatic use. However, the genetics data of antibiotic resistance among aquatic pathogens in Thailand is limited. Data from other countries could not be always used due to different antimicrobial use and geographical areas. In this case, genetics data of antibiotic resistance in aquatic pathogens needs to be systematically studied.

3. The information and materials obtained can be used for further studies as described below.

    3.1 Many multidrug resistance isolates did not contain class 1 integrons and lacked expression of AheB efflux system. These isolates could be used for genetic characterization of the mechanisms involved. This will help to better understand the resistance mechanisms among *A. hydrophila*.

    3.2 The data on genetics of antibiotic resistance among *A. hydrophila* isolated from aquatic environment are essential for develop the plan to reduce the antibiotic resistance originated from aquatic animals.

However, the results from this study are inadequate for creating the action plan. Therefore, the further studies of genetic of antibiotic resistance in aquatic pathogens are recommended. This will lead to better understanding the resistance dissemination among aquatic bacteria.

3.3 Several multidrug resistant strains also carried plasmid DNA. Therefore, the contribution of these plasmids in antibiotic resistance is recommended.

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APPENDICES

APPENDIX A

Cases record form of *A. hydrophila*

No.	ID	dd/mm/yy	Province	History	Organ for bacterial culture	Antimicrobials used
1	AH77	18/8/2007	Petchaburi	54 days old, lesions: fin rot, skin ulceration	kidney	Oxytetracycline
2	AH99	16/1/2008	Petchaburi	25 days old, lesions: lost scale, fin rot, ascites	kidney	Enrofloxacin
3	AH102	5/3/2008	Chachoengsao	60 days old, lesions: lost scale, fin rot, hemorrhage around the operculum, skin erosion, petechial hemorrhage of liver gastric and intestine	kidney	None
4	AH103	5/3/2008	Chachoengsao	60 days old, lesions: lost scale, fin rot, hemorrhage around the operculum, skin erosion, petechial hemorrhage of liver gastric and intestine	kidney	None
5	AH104	5/3/2008	Chachoengsao	30 days old, skin erosion	kidney	None
6	AH105	5/3/2008	Chachoengsao	60 days old	kidney	None
7	AH106	5/3/2008	Chachoengsao	30 days old, skin erosion	kidney	None
8	AH108	5/3/2008	Chachoengsao	15 days old, lesions: skin erosion, lethargy, gill edema, lost scale, fin rot	whole body (tissue homogenate)	Enrofloxacin
9	AH145	19/8/2008	Chachoengsao	female brooder stock, abscess at the snout, skin hemorrhage, ascites, submucosal edema, splenitis	kidney	sulfa/trimethoprim
10	AH146	7/7/2008	Chachoengsao	5 days old, skin erosion	whole body (tissue homogenate)	Enrofloxacin
11	AH147	19/8/2008	Chachoengsao	submucosal edema, damage of liver at the injection site	kidney	sulfa/trimethoprim
12	AH148	19/8/2008	Chachoengsao	ascites, liver abscess, splenitis	kidney	sulfa/trimethoprim
13	AH157	4/8/2008	Pratumtanee	sample 2,motality rate: 20%/day	kidney	NA
14	AH158	4/8/2008	Pratumtanee	sample 3,motality rate: 20%/day	kidney	NA
15	AH159	4/8/2008	Pratumtanee	sample 4,motality rate: 20%/day	kidney	NA
16	AH160	4/8/2008	Pratumtanee	sample 5,motality rate: 20%/day	kidney	NA
17	AH161	4/8/2008	Pratumtanee	sample 6,motality rate: 20%/day	kidney	NA
18	AH162	4/8/2008	Pratumtanee	sample 7,motality rate: 20%/day	kidney	NA
19	AH169	19/9/2008	Chachoengsao	female brooder stock: 3 years old	kidney	sulfa/trimethoprim
20	AM170	19/9/2008	Chachoengsao	petechial hemorrhage of intestine, pale liver, splenomegaly, hemorrhage and cyst at ovary	abscess	sulfa/trimethoprim
21	AH171	8/1/2009	Chachoengsao	8 days old, lethargy, spiralling	whole body (tissue homogenate)	NA
22	AH172	8/1/2009	Chachoengsao	15 days old, lethargy	whole body (tissue homogenate)	NA
23	AH173	8/1/2009	Chachoengsao	10 days old, healthy	whole body (tissue homogenate)	Enrofloxacin
24	AH174	8/1/2009	Chachoengsao	15 days old, lethargy, inflammations around the eyes	whole body (tissue homogenate)	Enrofloxacin

