GENOME CHARACTERIZATION OF QUINOLONE RESISTANCE ASSOCIATED GENES OF *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM COLUMNARIS DISEASED ASIAN SEA BASS (*LATES CALCARIFER*)

Miss Putita Chokmangmeepisarn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2018

การศึกษาคุณลักษณะทางจีโนมของยีนที่เกี่ยวข้องกับการดื้อต่อยากลุ่มควิโนโลนของเชื้อ *ฟลาโวแบคทีเรียม คอลัมแนร์* ที่แยกได้จากปลากะพงขาวที่เป็นโรคคอลัมนาริส

น.ส.พุธิตา โชคมั่งมีพิศาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	GENOME CHARACTERIZATION OF QUINOLONE RESISTANCE ASSOCIATED GENES OF *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM COLUMNARIS DISEASED ASIAN SEA BASS (*LATES CALCARIFER*)
By	Miss Putita Chokmangmeepisarn
Field of Study	Veterinary Pathobiology
Thesis Advisor	Assistant Professor CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P.
Thesis Co Advisor	Dr. Pattanapon Kayansamruaj, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Veterinary Science
(Professor ROONGROJE THANAWONGNUWECH, D.V.M., Ph.D., D.T.B.V.P.)

THESIS COMMITTEE

..... Chairman
(Associate Professor THEERAYUTH KAEWAMATAWONG, D.V.M, Ph.D., D.T.B.V.P.)

..... Thesis Advisor
(Assistant Professor CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P.)

..... Thesis Co-Advisor
(Dr. Pattanapon Kayansamruaj, D.V.M., Ph.D.)

..... Examiner
(Associate Professor NAPADON PIRARAT, D.V.M., Ph.D., D.T.B.V.P.)

..... External Examiner
(Teerapong Yata, M.Sc., Ph.D.)

..... External Examiner
(Ha Thanh Dong, M.Sc., Ph.D.)

พุธิตา โชคมั่งมีพิศาล : การศึกษาคุณลักษณะทางจีโนมของยีนที่เกี่ยวข้องกับการดื้อต่อยากลุ่มควิโนโลนของเชื้อ *ฟลาโวแบคทีเรียม คอลัมแนร์* ที่แยกได้จากปลากะพงขาวที่เป็นโรคคอลัมนาริส. (GENOME CHARACTERIZATION OF QUINOLONE RESISTANCE ASSOCIATED GENES OF *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM COLUMNARIS DISEASED ASIAN SEA BASS (*LATES CALCARIFER*)) อ.ที่ปรึกษาหลัก : ผศ. น.สพ. ดร.ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : น.สพ. ดร.พัฒนพล ขยันสำรวจ

ฟลาโวแบคทีเรียม คอลัมแนร์ เป็นแบคทีเรียแกรมลบที่ก่อให้เกิดโรคคอลัมนาริสในปลาน้ำจืดหลายชนิด ซึ่งการศึกษานี้เป็นรายงานการศึกษาคุณลักษณะ รวมทั้งการศึกษารูปแบบการดื้อต่อยาปฏิชีวนะของเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ ในปลากระพงขาวที่เพาะเลี้ยงในน้ำจืดในประเทศไทย เป็นครั้งแรก โดยเชื้อจำนวน 15 ไอโซเลท ถูกแยกได้จากปลากระพงขาวที่มีอาการของโรคคอลัมนาริส จากการศึกษาความสัมพันธ์ทางพันธุกรรม ระบุว่าเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ ในปลากระพงขาว จัดอยู่ใน genetic group ที่ 2 และ 4 จากผลการทดสอบความไวต่อยาปฏิชีวนะด้วยวิธี disk diffusion พบว่าเชื้อดื้อต่อกรดออกโซลินิค และกรดนาลิดิซิก ตามลำดับ นอกจากนี้ การศึกษาเพื่อหาค่าต่ำสุดในการยับยั้งการเจริญของเชื้อแบคทีเรีย (MIC) พบว่าในกลุ่มที่ดื้อต่อยาควิโนโลน มีค่า MIC ต่ออกรดออกโซลินิค และต่อกรดนาลิดิซิกสูงสุด คือ 16 μg/mL และ >64 μg/mL ตามลำดับ นอกจากนี้ ยังได้มีการมุ่งเน้นศึกษายีนที่เกี่ยวข้องกับกลไกการดื้อต่อยากลุ่มควิโนโลน โดยใช้ complete genome analysis จากการวิเคราะห์เทียบกับฐานข้อมูลยีนดื้อยา พบว่ายีนส่วนใหญ่เป็นยีนที่เกี่ยวข้องกับ efflux pump ที่ช่วยในกลไกลดปริมาณยาภายในเซลล์ และยังมีการศึกษาเพิ่มเติมในส่วนของการกลายพันธุ์ในส่วนคิวอาร์ดีอาร์ (quinolone resistance-determining regions: QRDRs) ของยีนไจร์เอ *(gyrA)* ไจร์บี *(gyrB)* พาร์ซี *(parC)* และพาร์อี (*parE)* อีกด้วย พบว่ามีการกลายพันธุ์ที่ตำแหน่ง 370 ในไจร์บี และ 389 ในพาร์อี ในกลุ่มเชื้อที่ดื้อต่อยาควิโนโลนทุกไอโซเลท แต่การกลายพันธุ์ที่ตำแหน่ง 83 บนไจร์เอ พบในกลุ่มดื้อยาทุกไอโซเลท ยกเว้น SP1805 ซึ่งมีค่า MIC ต่อกรดออกโซลินิค อยู่ในระดับต่ำ ซึ่งจากผลการวิเคราะห์ดังกล่าว สรุปได้ว่า การเกิดการกลายพันธุ์ที่ตำแหน่ง 370 ในไจร์บี และ 389 ในพาร์อี มีผลต่อกลไกการดื้อต่อกรดออกโซลินิค และกรดนาลิดิซิก ในขณะที่การเกิดการกลายพันธุ์ที่ตำแหน่ง 83 บนไจร์เอ มีผลต่อกลไกการดื้อต่อกรดออกโซลินิค แต่อย่างไรก็ตาม ในกลุ่มที่ไวต่อยาควิโนโลน

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5975309631 : MAJOR VETERINARY PATHOBIOLOGY

KEYWORD: Antimicrobial resistance Asian sea bass Flavobacterium columnare Mechanisms Quinolones

Putita Chokmangmeepisarn : GENOME CHARACTERIZATION OF QUINOLONE RESISTANCE ASSOCIATED GENES OF *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM COLUMNARIS DISEASED ASIAN SEA BASS (*LATES CALCARIFER*). Advisor: Asst. Prof. CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P. Co-advisor: Doctor Pattanapon Kayansamruaj, D.V.M., Ph.D.

Flavobacterium columnare is Gram negative bacteria which caused columnaris disease in various kinds of freshwater fishes. This study is the first report of *F. columnare* infection in freshwater culturing Asian sea bass in Thailand. Phenotypic characterization, antimicrobial susceptibility test and quinolone resistant-associated genes of *F. columnare* were performed. A total 15 of *F. columnare* were isolated from diseased fishes and phylogenetic analysis revealed that these isolates were belonged to genetic group 2 and 4. From disk diffusion test results, 5 and 6 isolates were resistant to oxolinic (OA) and nalidixic acid (NA) respectively. The highest MIC values to OA and NA were 16 and 64 μg/mL respectively. The complete genome analysis revealed that most resistant genes were responsible for efflux pump activity. Moreover, the mutations within quinolone resistance-determining regions (QRDRs) of *gyr*A, *gyr*B, *par*C, and *par*E were detected. The novel mutations at position 370 in *gyr*B and 389 in *par*E were founded in all quinolone resistant (QR) isolates. Moreover, mutation at position 83 in *gyr*A was responsible for OA resistant mechanism in QR isolates whereas mutations within *gyr*B, and *par*E were play role in both NA and OA resistant. On the other hands, the QS isolates were carried double mutations in *par*C at position 88 and 183. Thus, these mutations may not play important role in resistant against quinolones.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like express my special thanks to thesis advisor Asst. prof. Dr.Channarond Rodkhum for giving me an opportunity to study at this great institute Chulalongkorn University. I also thank for the good support and kindness from him. Moreover, I would like to thank my co-advisor Dr.Pattanapon Kayamsamruaj for the useful guidelines and all comments that make this project complete.

Besides, I am thankful for the scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Adulyadej, Fish Infectious Diseases Research Unit (FIDs RU), and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for financial support.

I would like to thank all scientists in Department of Microbiology especially Mrs.Waree Niyomtham for the useful suggestions and take a good care for me. I am thankful for graduated students for being a teacher and friend and encouragement. Finally, I would like to thank my family for thier love and support and help me to get through the hard time.

Putita Chokmangmeepisarn

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABO	Antibiotics
AML	Amoxycillin
AMR	Antimicrobial resistance
AMU	Antimicrobial use
AO	Anacker and Ordal
AR	Antimicrobial resistance
ARDB	Antibiotic Resistance Genes Database
ARG-ANNOT	Antibiotic Resistance Gene-Annotation
ARO	Antibiotic Resistance Ontology
ATCC	American Type Culture Collection
CAMHB	Cation-adjusted Mueller-Hinton Broth
CARD	The Comprehensive Antibiotic Resistance Database
CFU	Colony forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
DMHA	Diluted Mueller-Hinton agar
ENR	Enrofloxcacin
FFC	Florfenol
FQ	Fluoroquinolone ressitance
h	hours

LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
min	Minutes
mL	Milliliters
NA	Nalidixic acid
NGS	Next-generation sequencing
OA	Oxolinic acid
OT	Oxytetracycline
PCR	Polymerase Chain Reaction
PMQR	Plasmid-mediated Quinolone Resistance
QR	Quinolone resistance
QS	Quinolone sensitive
QRDRs	Quinolone Resistance-determining Regions
s	Seconds
SNPs	Snips
TYES	Tryptone yeast extract salts
WGS	Whole genome sequence
μg	Micrograms
μL	Microliters

CHAPTER I
Introduction

CHAPTER I
Introduction

1.1 Importance and Rational

A world fish consumption have been growing for decades. In 2016, the global aquaculture production was around 80.0 million tonnes and major of world production came from Asia. Thailand is one of the main aquaculture producing and top ten exporter countries in the world (FAO, 2018). A major cultured fish species in Thailand are tilapia, catfish, common silver barb, striped catfish, and Asian sea bass (DOF, 2017). The production was around 1 million tonnes and cost around 5.8 billion USD in 2016 and keep growing (FAO, 2018).

Asian sea bass (*Lates calcarifer*) is an economically important fish in many countries including Thailand. A number of freshwater sea bass farms have been increasing. However, the intensive production, raise many problems such as poor water quality, stress, and disease. The outbreaks of disease still being an issue in aquaculture production and affect the economic value.

Currently, columnaris disease, a disease caused by gram negative rod-shaped bacteria *'Flavobacterium columnare'*, remain being a problem to many freshwater fish farms in Thailand. The bacteria usually affects gills, skins, and fins result in tissue necrosis and produce a yellowish to white necrotic lesions in these organs. The disease can cause high mortality in wide range of freshwater fish species in both cold and warm water (Declercq et al., 2013b). There are several reports about this bacteria infecting various kinds of fish species in Thailand such as Nile tilapia, red tilapia, and catfish (Dong et al., 2015a; Dong et al., 2015b). Nevertheless, a study of *F. columnare* in Asian sea bass are not yet established.

A treatments for this disease is rely on antibiotics, chemical agents and management strategies due to lack of proficient commercial vaccine (LaFrentz et al.,

2018). Therefore, famers usually use antibiotics either prophylactic or therapeutic purposes (Rico et al., 2012). However, the improper usage, prolonged use, or illegal use of antibiotics can be a public health concern. The spread of antimicrobial resistance (AMR) not only within aquatic environment but also human. The human health hazard associate with antimicrobial use (AMU) in aquaculture including drug residues accumulation in aquaculture products and dissemination in environment, risen of antimicrobial resistance bacteria, and resistance genes transfer (WHO, 2007). Then, a studies of resistant mechanism and how resistant genes transfer and evolve should be established for monitoring and developing AMR control strategies (Watts et al., 2017).