No.	ID	dd/mm/yy	Province	History	Organ for bacterial culture	Antimicrobials used
25	AH175	8/1/2009	Chachoengsao	20 days old,	whole body (tissue homogenate)	Enrofloxacin
26	AH176	8/1/2009	Chachoengsao	20 days old, lethargy, mouth erosion	whole body (tissue homogenate)	Enrofloxacin
27	AH177	8/1/2009	Petchaburi	female brooder stock, 1 year old, skin ulceration and hemorrhage, lost scale	kidney	Enrofloxacin
28	AH178	8/1/2009	Petchaburi	female brooder stock, lost scale, hemorrhage around the mouth, fin and abdomen, submucosal edema, hemorrhage at the ovary, splenomegaly	kidney	Enrofloxacin
29	AH179	8/1/2009	Petchaburi	female brooder stock, lost scale,kin ulceration and hemorrhage, lost scale, anal swelling, submucosal edema, hemorrhage at the ovary and liver, splenomegaly, enlargement of gall bladder	kidney	Enrofloxacin
30	AH180	8/1/2009	Petchaburi	8 days old	whole body (tissue homogenate)	None
31	AH181	8/1/2009	Petchaburi	29 days old, let lethargy, skin darkening, exopthalmos	whole body (tissue homogenate)	Enrofloxacin
32	AH182	8/1/2552	Chachoengsao	5 days old	whole body (tissue homogenate)	Enrofloxacin
33	AH183	8/1/2552	Chachoengsao	10 days old,	whole body (tissue homogenate)	Enrofloxacin
34	AH184	14/11/2008	Petchaburi	female brooder stock , morbidity rate 100%	brain	NA
35	AH185	14/11/2008	Petchaburi	sample 2/1; female brooder stock,100% morbidity rate, ascites, skin ulceration and hemorrhage, petechial hemorrhage around the operculum, fin, genital organ and anus, lost scale, brown liver	brain	NA
36	AH186	14/11/2008	Petchaburi	sample 2/2; female brooder stock,100% morbidity rate, ascites, skin ulceration and hemorrhage, petechial hemorrhage around the operculum, fin, genital organ and anus, lost scale, brown liver	kidney	NA
37	AH187	14/11/2008	Petchaburi	sample 3/1; female brooder stock,100% morbidity rate, hemorrhage at the genital organ, submucosal edema, hemorrhage at the ovary, enlargement of gall bladder, splenomegaly, petechial hemorrhage of liver	brain	NA
38	AH188	14/11/2008	Petchaburi	sample 3/2; female brooder stock,100% morbidity rate, hemorrhage at the genital organ, submucosal edema, hemorrhage at the ovary, enlargement of gall bladder, splenomegaly, petechial hemorrhage of liver	kidney	NA

No.	ID	dd/mm/yy	Province	History	Organ for bacterial culture	Antimicrobials used
39	AH189	14/11/2008	Petchaburi	sample 4/1; female brooder stock,100% morbidity rate, skin ulceration and hemorrhage, petechial hemorrhage around the operculum, fin and anus, yellow liver, submucosal edema, hemorrhage at the ovary, enlargement of gall bladder, splenomegaly	brain	NA
40	AH190	14/11/2008	Petchaburi	sample 4/1; female brooder stock,100% morbidity rate, skin ulceration and hemorrhage, petechial hemorrhage around the operculum, fin and anus, yellow liver, submucosal edema, hemorrhage at the ovary, enlargement of gall bladder, splenomegaly	kidney	NA
41	AH191	14/11/2008	Petchaburi	sample 5/1; female brooder stock,100% morbidity rate, skin ulceration , brown liver, submucosal edema, enlargement of gall bladder, splenomegaly	brain	NA
42	AH192	14/11/2008	Petchaburi	sample 5/2; female brooder stock,100% morbidity rate, skin ulceration , brown liver, submucosal edema, enlargement of gall bladder, splenomegaly	kidney	NA
43	AH193	6/11/2008	Chachoengsao	eggs, hemorrhage at yolk, decreased hatching percentage	tissue homogenate	NA
44	AH194	6/11/2008	Chachoengsao	eggs	tissue homogenate	NA
45	AH195	6/11/2008	Chachoengsao	30 days old,	whole body (tissue homogenate)	sulfa/trimethoprim
46	AH196	6/11/2008	Chachoengsao	sample 4/1, 30 days old, lethargy, fin rot, skin ulceration	whole body (tissue homogenate)	sulfa/trimethoprim
47	AH197	6/11/2008	Chachoengsao	sample 4/2, 30 days old, lethargy, fin rot, skin ulceration	whole body (tissue homogenate)	sulfa/trimethoprim
48	AH198	6/11/2008	Chachoengsao	sample 6(1), 30 days old, lethargy	whole body (tissue homogenate)	sulfa/trimethoprim
49	AH199	6/11/2008	Chachoengsao	sample 6(2), 30 days old, lethargy	whole body (tissue homogenate)	sulfa/trimethoprim
50	AH200	3/3/2009	Chachoengsao	15 days old, lethargy, mouth erosion, fin rot	whole body (tissue homogenate)	Enrofloxacin

APPENDIX B

Bacterial growth media and chemical substances

1. Bacterial growth media

   1.1 Luria-Bertani (LB) (Difco)

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

   1.2 Tryptic Soy Agar (TSA) (Difco)

- Pancreatic digest of casein	17.0 g
- Enzymatic digest of soybean meal	3.0 g
- Dextrose	25.0 g
- Sodium chloride	5.0 g
- Dipotassium phosphate	2.5 g
- Agar	15.0 g