A common method for evaluated bacterial susceptibility in clinical practice is *in vitro* susceptibility testing such as disk diffusion, agar dilution, and broth dilution method. These methods have some disadvantages because of time consuming and many materials are required. In addition, different protocol are needed in different species especially genus *Flavobacteria* (Gieseker et al., 2016).

Quinolones is one of the drugs which use in both human and veterinary medicine. Increasing the usage of quinolones enhance the quinolone-resistant in aquatic and human pathogens worldwide (WHO, 2007; Tusevljak et al., 2013; Mata et al., 2018). Some quinolone resistant or resistant-associated genes and mechanisms have been investigated in the genus *Flavobacterium* such as mutation in the quinolone resistance-determining regions (QRDRs), the presence of efflux pumps (Clark et al., 2009; Zhang et al., 2017; Mata et al., 2018). However, the report of AMR in *F. columnare* still limited and the systematic study of quinolone resistant mechanisms not yet established.

Nowadays, genome sequencing technology have been developed and more costly reasonable. For instance, the next-generation sequencing (NGS) have a high throughput and rapid technique. In addition, a several free databases and bioinformatics tools were available (Ali, 2013). NGS can be applied for many fields of research e.g. phylogenetic relationship, genome evolution, horizontal gene transfer, and virulence or resistance genes identification (Ali, 2013). Some studies can used comparative genomics to tracking a multi-drug resistance mechanism, investigated

horizontal gene transfer and predicted the antimicrobial resistance-associated genes. These approaches are useful for monitoring and combating AMR (Zankari et al., 2012). Therefore, this study aim to determine the quinolone resistance-associated genes of *Flavobacterium columnare* isolated from diseased Asian sea bass in Thailand.

1.2 Objectives of Study

1. To characterize the antimicrobial susceptibility patterns of *Flavobacterium columnare* isolated from diseased Asian sea bass in Thailand.
2. To investigate the quinolone resistance-associated genes of *Flavobacterium columnare* isolated from diseased Asian sea bass in Thailand.

1.3 Keywords (Thai): การดื้อยา ปลากะพงขาว *ฟลาโวแบคทีเรียม คอลัมแนร์* กลไก ควิโนโลน
1.4 Keywords (English): Antimicrobial resistance, Asian sea bass, *Flavobacterium columnare*, Mechanisms, Quinolones

1.5 Hypothesis

There are others gene apart from topoisomerase IV encoded genes involving in quinolone resistance mechanisms in *Flavobacterim columnare*

CHAPTER II

Literature Review

Asian sea bass (*Lates calcarifer*) is a euryhaline fish species which can live in fresh water, brackish water and sea water (Kungvankij P, 1985). Due to its adaptability to variety of environment, easy to breed, fast growing, and high value, sea bass becomes economically important fish in many countries such as Thailand, Taiwan, Indonesia, Malaysia, and Australia (DOF, 2017). Culture of sea bass should be separate into three phase, hatchery, nursery and grow-out phase. Firstly, the eggs are rearing in hatchery tank with salinity around 30-32 ppt and it will hatch within 18 hours. After hatch, the larvae are transfer to nursery tank at about 30-31 ppt salinity. The salinity should be reduced gradually until freshwater condition before transfer the larvae to growth-out phase (Kungvankij P, 1985). Since Thailand succeed in breeding and culturing sea bass, a number of freshwater sea bass farms have been increasing. Hence, the intensive culture in order to get high production, can lead to occurrence of disease.

Flavobacterium columnare, a Gram negative long rod bacteria belonging to the family *Flavobacteriaceae*, was first descripted as causative agent of columnaris disease in catfishes in United States and named as *Bacillus columnaris* (Davis, 1922). The bacteria exhibit gliding motility and can produce yellow pigment of flexirubin. The colony morphology is flat and sticky adhere to agar (Bernardet and Bowman, 2006). The medium for *F. columnare* culture are various poor nutrient mediums such as Anacker and Ordal medium, Shieh's medium, and tryptone yeast extract salts medium (TYES) (Anacker and Ordal, 1955; Holt, 1987; Decostere et al., 1997). The bacterium has been distributed worldwide and affect important economical freshwater fish species such as channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis sp.*) and common carp (*Cyprinus carpio*) (Bootsma and Clerx, 1976; Bader et al., 2003; Suomalainen et al., 2006; Declercq et al., 2013b).

Columnaris disease, the disease causing by *F. columnare*, is a highly contagious disease which can cause high mortality rate up to 100% (Pacha and Ordal, 1967; Shoemaker et al., 2008). The pathogen commonly affect to gills, skin, and fins and lead

to gill necrosis, ulceration and fins erosion. The yellowish-brown lesions can be observed due to the pigment production of the bacteria. Sometimes the progression of lesion can encircle the fish body and also known as saddleback disease (Declercq et al., 2013b). This disease become an important disease because *F. columnare* can infect various kind of aquaculture species in wide range of habitats. However, the lack of effective vaccine promote the use of antibiotics to prevent and control the disease (LaFrentz et al., 2018).

A global antimicrobials use survey revealed that oxytetracycline and quinolone are the most common used in aquaculture (Tusevljak et al., 2013). In Thailand, a various kind of antibiotics (ABOs) have been permitted for use in aquaculture including amoxycillin, enrofloxacin, trimethroprim, sulfadimethoxine, sulfathiazole, sulfamonomethoxine, ormetroprim, and oxolinic acid (DOF, 2017). Although quinolones and fluoroquinolones are licensed for use in Thai aquaculture, another countries are prohibited because of the ability to promote the quinolone-resistant strains (WHO, 2007; Tusevljak et al., 2013). Susceptibility patterns of *F. columnare* isolated from 17 fish species were resistance to oxolinic acid and enrofloxacin in 16% and 10%, respectively while *F. columnare* isolated from red tilapia in Thailand shown the resistant to oxolinic acid and nalidixic acid (Declercq et al., 2013a; Mata et al., 2018).

Aquatic environment collected with high diversity of bacteria, resistance genes, and drug residues which can exert selective pressure to increase resistant bacteria. Besides, the ability to spread resistant bacteria through water system should be concern (Watts et al., 2017). From the study of antimicrobial determinants in aquatic pathogens and human pathogens shown the sharing of resistance genes or plasmids against many classes of antibiotics (Santos and Ramos, 2018). Occurrence of AMR limit the choice for disease treatment and become a global health concern. Thus, a study of antimicrobial resistance is necessary for monitoring and understanding AMR epidemiology, and resistance mechanisms (Zankari et al., 2012). There are two most common method for testing phenotypic antimicrobial susceptibility, disk diffusion and broth dilution test. Disk diffusion test is a simple qualitative method which determine the susceptibility by observing zone diameter. Broth dilution method is a quantitative

method which can determine the lowest drug concentration that inhibit bacterial growth or MIC (Reller et al., 2009). According to the Clinical and Laboratory Standards Institute (CLSI) guideline, *F. columnare* belonging to fastidious gliding bacteria group. The suggested media for susceptibility testing is diluted Mueller-Hinton medium in 1:5 proportion (CLSI, 2014; Gieseker et al., 2016). Nevertheless, the difficulty of this genus is its fastidious and clumping which can interfere result interpretation. Furthermore, these method also need to standardized more and the exactly criteria for result interpretation are absent (Gieseker et al., 2016).

Due to the limitations of phenotypic susceptibility tests, the resistance genes identification has been performed by another methods and the most common method is PCR (Zankari et al., 2012). From the previous study of quinolone resistant gene of *F. columnare* originating in Thailand, the strains which have high minimum inhibitory concentration (MIC) level against oxolinic acid and nalidixic acid were used to determine the quinolone resistant genes by using PCR. These strains shown the missense mutations within *parC* and *gyrA* gene which responsible for encode the quinolone target enzymes (DNA gyrase and topoisomerase IV). Mutations within these gene result in alter the quinolone targets and finally resistance to quinolone drugs (Mata et al., 2018).

Quinolone is a group of antimicrobial agents which synthesized from nalidixic acid derivatives and have a broad-spectrum against gram negative and positive bacteria. First-generation quinolones, nalidixic acid and oxolinic acid, were used to treat gram negative bacterial infection (Jeffrey and Christian, 1996). After that, second-generation quinolones (norfloxacin, ciprofloxacin, ofloxacin) were developed with addition of fluorine in quinolone structure to extend the antimicrobial activity and pharmacokinetics. Then, this second-generation quinolones were later called fluoroquinolones and extended to gram positive bacterial infection. Enrofloxacin is the first fluoroquinolone which introduced in veterinary medicine (Riviere and Papich, 2009). Recently, enrofloxacin are approved for extralabel use in food animals for instance cattle, swine, shrimp, and fishes but already withdrawn from poultry because of bacterial resistance concerns (FDA, 2018). In addition, WHO described the enrofloxacin as critically importance drugs because it often use in both animal and

human medicine (WHO, 2006). Recently, the third-generation quinolones such as sparfloxacin, levofloxacin, grepafloxacin, moxifloxacin, and gatifloxacin were developed and have more potential to eradicate gram positive bacteria (Aldred et al., 2014).

Quinolones action is inhibiting type II topoisomerase enzyme activity, DNA gyrase and topoisomerase IV, which play crucial role in DNA synthesis. DNA gyrase act by relaxing superhelical twist at replication fork whereas topoisomerase IV segregating daughter chromosomes during cell division process (Hawkey, 2003). Quinolone disrupts activity of these enzymes by binding the enzyme-DNA complexes lead to DNA breaks and quinolones also prevent re-ligation of DNA. All of these actions result in bacteriostatic or even cell death (Aldred et al., 2014).

There are three main mechanisms involving in quinolone resistance. First, alter the drug targets by target-site gene mutations. Mutations of the genes that encode DNA gyrase and topoisomerase IV such as *gyrA*, *gyrB*, *parC*, and *parE* usually occur within the quinolones resistance-determining regions (QRDRs) leading to the type II topoisomerase subunits structure alteration. This action aims to change the quinolone targets leading to reduce drug affinity and effectiveness (Bearden and Danziger, 2001). According to previous studies, quinolones seem to have different target preferences. In general, DNA gyrase is the primary target of quinolones in gram negative bacteria while in gram positive bacteria is topoisomerase IV. In gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* the first step of mutation generally occur in *gyrA* follows by secondary target, *parC*, and the combination of two steps mutation cause a higher level of MIC (Everett et al., 1996; Nakano et al., 1997). However, this pattern is variable, some studies founded that the primary targets is depends on quinolone types due to its chemical structure (Pan et al., 2001; Hawkey, 2003). These results can infer that the correlation between quinolone targets and bacterial species need to be evaluated individually.

Second, reduce drug accumulation by chromosomal mutation. The mechanisms which help to reduce intracellular drug concentration is rely on two actions, decrease drug uptake and increase efflux pump. The gram negative bacteria have the outer membrane component that prevent a hydrophilic drugs to diffuse

called lipopolysaccharide (LPS). Then, the drugs have to enter the cell via protein channel called porin. Mutation within genes that regulate these mechanisms can prevent drugs to enter bacterial cells (Hawkey, 2003; Aldred et al., 2014).

Third, Plasmid-mediated quinolone resistance. Plasmid are a fragments of DNA which can harbor many mobile genetic element and resistance genes. In contrast with target-mediated resistance, this mechanism can transfer horizontally. A quinolone resistance genes located on plasmid referred as plasmid-mediated quinolone resistance (PMQR). The first genes family is *qnr* which encoded Qnr proteins such as McbG and MfpA. These proteins can reduce the DNA-enzyme binding activity. The second is *aac(60)-lb-cr* which is a point mutations in aminoglycoside acetyltransferase. This protein can destruct some kind of quinolones and fluoroquinolones. The last genes is efflux pumps such as *oqxAB* and *qepA* (Hawkey, 2003; Aldred et al., 2014). However, to our knowledge, the occurrence of *F. columnare* plasmid were not founded (Zhang et al., 2016; Tekedar et al., 2017). Thus, the plasmid-mediated quinolone resistance mechanisms may not affect quinolone-resistance activity in *F. columnare*.