   1.3 Muller-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

2. Chemical substance

   2.1 MasterMix (Fermentas)

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

2.2 50X TAE (Tris-Acetate buffer) 1,000 ml contains

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

2.3 Agarose gel (Sigma-Aldrish®)

- Agarose (ultrapure)	1.2 g
- Adjusted 1X TBE volume to 100 ml	

2.4 Ethidium Bromide 10mg/ml (Sigma-Aldrish®)

- Ethidium bromide	1.0 g
- Distilled water	1,000.0 ml

2.5 DNaseI, Rnase-free (Fermentas)

- DNaseI, Rnase-free 1U/µl	1 µl
- 10X reaction buffer with $MgCl_2$	1 µl

2.6 SuperScript™III Reverse Transcriptase (Invitrogen)

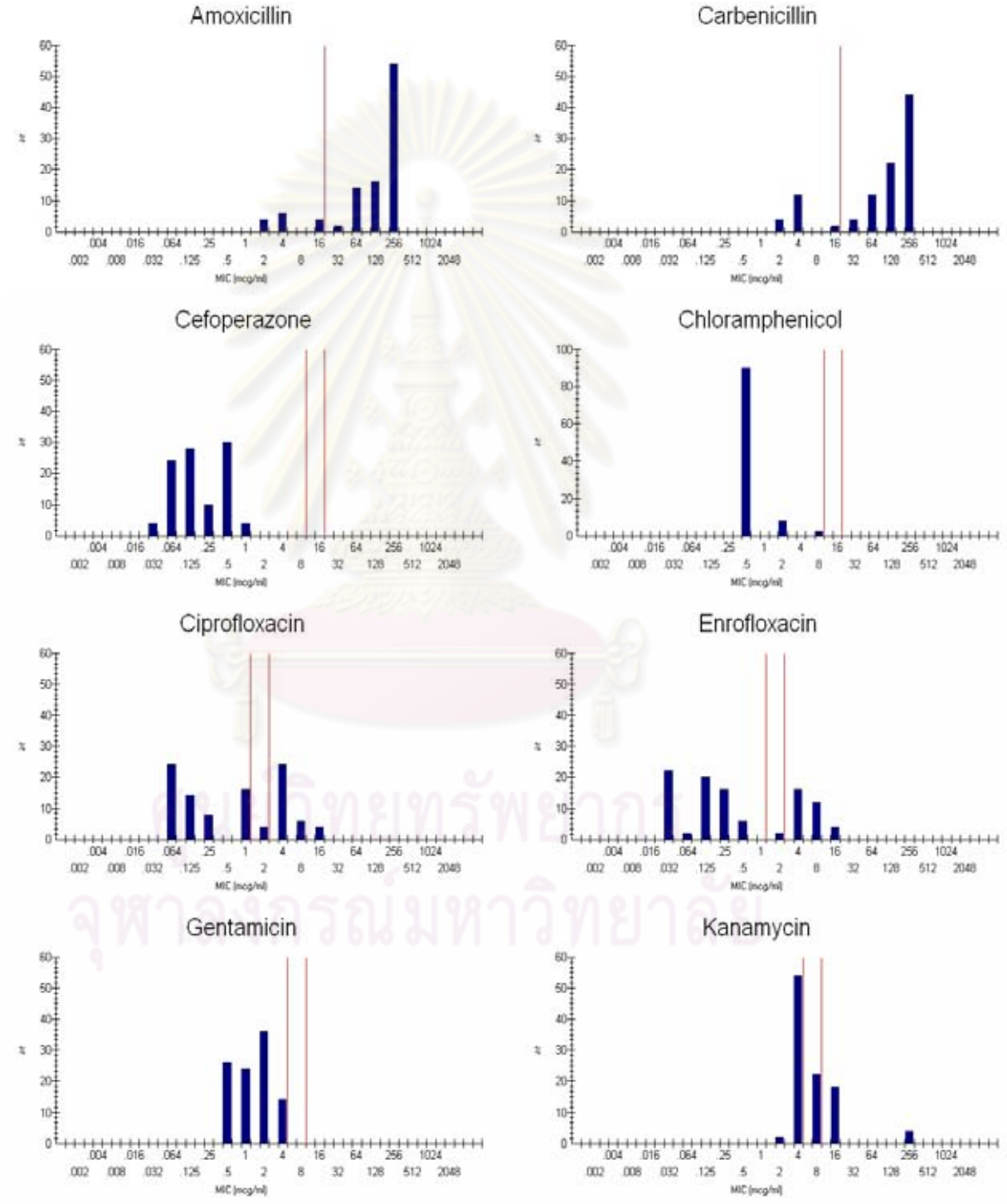
- SuperScript™ III RT 200 U/µl	1 µl
- 5X First-Strand Buffer	4 µl
- 0.1 M DTT	1 µl

2.7 Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A.)

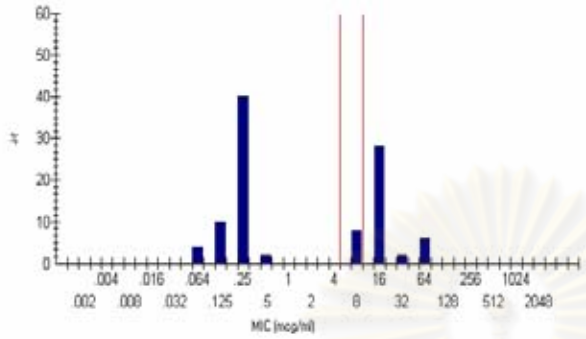
- 2X QUANTISYG	10 µl

APPENDIX C

Distribution of the MIC values of *A. hydrophila*



Oxytetracycline



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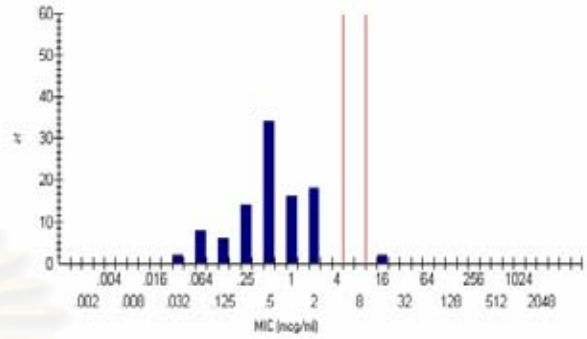
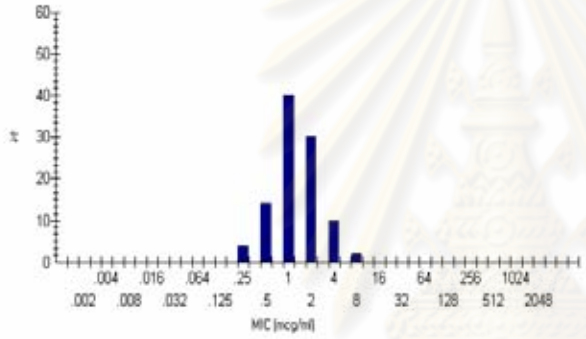
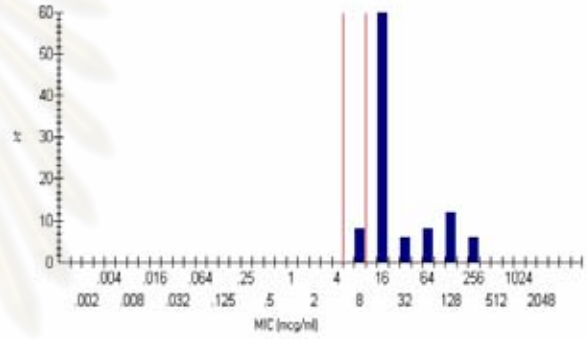