Even though low-throughput based methods such as PCR are more convenient and gain more insight into AMR, limited number of resistance genes can be founded. Mostly, these genes were selected because play a main role in resistance mechanisms. By the way, the absent of resistance genes cannot claim that the bacteria are sensitivity to the drugs. Maybe some novel genes which not yet summit to databases or genes which indirectly affect resistance phenotypes remains ambiguous (Ellington et al., 2017). Besides, complete evaluation of resistance genes by conventional method is cost and time-consuming (Zankari et al., 2012).

Nowadays, a performance of next-generation sequencing have been improving while the cost and time have been decreasing. Likewise, a several bioinformatics tools have been developing and also free-access databases. The resistome analysis (The analysis of entire resistance genes) by using whole genome sequencing (WGS) can provide complete information because both resistance genes which existed in databases and novel genes without previous study of phenotype characteristics can

be identified. In addition, WGS also can identify mutations and investigate resistance traits (Xavier et al., 2016; Ellington et al., 2017).

A several antimicrobial online databases contribute a lots of resistance genes and resistance associated genes and another aspect such as antibiotic resistance ontology (ARO) and single nucleotide polymorphism (SNP). A databases such as Antibiotic Resistance Genes Database (ARDB), Comprehensive Antibiotic Resistance Database (CARD), ResFinder, and Antibiotic Resistance Gene Annotation (ARG-ANNOT) are the popular data resources (Xavier et al., 2016; Ellington et al., 2017).

CHAPTER III

Materials and Methods

3.1 Sample collection and bacterial isolation

Freshwater culturing Asian sea bass were obtained from 2 farms in Chachoengsao and Samutprakarn province. Only fish exhibiting columnaris-like clinical signs such as gill necrosis, fin erosion, tail rot, skin ulceration, and saddle back lesion were collected for bacterial isolation. Gill and skin were collected and streaked directly to Anacker and Ordal agar (AOA) plates then transported to the laboratory and incubated at 28°C for 48 hours (Anacker and Ordal, 1955). After that, suspected colonies of each isolates were selected and subcultured in AO broth with constant shaking at 160 rpm at 28°C for 48 hours. The bacterial suspension were kept for bacterial stocks by mixing with 10% glycerol and 20% fetal bovine serum and preserved at -80°C for further use.

3.2 DNA extraction

All *F. columnare* isolates were cultured in AO broth and incubated at 28 °C for 48 h with constant shaking at 160 rpm. Then, the bacterial suspension were subjected to DNA extraction. The genomic DNA of bacteria were extracted by Wizard® Genomic DNA purification kit (Promega, USA) according to manufacturer's instruction and keep in -20°C until use.

3.3 Bacterial identification

3.2.1 Identification by phenotypic characterization

All bacterial isolates which have characteristics of *F. columnare* colony morphology such as yellow, rhizoid, or sticky adherent were subjected to bacterial Gram staining to observe bacterial morphology. Motility, catalase and cytochrome oxidase test also performed. The growth capacity on tryptic soy agar (TSA) and MacConkey agar (MAC) were observed. The flexirubin production also determined by using 20% KOH. Decarboxylase test were performed using modified Anacker and Ordal

(MAO) broth containing 1 % L-lysine, L-ornithine, or L-arginine (Bernardet and Bowman, 2006).

3.2.2 Identification by PCR and sequencing

Species identification were performed by using species-specific primers; FCISRFL (5'-TGCGGCTGGATCACCTCCTTTCTAGAGACA-3') and FCISRR1 (5'-TAATYRCTAAAGATGTTCTTTCTACTTGTTTG-3'). A total 25 µL of PCR mixture were placed in thermal cycler and programmed as follows initial denaturation at 94°C for 5 min, 30 cycles of amplification at 45°C for 45 s, and extension at 72 °C for 7 min (LaFrentz et al., 2017). The PCR products were subjected to gel electrophoresis with 1% agarose gel stained with Red Safe™ staining solution (Intron, Korea) and observed under UV light. The PCR products were extracted by using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and sent for sequencing by 1st BASE DNA sequencing services (Malaysia). Then, the sequences were blasted against National Center for Biotechnology Information (NCBI) database.

3.4 Phylogenetic analysis

Phylogenetic analysis was based on the protocol described by LaFrentz et al which is divided *F. columnare* into four distinct genetic group (genetic group 1-4) (LaFrentz et al., 2018). A total 30 16s rRNA gene sequences of *F. columnare*, *F. psychrophilum,* and *F. johnsoniae* were used for generate the phylogenetic tree. Twenty-two published 16s rRNA sequences of *F. columnare* were downloaded from NCBI and 8 sequences were extracted from *F. columnare* genomes of this study (Table A1). All sequences were aligned and trimmed by using the Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al., 2016). There were a total of 1,341 positions in the final dataset and all positions containing gaps and missing data were eliminated. The best nucleotide substitution model were tested by using MEGA7 and the best model with the lowest Bayesian Information Criterion (BIC) scores were used for phylogenetic analysis. The 16s rRNA gene-based tree were constructed using the maximum likelihood method based on the Kimura 2-parameter model (K2+G) with 1,000 replicates (Kimura, 1980).

Moreover, the multilocus phylogenetic analysis (MLPA) also indicated that *dna*K gene can be a represented locus for genetic group classification. Therefore, a phylogenic tree of *dna*K genes were constructed by using the same software and condition as described above. Twenty *dna*K gene sequences of *F. columnare* and sequences from *F. psychrophilum* and *F. johnsoniae* were retrieved from NCBI database. The *dna*K gene sequences of 8 *F. columnare* isolates were extracted from the genomes from this study (Table 1). There were a total of 661 positions in the final dataset and all positions containing gaps and missing data were eliminated. The *dna*K gene-based tree were constructed using the maximum likelihood method based on the general time reversible model (GTR+G+I) with 1,000 replicates (Nei and Kumar, 2000).

3.3 Antimicrobial susceptibility tests

3.3.1 Disk diffusion test

Total 8 discs containing antimicrobial agents were used for testing including oxytetracycline (OT, 30 μg), amoxicillin (AML, 10 μg), florfenicol (FFC, 30 μg), and 4 kind of quinolone drugs including oxolinic acid (OA, 30 μg), nalidixic acid (NA, 10 μg), enrofloxacin (ENR, 10 μg), norfloxacin (NOR, 5 μg), and ciprofloxacin (CIP, 10 μg) (Oxoid, UK). The test were performed by using 1:5 diluted Mueller-Hilton agar (DMHA) as recommend in CLSI guideline VET03-A (CLSI, 2006). All *F. columnare* isolates were cultured in AO broth and incubated at 28 °C for 48 h with constant shaking at 160 rpm. Then, the bacterial suspension were adjusted the turbidity equal to 0.5 McFarland standard (1.5×10^8 CFU/ml) followed by streaking on 1:5 DMHA and antimicrobials discs were placed on agar (3-4 discs/plate). The reference strain, *Escherichia coli* ATCC 25922 were included for quality control. Then, all DMHA plates were incubated at 28 °C for 24 h before measure the inhibition zone diameters.

3.3.2 Minimum inhibitory concentration (MIC) testing

A broth microdilution method were used to determine the MIC value of 3 quinolone drugs (enrofloxacin, ciprofloxacin, oxolinic acid, and nalidixic acid) as descript in CLSI guideline VET04 (CLSI, 2014). The antimicrobial stock solution (10,240 μg/mL) of 4 quinolone drugs were prepared by dissolving in 1 M NaOH (oxolinic acid,

nalidixic acid, enrofloxacin) (Sigma-Aldrich, USA) and adjust by sterile distilled water in total volume 25 ml and store at -20 °C. The antimicrobial stocks were two-fold diluted into 10 concentration (from 0.125 to 64 μg/mL) before MIC testing. The 1:5 Diluted cation-adjusted Mueller-Hinton broth (CAMHB) were used for performing the test. The media were prepared by adding stock solution (10 mg/L) of calcium chloride and magnesium chloride to get 4 mg/L of Ca^{2+} and 2 mg/L of Mg^{2+} in the final concentration (CLSI, 2014; Gieseker et al., 2016).

F. columnare isolates were growth in AO broth and incubated at 28 °C with constant shaking at 160 rpm. After incubate 24 hours, the bacterial suspension were adjusted the turbidity equal to 0.5 McFarland standard (bacterial concentration 1.5 x 10^8 CFU/mL) and dilute 1:100 with 1:5 CAMHB to get bacterial concentration as 1.5 x 10^6 CFU/mL before inoculate into 96-well plates. Each well consisted bacterial suspension and antimicrobial solutions in 1:1 proportion (final bacterial concentration 7.5 x 10^5 CFU/mL). The wells which have only CAMHB and bacterial suspension without antimicrobial agents were negative and positive control respectively. The reference strain, *Escherichia coli* ATCC 25922, were used as quality control. The MIC values were interpreted by observing the lowest concentration of ABOs which have no visible growth of bacteria.

The isolates which have MIC value from 4 to 8 μg/mL and higher than 8 μg/mL were considered as quinolone sensitive (QS) and quinolone resistance (QR) group respectively (Mata et al., 2018). Four and three *F. columnare* isolates from QS and QR group were selected respectively and subjected to whole genome sequencing.

3.4. Genome sequencing, assembly, and annotation

Genomic DNA of 7 *F. columnare* isolates including CC1802, CC1803, CC1805, CC1808 from QS group and SP1802, SP1805, SP1809 from QR group were extracted with same method as described in 3.2 and DNA quantity and purity were measured by using Colibri microvolume spectrophotometer (TITEK BERTHOLD, Germany). Then, DNA were submitted to next-generation sequencing by Vishuo Biomedical (Thailand) LTD. The library preparations were constructed following the manufacturer's protocol (NEBNext® UltraTM DNA Library Prep Kit for Illumina®). Sequencing was performed on

the Illumina HiSeq platform, in a 2x150bp paired-end (PE) configuration. After that, poor quality raw reads were be filtered out by CLC Genomic Workbench. Then, the de novo assembly were performed by using SPAdes genome assembler to get contigs and combine into scaffolds by SSPACE. After that, the gap will be filled by GapFiller. Finally, the scaffolds will be annotated with Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org/rast.cgi) (Aziz et al., 2008; Overbeek et al., 2014).

3.5. Analysis of resistome

The genomes were blasted against two resistance genes databases. First, identify resistance genes, and SNP by using the *Comprehensive* Antibiotic Resistance Database *(*CARD*;* https://card.mcmaster.ca). CARD is an antibiotics resistance database which include resistance genes, their products, and associated phenotypes. The antibiotics resistance concepts of CARD are organized by the Antibiotic Resistance Ontology (ARO) which provide the information about resistance genes, resistance mechanisms, drug targets, antibiotics, and their relationships with ontology terms. In addition, CARD also have extended tool for predict antibiotic resistance genes and targeted drug classes called the Resistance Gene Identifier (RGI) (McArthur et al., 2013). All contigs length over than 500 bp were submitted to CARD server. The selection criteria were included perfect, strict, and loose hits. Perfect hits mean full length of our sequences match 100% to reference sequences in the database. Strict hits refer to the sequence that match over than the bit-score of the curated BLAST bit-score cutoff. Loose hits refer to the sequence that match below the bit-score of the curated BLAST bit-score cutoff which is provide detection of new AMR genes (McArthur et al., 2013).

Second, identify the acquired resistance genes by ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/). This is a database which obtained information of acquired antimicrobial resistance genes. A raw reads and contigs can be submit to the database and no minimum sequence length are required (Zankari et al., 2012). All contigs length over than 500 bp were submitted to ResFinder server. Both chromosomal mutations and acquired AMR genes were searched against the genomes.

CHAPTER IV

Results

4.1 Sample collection and bacterial isolation

All fishes showed a clinical signs of columnaris disease including gill necrosis and fin erosion. Only one fish was showed saddle back lesion which is the typical sign of this disease (Figure 1). Total 15 isolates including 8 isolates from Chachoengsao and 7 isolates from Samutprakarn were recovered (Table 1). The isolates from Chachoengsao and Samutprakarn were abbreviated as “CC” and “SP” respectively.