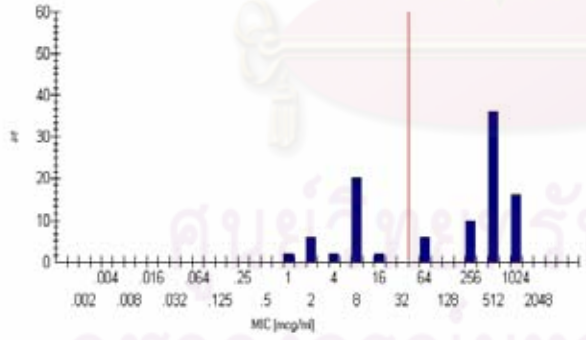
Rifampin



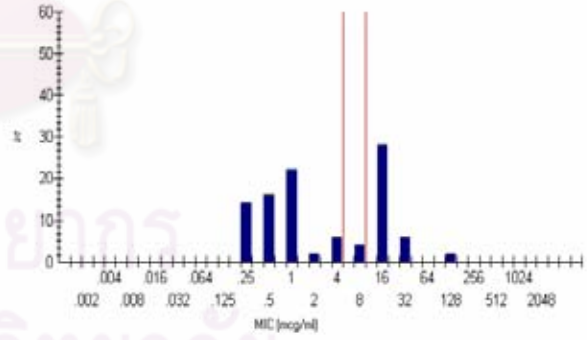
Streptomycin



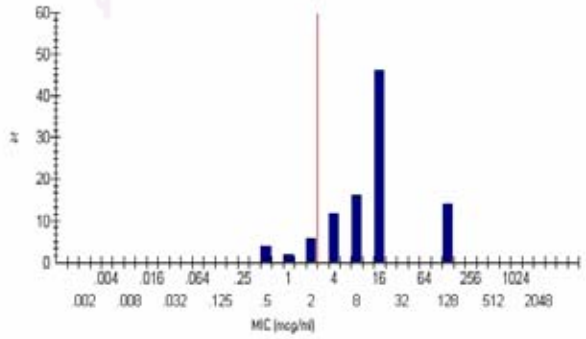
Sulfamethoxazole



Tetracycline



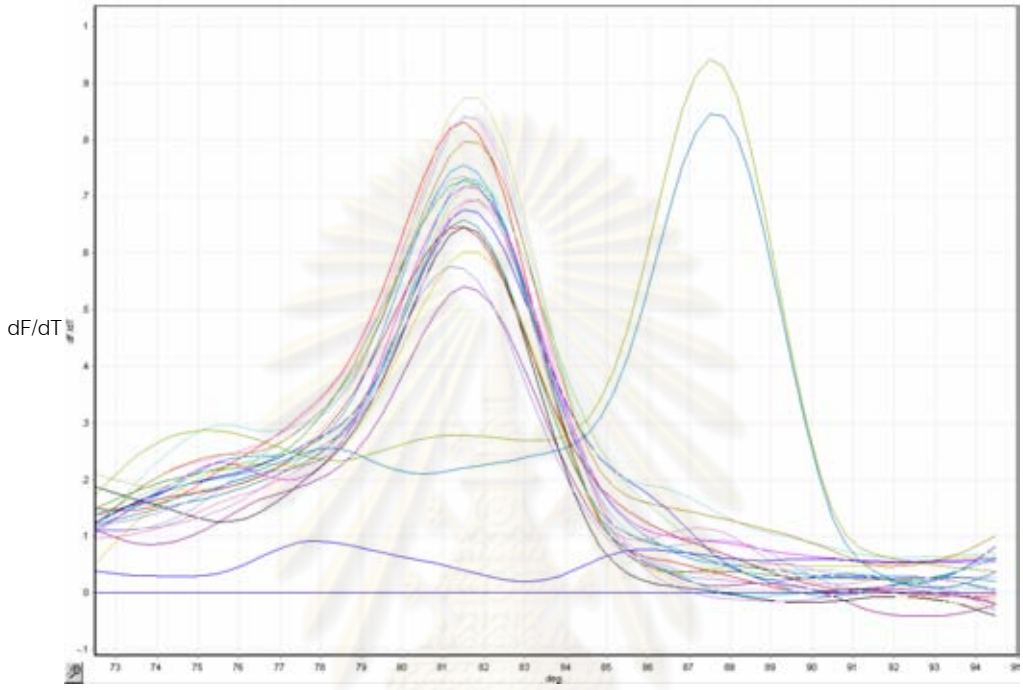
Trimethoprim



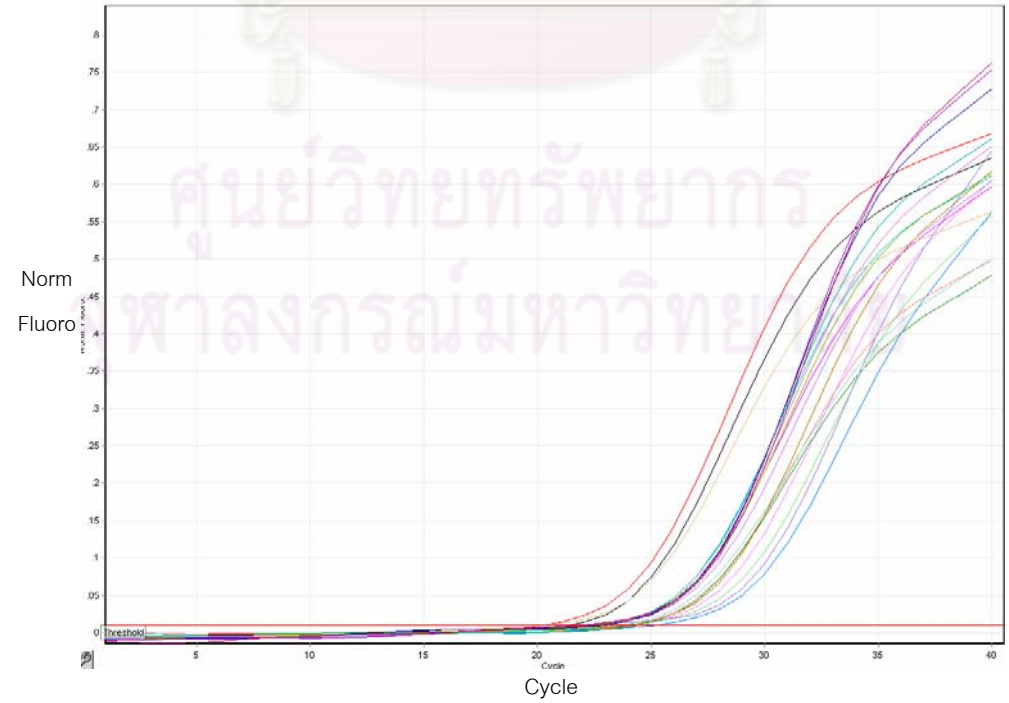
APPENDIX D

Real-time Quantitative Reverse Transcription (qRT) PCR

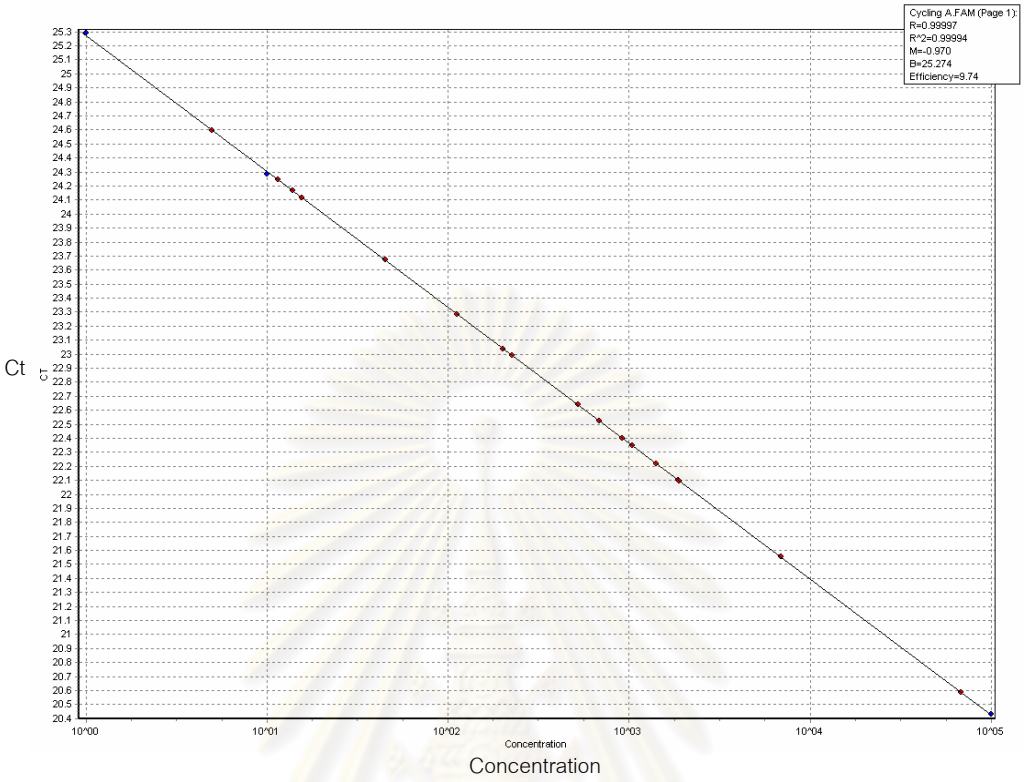
1. Melt curve analysis



2. Quantitation analysis



3. Standard curve (r^2>0.999)



APPENDIX E

Mutations observed in *aheR* gene in AH106

Mutation	
Nucleotide substitution	Amino acid substitution
A-120-C	Silent mutation
T-135-A	Silent mutation
T-228-C	Silent mutation
G-282-A	Silent mutation
G-284-A	Glu-95-Gly
C-289-A G-291-C	Leu-97-Ile
G-292-A	Val-98-Ile
A-297-G	Silent mutation
C-300-A	Silent mutation
T-301-A	Cys-101-Ser
A-307-G C-309-G	Asn-103-Glu
G-312-A	Silent mutation
G-363-A	Silent mutation
A-366-G	Silent mutation
G-486-A	Silent mutation
A-525-C	Silent mutation
A-567-G	Silent mutation
A-615-C	Silent mutation

APPENDIX F

Mutations observed in the *gyrA* and *parC* gene sequences from the quinolone resistant *A. hydrophila* isolates (*n*=13)

Gene	Mutation		No. (%)
	Nucleotide substitution	Amino acid substitution	
gyrA	C-222-A	Silent mutation	1(7.7)
	C-246-T	Silent mutation	13(100)
	G-248-C,T C-249-T	Ser-83-Ile	12, 1(100)
	C-252-T	Silent mutation	13(100)
	C-267-T	Silent mutation	13(100)
	T-270-C	Silent mutation	13(100)
	C-273-A	Silent mutation	13(100)
	G-333-A	Silent mutation	13(100)
	C-354-G	Silent mutation	13(100)
	C-357-G	Silent mutation	8(61.5)
	C-366-T	Silent mutation	8(61.5)
	C-408-T	Silent mutation	11(84.6)
	G-414-C	Silent mutation	1(7.7)
	A-441-G	Silent mutation	13(100)
	C-456-T	Silent mutation	1(7.7)
	A-471-G, T	Silent mutation	8, 5(100)
	C-480-T	Silent mutation	13(100)
	T-501-G	Silent mutation	13(100)
	G-510-C	Silent mutation	13(100)

Gene	Mutation		No. (%)
	Nucleotide substitution	Amino acid substitution	
parC	G-152-A	Silent mutation	1(7.7)
	A-158-C	Silent mutation	6(46.2)
	G-159-A	Silent mutation	5(38.5)
	C-164-A	Silent mutation	4(30.8)
	C-167-G	Silent mutation	12(92.3)
	C-170-G	Silent mutation	13(100)
	T-185-C	Silent mutation	13(100)
	G-188-A, T	Silent mutation	2, 1(23.1)
	G-206-A	Silent mutation	2(15.4)
	C-218-T	Silent mutation	4(30.8)
	G-221-A	Silent mutation	4(30.8)
	G-230-A	Silent mutation	4(30.8)
	C-239-A	Ser-80-Ile	13(100)
	G-248-A	Silent mutation	13(100)
	C-269-G	Silent mutation	13(100)
	G-284-A	Silent mutation	1(7.7)
	A-287-G	Silent mutation	13(100)
	G-290-A	Silent mutation	1(7.7)
	G-302-A	Silent mutation	1(7.7)
	G-311-A	Silent mutation	13(100)
	C-320-A	Silent mutation	13(100)
	C-335-G	Silent mutation	13(100)

BIOGRAPHY

Miss Mintra Lukkana was born on April 14, 1982 in Bangkok, Thailand. She got the degree of Doctor of Veterinary Medicine (2nd Class Honors) from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2006. After that, she has worked at Office of Commodity and System Standard, National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives. She enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2008.