Table 1 *F. columnare* isolates used in this study

No.	Isolates	Region	Organ	Year isolated
1	CC1801	Chachoengsao	Skin	2018
2	CC1802	Chachoengsao	Skin	2018
3	CC1803	Chachoengsao	Skin	2018
4	CC1804	Chachoengsao	Skin	2018
5	CC1805	Chachoengsao	Skin	2018
6	CC1806	Chachoengsao	Skin	2018
7	CC1807	Chachoengsao	Skin	2018
8	CC1808	Chachoengsao	Skin	2018
9	SP1801	Samutprakarn	Gill	2019
10	SP1802	Samutprakarn	Gill	2019
11	SP1803	Samutprakarn	Skin	2019
12	SP1805	Samutprakarn	Skin	2019
13	SP1806	Samutprakarn	Gill	2019
14	SP1808	Samutprakarn	Gill	2019
15	SP1809	Samutprakarn	Skin	2019

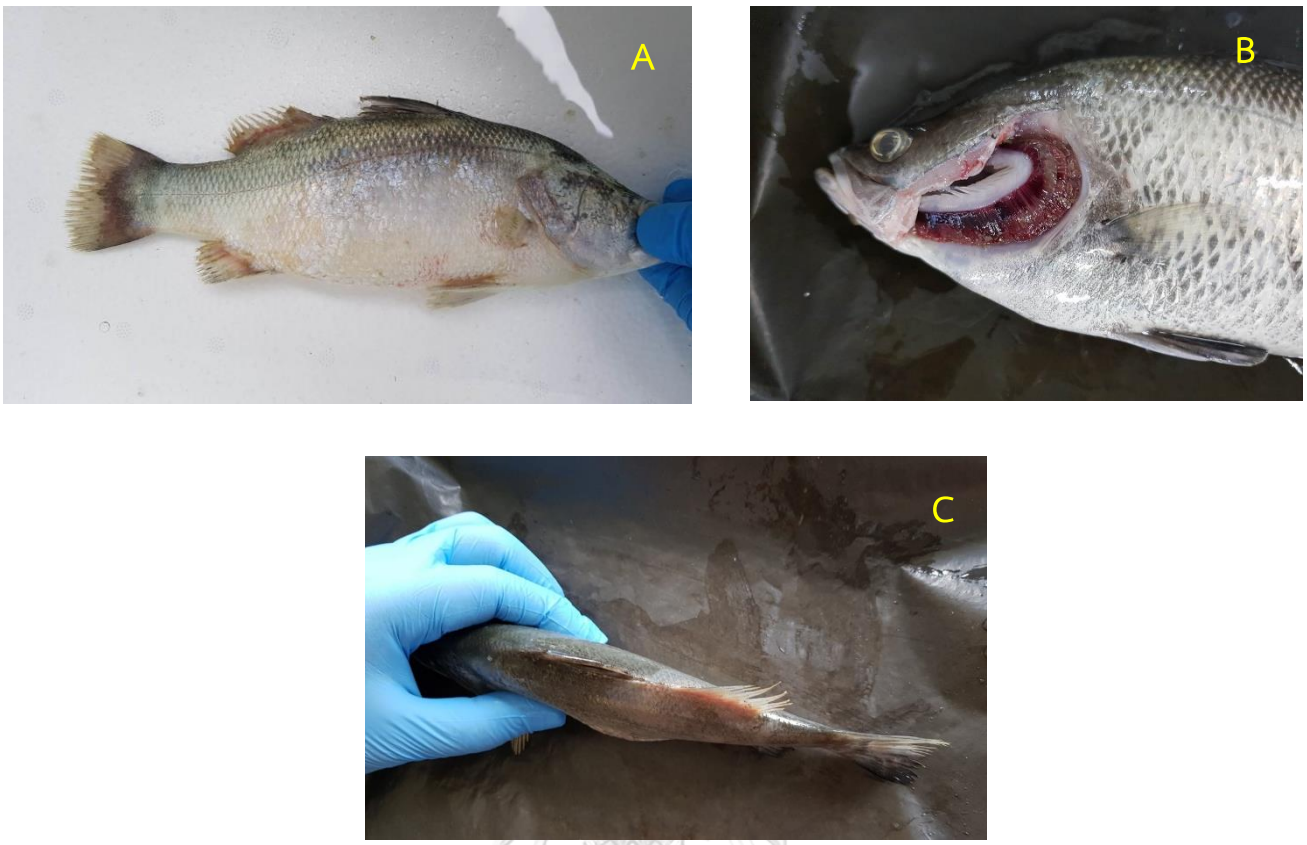

Figure 1 Asian sea bass with fin and tail rod (A), gill necrosis (B), and saddleback lesion (C)

4.2 Bacterial identification

All isolates produced yellow, flat, rhizoid, and strongly adherent colonies on AO agar. The bacteria were Gram-negative slender long rod-shaped and incapable of growing on TSA and MAC. Flexirubin pigment was presented when tested with 20% KOH. Positive results to motility, cytochrome oxidase, and catalase test and negative results to all decarboxylase tests were observed in all isolates (Table 2).

Table 2 Biochemical characteristics of all isolates in this study and *F. columnare*

Characteristics	Results	*F. columnare*
Gram	Negative	Negative
Morphology	Slender long rod	Slender long rod
Growth on TSA	-	-
Growth on MAC	-	-
Flexirubin pigment	+	+
Catalase	+	+
Cytochrome oxidase	+	+
Gliding motility	+	+
Decarboxylase test		
Arginine	-	-
Lysine	-	-
Ornithine	-	-

The PCR amplification of all isolates obtained one fragment of 400-500 bp by using species-specific primers (Figure 2). After that, the nucleotide sequence of fragments were blast against NCBI database and showed 99-100% identity to *F. columnare*. Thus, the bacterial characteristics and sequencing results indicated the bacterial species as *F. columnare*.

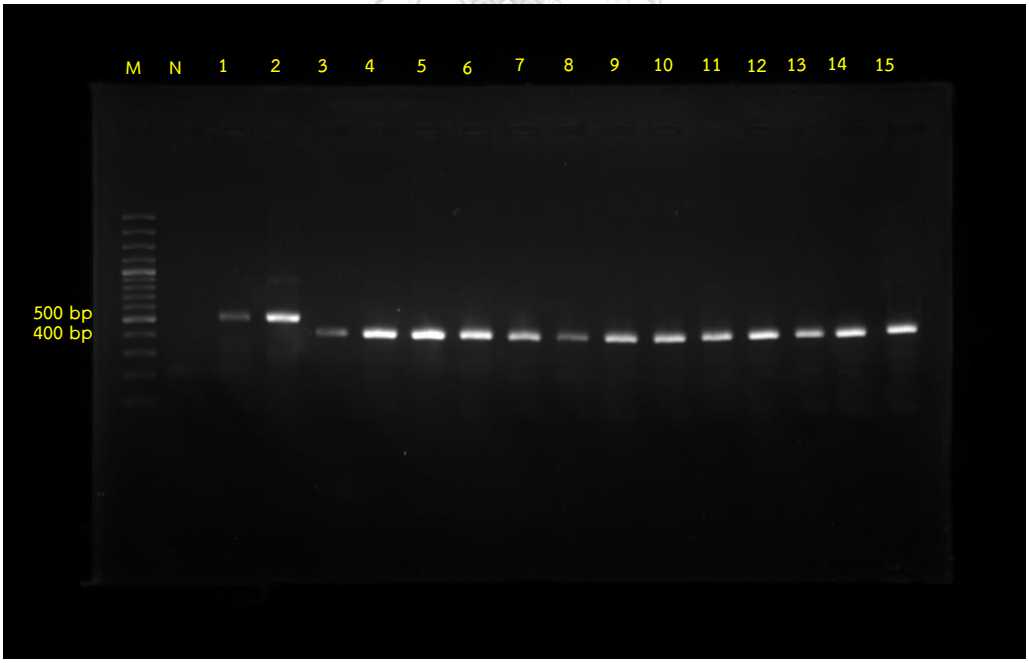

Figure 2 PCR amplification of all *F. columnare* isolates by using species-specific FCISRFL and FCISRR1 primers, Lane M: DNA marker, Lane N: negative control, Lane 1-15: SP1801, SP1802, SP1803, SP1805, SP1806, SP1808, SP1809, CC1801, CC1802, CC1803, CC1804, CC1805, CC1806, CC1807, and CC1808 respectively.

The 16s rRNA gene sequences of all *F. columnare* isolates were both download from public database and retrieved from genome in this study. The Kimura 2-parameter model (K2+G) was used for constructed the phylogenetic tree from 16s rRNA gene. From phylogenetic analysis, the outgroup (*F. psychrophilum* and *F. johnsoniae*) were distinct from *F. columnare* and 4 distinct genetic groups were generated among *F. columnare* isolates as supported by bootstrap values >80 (Figure 3). All Samutprakarn and Chachoengsao isolates were classified into genetic group 2 and 4 respectively.

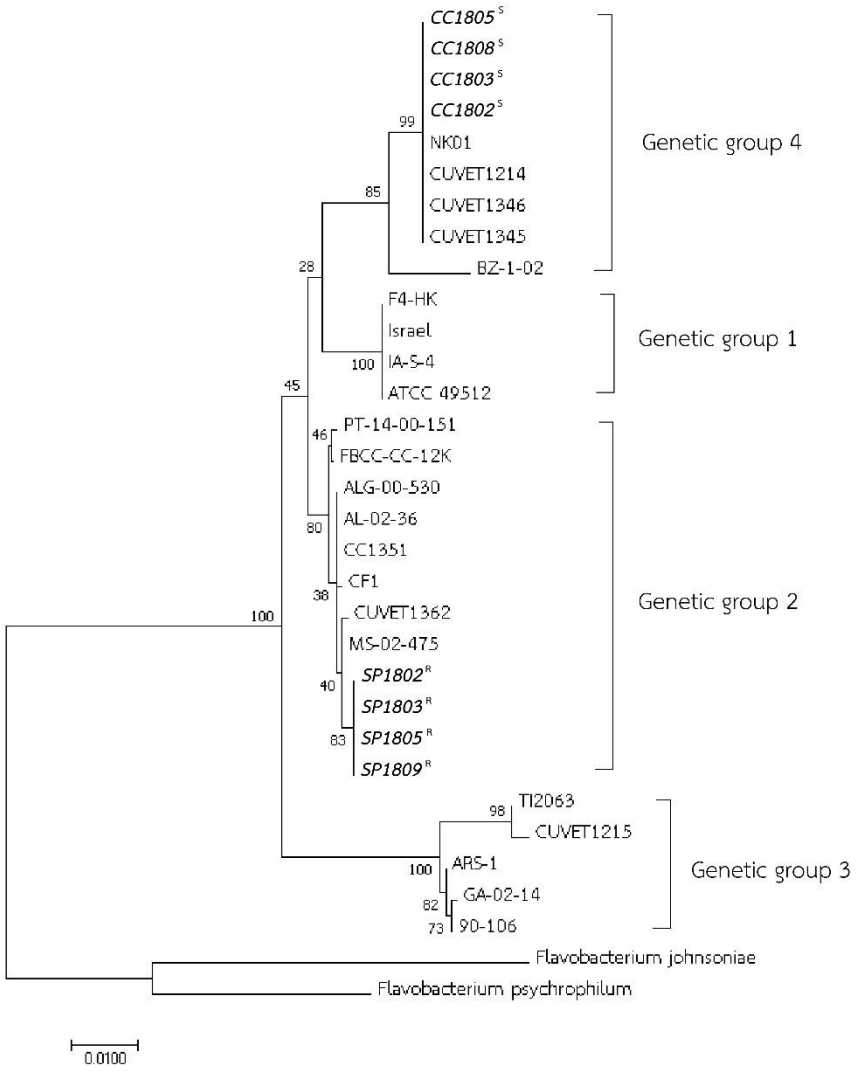

Figure 3 Phylogenetic tree based on 16s rRNA gene of 30 *F. columnare* isolates and rooted with *F. psychrophilum* and *F. johnsoniae*. All positions containing gaps and missing data were eliminated. There were a total of 1,341 positions in the final dataset. The bootstrap values were shown at the node of each branches. The *F. columnare* isolates of this study were presented in bold italic font.

R: quinolone resistant group, S: quinolone sensitive group

The *dna*K-based tree also constructed according to the study of phylogenetic relationship of *F. columnare* genetic diversity (Lafrentz et al, 2018). The general time reversible model (GTR+G+I) was used for constructed the tree. The phylogenetic analysis were indicated 4 genetic groups among *F. columnare* isolates which supported

by bootstrap values >99 and the outgroup were distinct from *F. columnare*. The results were correspond to 16s rRNA-based tree, Samutprakarn isolates were belong to genetic group 2 while Chachoengsao isolates were belong to genetic group 4 (Figure 4).

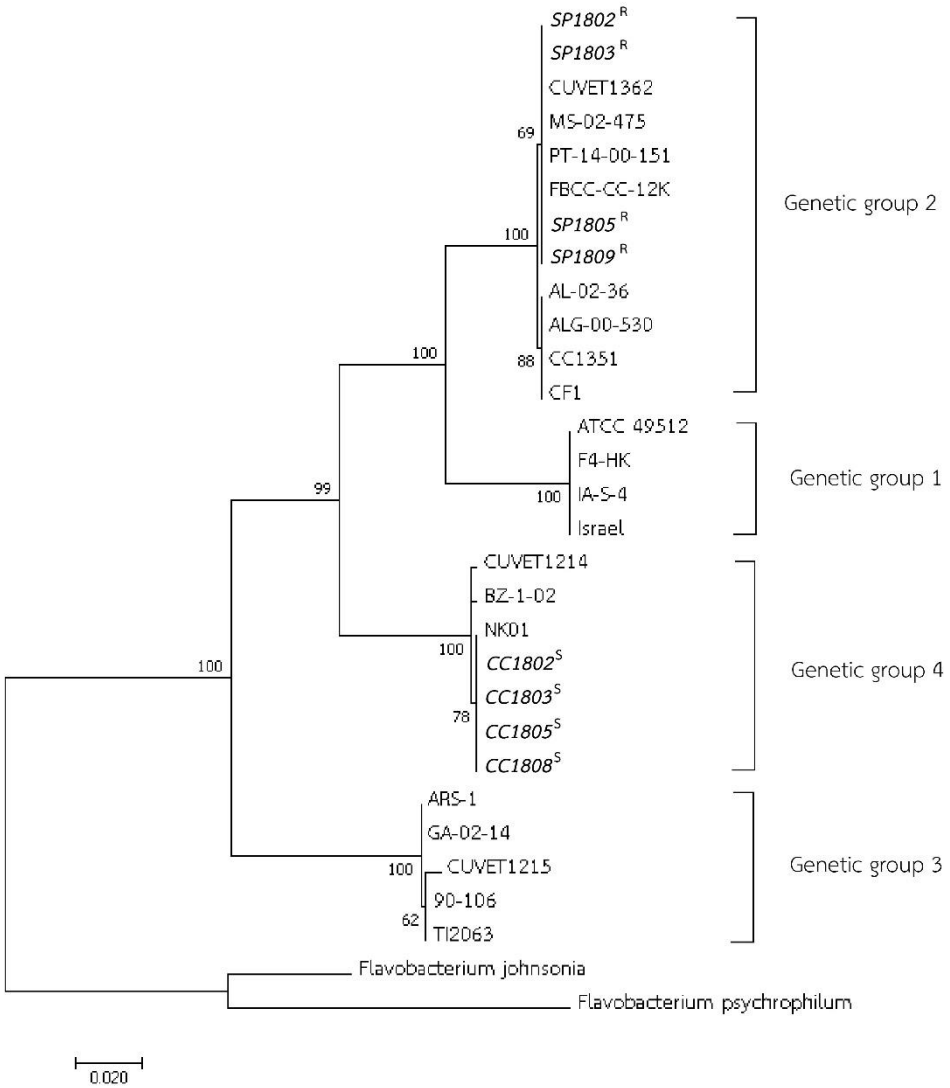

Figure 4 Phylogenetic tree based *dna*K gene of 28 *F. columnare* isolates and rooted with *F. psychrophilum* and *F. johnsoniae*. All positions containing gaps and missing data were eliminated. There were a total of 661 positions in the final dataset. The bootstrap values were shown at the node of each branches. The *F. columnare* isolates of this study were presented in bold italic font.

R: quinolone resistant group, S: quinolone sensitive group

4.3 Antimicrobial susceptibility tests

4.3.1 Disk diffusion test

Seven kinds of antimicrobial disk were used to evaluate a susceptibility patterns of *F. columnare* isolates. The antimicrobial susceptibility patterns from disk diffusion test were showed in table 3. The interpretations of inhibition zone diameter were based on breakpoint criteria from previous study (Mata et al., 2018). All isolates were sensitive to ciprofloxacin (CIP), enrofloxacin (ENR), oxytetracycline (OT), amoxicillin (AML), and florfenicol (FFC). Five isolates were resistant to both oxolinic acid (OA) and nalidixic acid (NA). Interestingly, all of isolates from Chachoengsao were susceptible to all tested antibiotics while most of Samutprakarn isolates were resistant to quinolone drugs. Five isolates (SP1801, SP1802, SP1803, SP1806, and SP1809) were resistant to oxolinic acid and six (SP1801, SP1802, SP1803, SP1805, SP1806, and SP1809) isolates were resistant to nalidixic acid. Among them, almost all isolates were resistant to both of antibiotics except SP1805 which only resistant to nalidixic acid. Then, all Chachoengsao (CC) and Samutprakarn (SP) isolates were referred to quinolone sensitive (QS) and quinolone resistant (QR) group respectively.

Table 3 Inhibition zone diameters and interpretations of *F. columnare* by disk diffusion method

Isolates	Inhibition zone diameters with millimeter unit (interpretation)*						
	OA	NA	ENR	CIP	OT	AML	FFC
CC1801	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1802	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1803	30 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1805	30 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1806	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1807	35 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1808	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
CC1809	30 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
SP1801	9 (R)	9 (R)	31 (S)	30 (S)	>40 (S)	>40 (S)	>40 (S)
SP1802	10 (R)	9 (R)	34 (S)	30 (S)	>40 (S)	>40 (S)	>40 (S)
SP1803	11 (R)	9 (R)	35 (S)	30 (S)	>40 (S)	>40 (S)	>40 (S)
SP1805	30 (S)	9 (R)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
SP1806	10 (R)	9 (R)	31 (S)	35 (S)	>40 (S)	>40 (S)	>40 (S)
SP1808	28 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)	>40 (S)
SP1809	11 (R)	9 (R)	32 (S)	34 (S)	>40 (S)	>40 (S)	>40 (S)

OA: oxolinic acid (30 μg), NA: nalidixic acid (10 μg), ENR: enrofloxacin (10 μg), CIP: ciprofloxacin (10 μg), OT: oxytetracycline (30 μg), AML: amoxicillin (10 μg), FFC: florfenicol (30 μg), * Interpretations were based on previous study (Mata et al., 2018)

4.3.2 Minimum inhibitory concentrations (MIC)

The MIC values of oxolinic acid, nalidixic acid and enrofloxacin were shown in table 5. The MIC values of oxolinic acid, nalidixic acid, and enrofloxacin were varied from 0.25 – 32 μg/mL, <0.125 - >64 μg/mL, and <0.125 – 1 μg/mL respectively. The MIC values of nalidixic acid was the widest range. The highest values was >64 μg/mL and presented in all SP isolates. According to the break points criteria from previous study, six isolates were categorized as a high MIC level group of oxolinic acid (Mata et

al., 2018). Conversely, all isolates shown susceptibility to enrofloxacin indicated by the low MIC values. The MIC results were complied with disk diffusion. Thus, All *F. columnare* isolates were divided into 2 groups same as disk diffusion method, the QS group were contained all CC isolates and 4 isolates including CC1802, CC1803, CC1805, and CC1808 were selected whereas the QR group were contained all SP isolates and 3 isolates including SP1802, SP1805, and SP1809 were selected for whole genome sequencing and further analysis.

Table 4 Minimum inhibitory concentrations (MIC) of quinolones

	MIC values (μg/mL) and MIC level*			
Isolates	OA	NA	ENR	Group
CC1801	0.25 (low)	2 (low)	<0.125 (low)	QS
CC1802	0.125 (low)	2 (low)	<0.125 (low)	QS
CC1803	0.25 (low)	0.25 (low)	<0.125 (low)	QS
CC1804	0.25 (low)	0.25 (low)	<0.125 (low)	QS
CC1805	0.25 (low)	4 (low)	<0.125 (low)	QS
CC1806	0.25 (low)	4 (low)	<0.125 (low)	QS
CC1807	0.25 (low)	4 (low)	<0.125 (low)	QS
CC1808	8 (high)	<0.125 (low)	0.25 (low)	QR
SP1801	32 (high)	>64 (high)	1 (low)	QR
SP1802	32 (high)	>64 (high)	1 (low)	QR
SP1803	8 (high)	>64 (high)	1 (low)	QR
SP1805	0.5 (low)	>64 (high)	0.25 (low)	QR
SP1806	8 (high)	>64 (high)	1 (low)	QR
SP1808	0.5 (low)	>64 (high)	0.5 (low)	QR
SP1809	16 (high)	>64 (high)	1 (low)	QR

OA: oxolinic acid, NA: nalidixic acid, ENR: erofloxacin, QS: quinolone sensitive, QR: quinolone resistant, * MIC breakpoint criteria was based on previous study (Mata et al., 2018)

4.4 Genome features

The genomes of 7 *F. columnare* isolates were sequenced by Illumina® Hiseq platform. The general features of all genome sequences were generated from RAST server and shown in table 6. Total 89 – 124 contigs (size >500 bp) were generated by SPAdes genome assembler. The genomes size were 3.1 – 3.3 Mb and contained GC 29.9 – 30.7%. Total number of 2952 – 3068 coding sequences (CDSs) and 62 – 70 RNAs were predicted.

Table 5 General features of *F. columnare* genomes

	CC1802	CC1803	CC1805	CC1808	SP1802	SP1805	SP1809
Genome size (bp)	3,387,405	3,386,767	3,387,732	3,386,568	3,186,628	3,187,301	3,188,351
GC content (%)	29.9	29.9	29.9	29.9	30.7	30.7	30.7
No. of contigs	91	89	88	91	118	124	124
No. of subsystem	243	243	243	243	244	244	244
No. of CDS	3068	3068	3067	3062	2941	2935	2952
No. of RNAs	68	69	70	66	67	62	62

4.5 Resistome analysis

4.5.1 Overview of antimicrobial resistance genes

The 7 whole genomes of *F. columnare* were submitted to CARD to predict AMR genes. Blast results shown that AMR gene profiles among *F. columnare* isolates in each group were similar. Total 165 and 173 genes were predicted from QS and QR group respectively and all of genes were loose hit according to resistance gene identifier (RGI) criteria. In sensitive group, the number of AMR genes against macrolide (20 hits), tetracyclin (20 hits), fluoroquinolone 20 (hits) were highest followed by glycopeptide (15) and peptide antibiotic (13). In resistant group, the number of AMR genes against tetracycline (24 hits) was highest followed by fluoroquinolone (22 hits), macrolide (22 hits), peptide (12 hits), and glycopeptide antibiotic (11 hits) respectively. The details of all AMR genes were shown in figure 5 and 6.

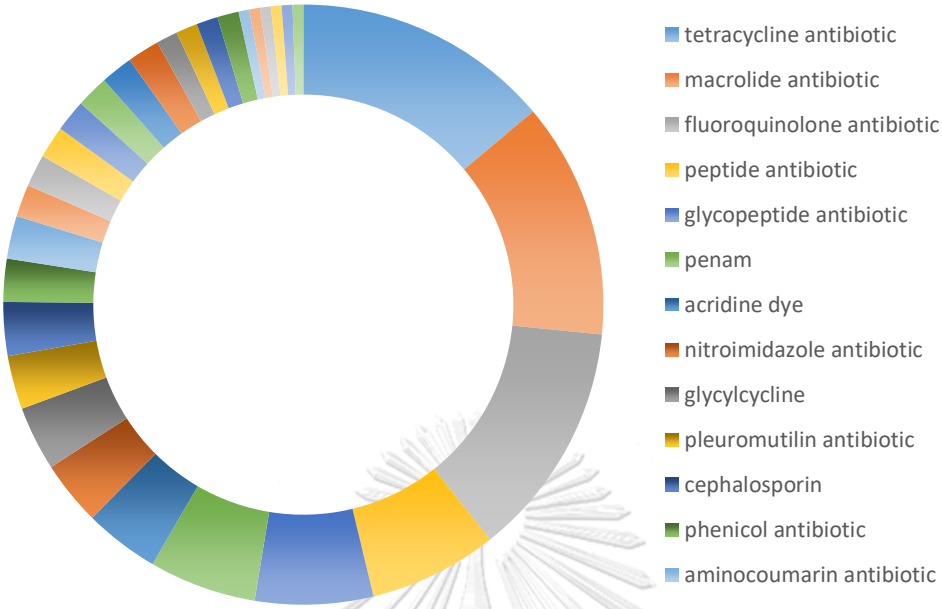

Figure 5 Antimicrobial resistance genes of sensitive group detected from CARD

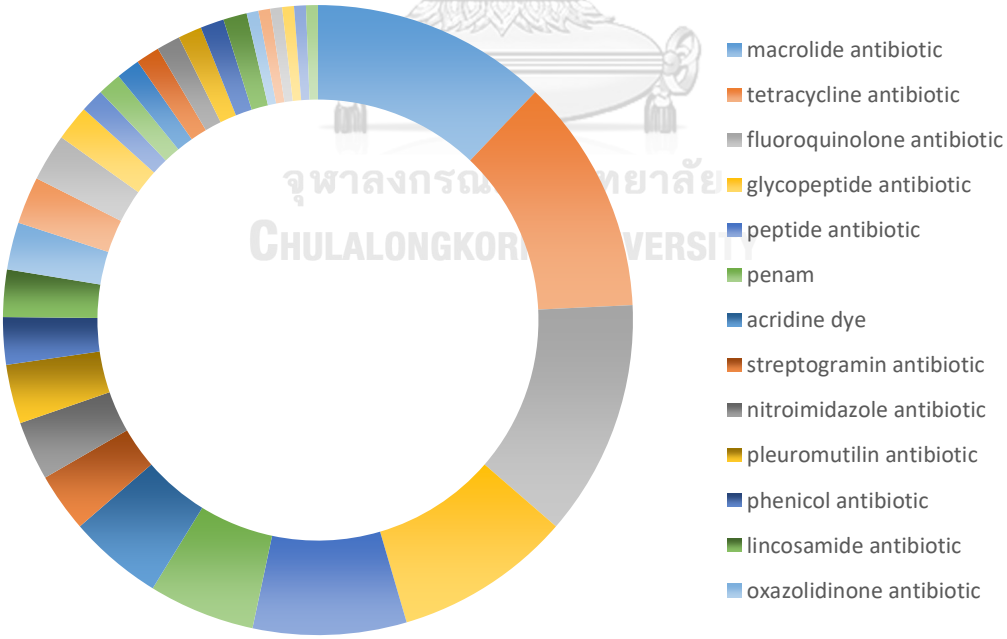

Figure 6 Antimicrobial resistance genes of resistant group detected from CARD

4.5.2 Quinolone resistance genes

Total 45 quinolone resistance (QR) genes were predicted from CARD (Table A2). Forty-three and Forty-two QR genes were predicted from sensitive and resistant group respectively. After that, all 45 QR gene sequences were retrieved from CARD and blasted against *F. columnare* genomes by using Blast2GO® software. The percentage of similarity were shown in heat map (Figure 7). The genes which have similarity more than 50% were considered as QR-associated gene. In conclusion, 15 and 28 QR genes were considered as QR-associated genes in sensitive and resistant isolates respectively (Table 6). Moreover, CARD also identified SNPs within QRDR genes which is conferring resistance to fluoroquinolone. One SNP (K134R) were identified within *par*C of CC isolates. Two SNPs were identified (K134R and S83F) were identified within *par*C of SP isolates.

Table 6 AMR genes which considered as quinolone resistant-associated genes

Genes	Sensitive group	Resistant group
*ade*N	✓	✓
*arl*R	✓	✓
*arl*S	✗	✓
*cme*A	✗	✓
*efr*A	✓	✓
*efr*B	✓	✓
*emr*B	✓	✓
*arl*R	✗	✓

FQR: fluoroquinolone resistance

*arl*S	✗	✓
*cme*A	✗	✓
*E.coli acr*R mutant	✗	✓
*efr*A	✗	✓
*efr*B	✗	✓
*emr*B	✗	✓
*evg*A	✓	✓
*evg*S	✓	✓
*gyr*B conferring FQR	✓	✓
*hmr*M	✗	✓
*mar*R mutant	✗	✓
*mex*B	✗	✓
P. aeruginosa CpxR	✓	✓
*par*C conferring FQR	✓	✓
*par*E confering FQR	✓	✓
*pat*B	✓	✓
*qac*A	✓	✓
*ram*R mutant	✗	✓
*sme*E	✓	✓
*sox*S mutant	✓	✓

Figure 7 Heat map with similarity percentage of all QR genes

SP1809	SP1805	SP1802	CC1808	CC1805	CC1803	CC1802	
49	49	49	47	47	47	47	*acr* S
46	46	46	47	47	47	47	*ade* L
67	67	67	72	72	72	72	*ade* N
54	54	54	58	58	58	58	*arl* R
54	54	54	50	50	50	50	*arl* S
48	48	48	48	48	48	48	*cde* A
68	68	68	44	44	44	44	*cme* A
44	44	44	44	44	44	44	*cme* B
45	45	45	43	43	43	43	*cme* C
48	48	48	49	49	49	49	CRP
44	44	44	43	43	43	43	E. cloacae acrA
49	49	49	50	50	50	50	*E. coli* acrA
56	56	56	52	52	52	52	*E. coli acr* R mutant
47	47	47	46	46	46	46	*efm* A
58	58	58	58	58	58	58	*efr* A
58	58	58	56	56	56	56	*efr* B
44	44	44	45	45	45	45	*emr* A
50	50	50	50	50	50	50	*emr* B
55	55	55	56	56	56	56	*evg* A
55	55	55	55	55	55	55	*evg* S
49	49	49	49	49	49	49	*gad* W
71	71	71	52	52	52	52	*gyr* B conferring FQR
50	50	50	49	49	49	49	*hmr* M
46	46	46	46	46	46	46	*lrf* A
51	51	51					*mar* R mutant
56	56	56	49	49	49	49	*mex* B
			45	45	45	45	*mex* F
43	43	43	42	42	42	42	*mex* H
45	45	45	45	45	45	45	*mex* V
51	51	51	55	55	55	55	*P. aeruginosa* CpxR
59	59	59	56	56	56	56	*par* C conferring FQR
59	59	59	59	59	59	59	*par* E confering FQR
79	79	79	76	76	76	76	*pat* B
48	48	48	49	49	49	49	*pmr* A
50	50	50	50	50	50	50	*qac* A
48	48	48	49	49	49	49	*qac* B
45	45	45	45	45	45	45	QepA2
37	37	37					QnrS2
41	41	41	41	41	41	41	QnrS4
40	40	40	39	39	39	39	QnrVC7
53	53	53	49	49	49	49	*ram* R mutant
			50	50	50	50	*sdi* A
			44	44	44	44	*sme* D

4.5.3 QRDR mutations

The web tool "ResFinder" was used to identify chromosomal mutation and acquired AMR genes. The results indicated that all isolates carried mutations within QRDR genes including *gyr*A, *gyr*B, *par*C, *par*E. Therefore, all 4 QRDR gene sequences (*gyr*A, *gyr*B, *par*C, *par*E) were retrieved from the whole genomes of all isolates and sequences of OXO-susceptible isolate CUVET 1213 under accession number KP403258 (*gyr*A), KP403263 (*gyr*B), KP403268 (*par*C) and KP403273 (*par*E) also included for mutation detection (Mata et al., 2018). All sequences were aligned and translated into amino acid by MEGA7 software. QRDR region on *gyr*A, *gyr*B, *par*C, and *par*E was located between nucleotide position 93 - 173, 291 - 441, 148 – 990, and 1099 – 1597 respectively. The detected mutations within QRDRs of on *gyr*A, *gyr*B, *par*C, and *par*E

were summarized in table 7 which included the results from previous study (Mata et al., 2018). The mutation at position 83, Ser (TCT) to Phe (TTT), in *gyr*A was founded in SP1802 (OA MIC = 8 μg/mL, NA MIC = >64 μg/mL) and SP1809 (OA MIC = 16 μg/mL, NA MIC = >64 μg/mL) (Figure 8). All QR isolates were carried point mutations within *gyr*B at position 370, Ser (CTA) to Asn (TTA), and *par*E at position 389, Arg (AGA) to Lys (AAG) (Figure 9, 10). For QS isolates, all of them carried double point mutation with *par*C at position 88, His (CAC) to Tyr (TAC), and 183, Ala (GCA) to Pro (CCA) (Figure 11, 12).

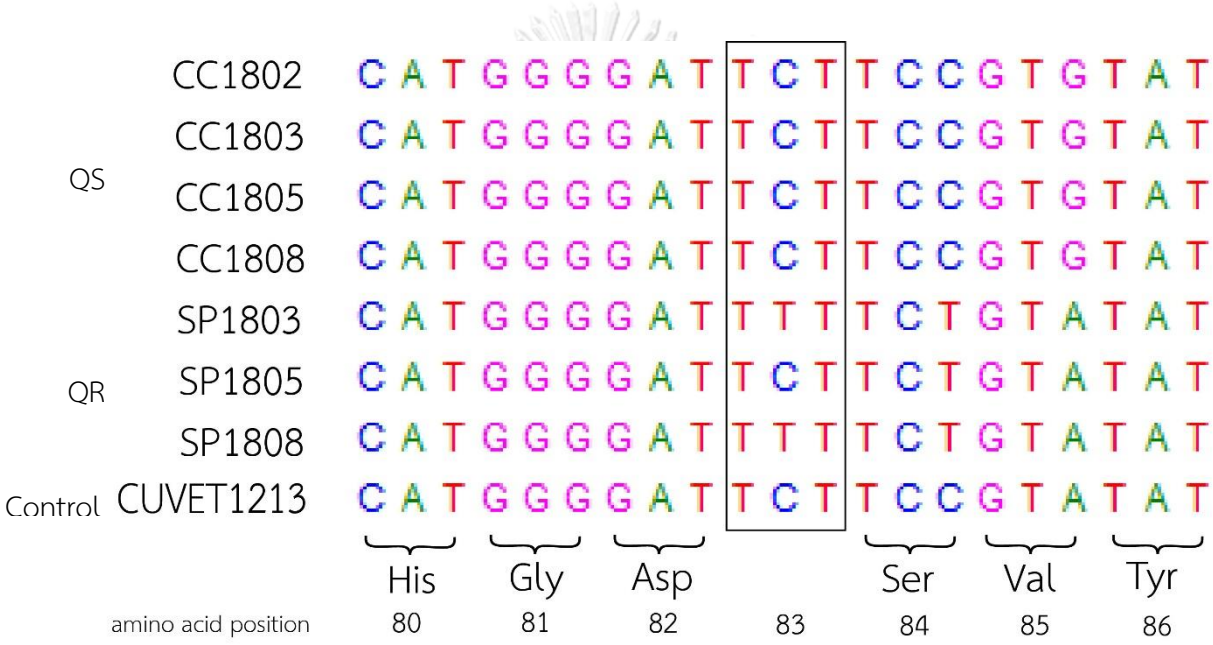

Figure 8 Multiple sequence alignment of QRDR of *gyr*A showed point mutation at position 83 (TCT→TTT)

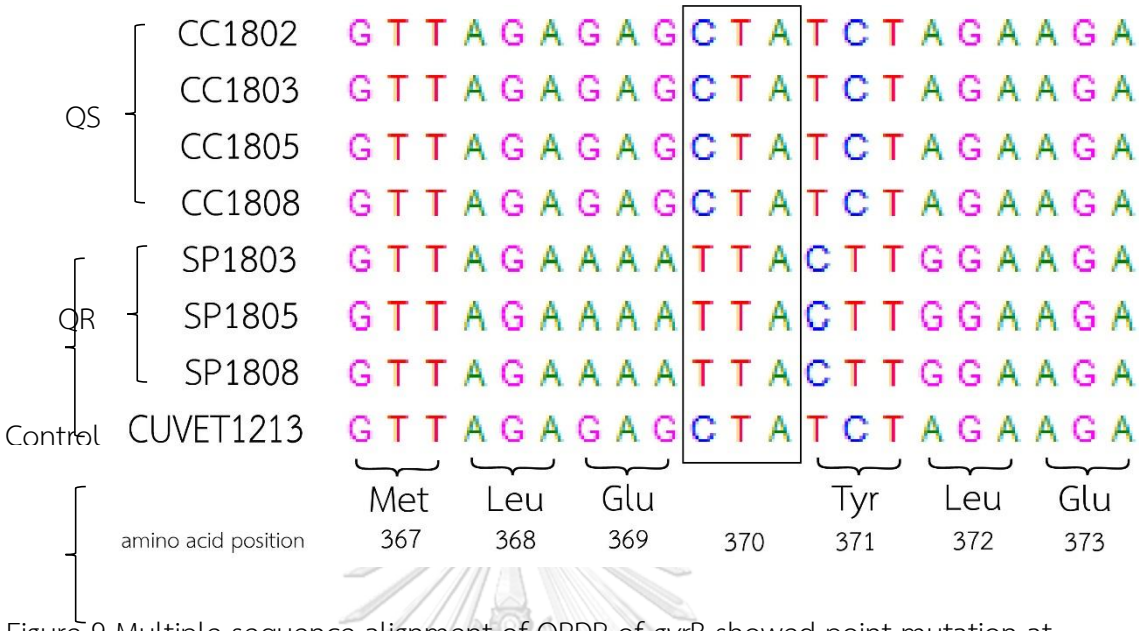

Figure 9 Multiple sequence alignment of QRDR of *gyr*B showed point mutation at position 370 (CTA→TTA)

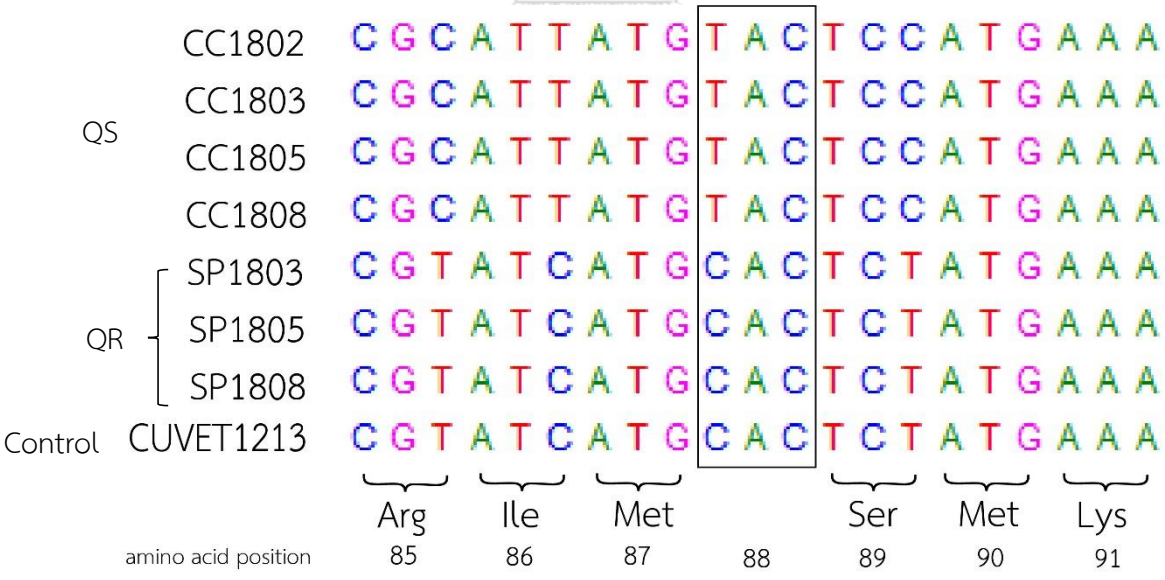

Figure 10 Multiple sequence alignment of QRDR of *par*C showed point mutation at position 88 (CAC→TAC)

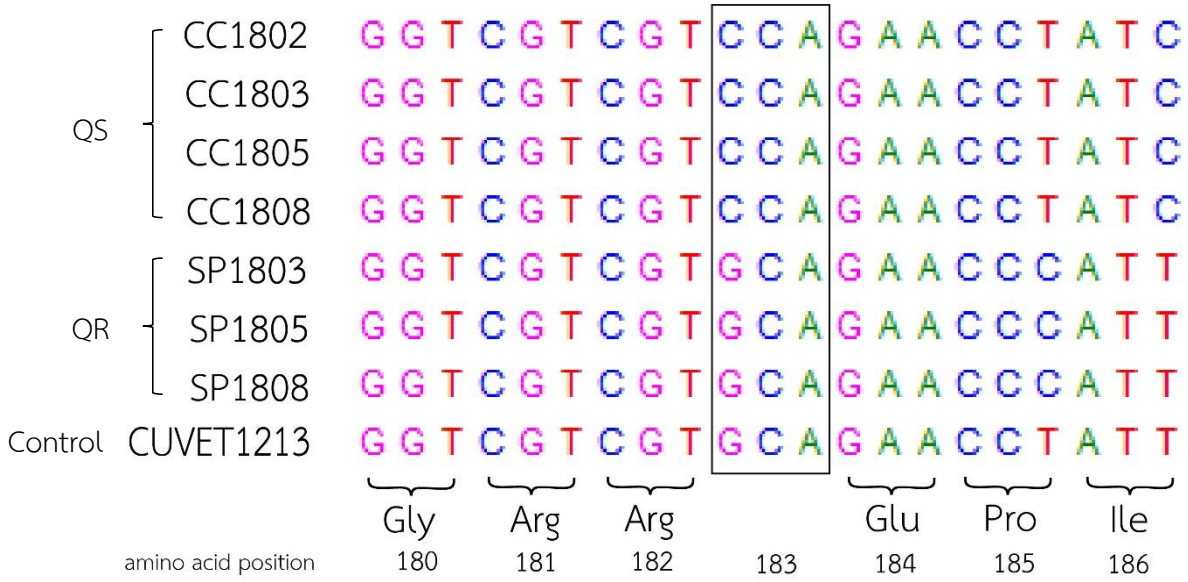

Figure 11 Multiple sequence alignment of QRDR of *par*C showed point mutation at position 183 (GCA→CCA)

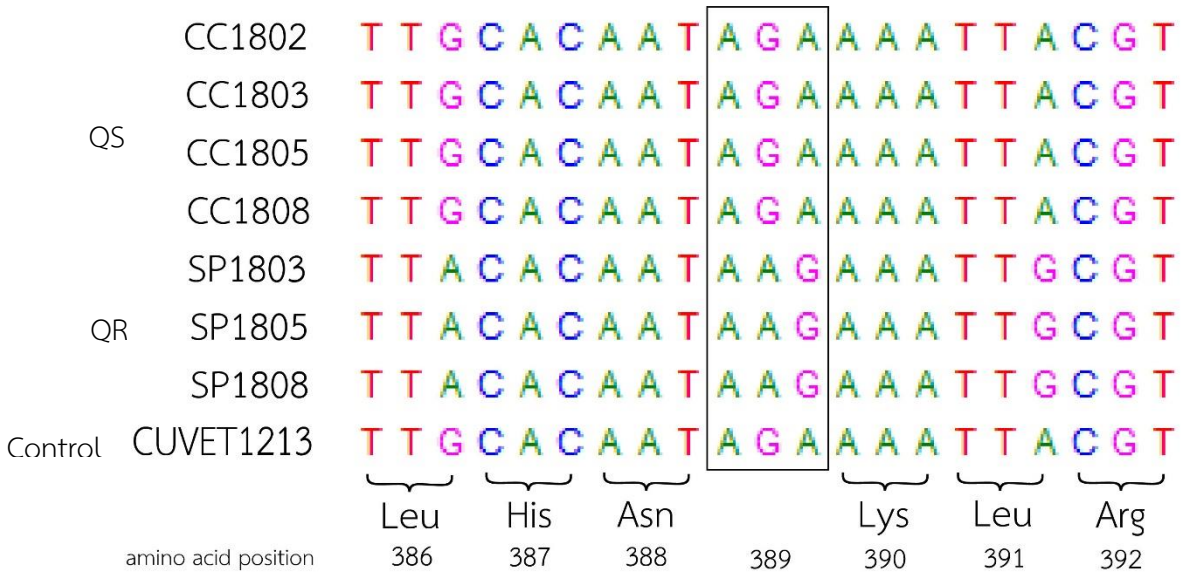

Figure 12 Multiple sequence alignment of QRDR of *par*E showed point mutation at position 389 (AGA→AAG)

Table 7 Detected mutations within QRDR region of *gyr*A, *gyr*B, *par*C, and *par*E genes

Isolates	MIC (μg/mL)			Mutation in QRDR			
	OA	NA	ENR	*gyr*A	*gyr*B	*par*C	*par*E
CUVET 1213	<0.125	-	-	-	-	-	-
CC1802	0.125	2	<0.125	-	-	His⟶88Tyr Ala⟶183Pro	-
CC1803	0.25	0.25	<0.125	-	-	His⟶88Tyr Ala⟶183Pro	-
CC1805	0.25	4	<0.125	-	-	His⟶88Tyr Ala⟶183Pro	-
CC1808	8	<0.125	0.25	-	-	His⟶88Tyr Ala⟶183Pro	-
SP1802	8	>64	1	Ser83⟶Phe	Ser370⟶Asn	-	Arg⟶389Lys
SP1805	0.5	>64	0.25	-	Ser370⟶Asn	-	Arg⟶389Lys
SP1809	16	>64	1	Ser83⟶Phe	Ser370⟶Asn	-	Arg⟶389Lys
CUVET 1361	2	-	-	-	-	His⟶87Tyr	-
CUVET 1343	4	-	-	Ser83⟶Phe	-	His⟶87Tyr	-
CUVET 1374	8	-	-	Ser83⟶Ala	-	His⟶87Tyr	-
CUVET 1339	16	-	-	Ser83⟶Ala	-	-	-

Ser: serine, Phe: phenylalanine, Asn: asparagine, His: histidine, Arg: arginine, Lys: lysine, Pro: prolene

CHAPTER V

Discussion

Columnaris disease is a disease causing by Gram negative bacteria called *F. columnare*. This bacteria can infect a wide range of freshwater fish species and threaten to aquaculture production. In Thailand, *F. columnare* has been reported in Nile tilapia, red tilapia, and catfish (Dong et al., 2015a; Dong et al., 2015b). Recently, this study firstly reported the occurrence of *F. columnare* in freshwater culturing Asian sea bass in Thailand. The clinical signs and phenotypic characteristics of *F. columnare* isolated from Asian sea bass were similar to the *F. columnare* isolated from tilapia in Thailand (Dong et al., 2015a). Phylogenetic analysis indicated that *F. columnare* isolated from Asian sea bass were belong to genetic group 2 and 4 which is the dominant genetic group of *F. columnare* isolates in Thailand.

Quinolones is one of the antibiotics that approved for use in Thai aquaculture and commonly used to treat columnaris disease such as OA and ENR. The antimicrobial susceptibility patterns of *F. columnare* isolated from Thai red tilapia in 2013 were shown resistant to quinolone drugs including OA (26%) and NA (15%) (Mata et al., 2018). Same as this study, resistance to first generation of quinolone were founded including OA (33.33%) and NA (40%). The MIC values of quinolones including OA, NA, and ENR were determined according to CLSI guideline VET04 (CLSI, 2014). The MIC values of NA shown the highest values (>64 μg/mL) followed by OA (32 μg/mL) which 1-2 folds higher than previous study (Mata et al., 2018). These results indicated that quinolone resistant in *F. columnare* seem to be common in Thailand. Therefore, first generation of quinolone may not treat the disease effectively. However, none of resistance to the others drug class such as fluoroquinolone, tetracycline, beta-lactam, and fenicol were founded. These kinds of drugs can be another choices.

The genomes of *F. columnare* which represented sensitive and resistant to quinolones were sequenced and blasted against AMR databases. The results from

CARD were predicted total AMR 45 genes (Table A2). Each group contained almost the same AMR genes and only 2 -3 genes were different. Therefore, all genes were blasted against *F. columnare* genomes via Blast2Go® software to determine similarity percentage and heat map were generated (Figure 7). From the blasted results, total 15 and 28 QR-associated genes which have identity more than 50% were considered as QR-associated genes of sensitive and resistant group respectively (Table 6). Although first blast results from CARD were almost similar between 2 groups, the resistant group carried higher number when investigated the % identity. Most of QR-associated genes identified in *F. columnare* genomes belonged to resistance-nodulation-cell division (RND) antibiotic efflux pump.

Resistance-Nodulation-Division (RND) are the family of efflux pump which is the transporter of metabolites, organic compounds, or antimicrobial substance. This efflux pump family are commonly found in Gram negative bacteria and consist of 3 parts called tripartite. These complex are including inner membrane protein, outer membrane protein, and membrane fusion protein (Daury et al., 2016). From the previous studies, RND tripartite in *E. coli* and *P. aeruginosa* are AcrAB-TolC and MexAB-OprM respectively (Nikaido, 2009). Besides, the study of *F. columnare* Pf1 complete genome sequences also identified 32 RND-type efflux pump related genes related with aminoglycoside resistant (Zhang et al., 2017).

Moreover, many QR genes with mutation conferring quinolone resistant were predicted such as *acr*R, *ram*R, *sox*S, *gyr*A, *gyr*B, *par*C, and *par*E. One of the main mechanisms of quinolone resistance is target-site gene mutation. The quinolone targets are including DNA gyrase subunit A, DNA gyrase subunit B, topoisomerase IV subunit A, and topoisomerase IV subunit B which encoded by *gyr*A, *gyr*B, *par*C, and *par*E gene respectively. Several studies have been reported the prevalence of mutations within QRDR region of quinolone target genes that mutations within *gyr*A and *par*C are the most frequently founded and these mutations affect the level of resistant significantly. (Nakano et al., 1997; Eaves et al., 2004; Minarini and Darini, 2012;

Mata et al., 2018). Therefore, these genes sequences were retrieved from the genomes and aligned to gain more insight into mutations within quinolone target genes.

One point mutation at position 83 (Ser83⟶Phe) were detected on *gyr*A QRDR of 2 resistant isolates (SP1802 and SP1809). Interestingly, the mutations not detected in SP1805 which belonged to resistant group. All resistant isolates were performed the same MIC values of NA (>64 μg/mL) but SP1805 have different in the MIC of OA which is 5 - 6 folds lower than the others. This position were commonly founded in many Gram negative bacteria and conferred resistant to fluoroquinolone (Hallett and Maxwell, 1991; Nawaz et al., 2015; Campioni et al., 2017). The amino acid position between 67 and 106 is located on N-terminal of *gyr*A which is the quinolones binding pocket (Yoshida et al., 1990). Thus, alteration of amino acid at amino acid position 83 especially the change of amino acid group from polar (Ser) to nonpolar (Phe) could strongly effect the binding ability of quinolone. Besides, all resistant isolates were carried one mutation at position 370 (Ser370⟶Asn) and 388 (Arg388⟶Lys) on *gyr*B and *par*E QRDR respectively (Table9). The mutation at position 388 was reported in high-level resistance to CIP *Pseudomonas auruginosa* (Wang et al., 2014). Since the study of Mata et al. proposed that mutations in *gyr*B or *par*E may not involve with resistant activity against oxolinic acid in *F. columnare*, this study founded that mutations in these genes were effect the rise of MIC values to nalidixic acid. Double mutation within *par*C (His88⟶Tyr, Ala183⟶Pro) were detected in all QS isolates. The mutation at amino acid position 80 were reported in several studies. Some of them suggested that mutations in *par*C are not the primary target of fluoroquinolone but could enhance the resistant activity when combine with mutations in another genes (Bagel et al., 1999; Salma et al., 2013). The previous study of Mata et al proposed that mutations in *par*C are the major target of quinolone. However, most of isolates from this study were carried mutations in both gyrA and parC. Only one isolates carried mutation in *par*C and also showed low MIC values (OA MIC = 2 μg/mL) (Table 7). In this study all QS isolates were carried none of mutations in the others genes except

*par*C and also presented lower MIC values to quinolones. These results indicated that *par*C are not the primary target of quinolone in *F. columnare*.

Quinolone resistance in *F. columnare* is based on 2 main mechanisms; reduce drug accumulation and alter the drug targets. Overexpression of efflux pumps and reduce membrane permeability commonly founded in multidrug resistant bacteria to reduce drug accumulation. *F. columnare* resistant isolates carried a few kinds of efflux pump family and mutated some genes that involve with reduced permeability to antibiotic. To alter quinolone targets, mutations usually occur on QRDR of quinolone target genes. In this study, novel mutation within QRDRs of *gyr*B (Ser370⟶Asn) and *par*C (Ala183⟶Pro) were discovered and *gyr*A seem to be the primary target of quinolone. However, further research of protein structure and how the drug bind to target should be established to investigate schematic protein interaction model that may affect quinolone resistant activity.

Conclusion

This study performed a characterization and susceptibility patterns of *F. columnare* isolated from Asian sea bass. The phenotypic characters were similar to *F. columnare* isolated from others freshwater fish species. The susceptibility patterns

indicated that most of *F. columnare* were resistant to quinolones including OA and NA and none of isolates were resistant to fluoroquinolone (CIP, ENR). The MIC values from broth microdilution method were related with disk diffusion that most of isolates performed high-level MIC to OA and NA. The analysis of overall quinolone resistance-associated genes shown that many efflux pump genes were involved with quinolone resistance mechanisms and mutations within QRDRs of *gyr*A are the primary target of quinolone in *F. columnare*.

Advantages of Study

1. Monitoring the antimicrobial resistance situation and giving awareness of antimicrobial use in Thai aquaculture.

2. Provide the information of quinolone resistance mechanisms which helpful for setting prevent and control strategies for antimicrobial resistance.

REFERENCES

VITA

NAME	Putita Chokmangmeepisarn
DATE OF BIRTH	1 Feb 1994
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Pathobiology program, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University
HOME ADDRESS	Bangkok, Thailand
PUBLICATION	Poster presentation in the topic of Characterization and Antimicrobial susceptibility of F. columnare isolated from freshwater culturing Asian sea bass (Lates Calcarifer) in Thailand in the The 18th Chulalongkorn University Veterinary Conference 2019 (CUVC2019) Proceeding in the topic of Characterization and Antimicrobial susceptibility of F. columnare isolated from freshwater culturing Asian sea bass (Lates Calcarifer) in Thailand in the Thai Journal of Veterinary Medicine Vol 49 No 1 (2019)

Aldred KJ, Kerns RJ and Osheroff N 2014. Mechanism of quinolone action and resistance. Biochem. 53(10): 1565-1574.

Ali A 2013. Microbial Comparative Genomics: An Overview of Tools and Insights Into The Genus *Corynebacterium*. J Bacteriol & Parasitol. 04(02).

Anacker RL and Ordal EJ 1955. Study of a Bacteriophage Infecting the *Myxobacterium Chondrococcus Columnaris*. J Bacteriol. 70(6): 738-741.

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A and Zagnitko O 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics. 9: 75.

Bader JA, Shoemaker CA and Klesius PH 2003. Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. J Microbiol Methods. 52(2): 209-220.

Bagel S, Hullen V, Wiedemann B and Heisig P 1999. Impact of *gyr*A and *par*C mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob Agents Chemother. 43(4): 868-875.

Bearden DT and Danziger LH 2001. Mechanism of action of and resistance to quinolones. Pharmacotherapy. 21(10 Pt 2): 224S-232S.

Bernardet J and Bowman J 2006. The genus *Flavobacterium*. In: The Prokaryotes: A Handbook on the Biology of Bacteria: Volume 7: Proteobacteria: Delta and Epsilon Subclasses. M Dworkin, S Falkow, E Rosenberg, KH Schleifer, and E Stackebrandt (eds). New York: Springer Science+Business Media, LLC. 481-531.

Bootsma R and Clerx JPM 1976. Columnaris disease of cultured carp *Cyprinus carpio* L. Characterization of the causative agent. Aquaculture. 7(4): 371-384.

Campioni F, Souza RA, Martins VV, Stehling EG, Bergamini AMM and Falcao JP 2017. Prevalence of *gyr*A Mutations in Nalidixic Acid-Resistant Strains of *Salmonella Enteritidis* Isolated from Humans, Food, Chickens, and the Farm Environment in Brazil. Microb Drug Resist. 23(4): 421-428.

Clark SE, Jude BA, Danner GR and Fekete FA 2009. Identification of a multidrug efflux pump in *Flavobacterium johnsoniae*. Vet Res. 40(6): 55.

CLSI 2006. Method for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals. CLSI document VET03-A. In: Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

CLSI 2014. Methods for Broth Dilution Suscecptibility Testing of Bacteria Isolated From Aquatic Animals. CLSI document VET04-A2. In: Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

Daury L, Orange F, Taveau JC, Verchere A, Monlezun L, Gounou C, Marreddy RK, Picard M, Broutin I, Pos KM and Lambert O 2016. Tripartite assembly of RND multidrug efflux pumps. Nat Commun. 7: 10731.

Davis H 1922. A new bacterial disease of fresh-water fishes. Vol 38. In: United States Bureau of Fisheries Bulletin, Washington DC.

Declercq AM, Boyen F, Van den Broeck W, Bossier P, Karsi A, Haesebrouck F and Decostere A 2013a. Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. J Fish Dis. 36(1): 45-55.

Declercq AM, Haesebrouck F, Van den Broeck W, Bossier P and Decostere A 2013b. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Vet Res. 44: 27.

Decostere A, Haesebrouck F and Devriese LA 1997. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. J Clin Microbiol. 35(1): 322-324.

DOF 2017. Fisheries Statistics of Thailand 2015. Vol 55. In: Department of Fisheries.

Dong HT, LaFrentz B, Pirarat N and Rodkhum C 2015a. Phenotypic characterization and genetic diversity of *Flavobacterium columnare* isolated from red tilapia, Oreochromis sp., in Thailand. J Fish Dis. 38(10): 901-913.

Dong HT, Nguyen VV, Phiwsaiya K, Gangnonngiw W, Withyachumnarnkul B, Rodkhum C and Senapin S 2015b. Concurrent infections of *Flavobacterium columnare* and

Edwardsiella ictaluri in striped catfish, *Pangasianodon hypophthalmus* in Thailand. Aquaculture. 448: 142-150.

Eaves DJ, Randall L, Gray DT, Buckley A, Woodward MJ, White AP and Piddock LJ 2004. Prevalence of mutations within the quinolone resistance-determining region of *gyr*A, *gyr*B, *par*C, and *par*E and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrob Agents Chemother. 48(10): 4012-4015.

Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden MTG, Hopkins KL, Iredell J, Kahlmeter G, Koser CU, MacGowan A, Mevius D, Mulvey M, Naas T, Peto T, Rolain JM, Samuelsen O and Woodford N 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect. 23(1): 2-22.

Everett MJ, Jin YF, Ricci V and Piddock LJ 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrob Agents Chemother. 40(10): 2380-2386.

FAO 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome.

FDA 2018. "Subject: Extralabel Use and Antimicrobials" (online). Available: https://www.fda.gov/animalveterinary/safetyhealth/antimicrobialresistance/ucm421527.htm.

Gieseker CM, Crosby TC, Mayer TD, Bodeis SM and Stine CB 2016. Development of Similar Broth Microdilution Methods to Determine the Antimicrobial Susceptibility of *Flavobacterium columnare* and *F. psychrophilum*. J Aquat Anim Health. 28(1): 27-38.

Hallett P and Maxwell A 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. Antimicrob Agents Chemother. 35(2): 335-340.

Hawkey PM 2003. Mechanisms of quinolone action and microbial response. J Antimicrob Chemother. 51 Suppl 1: 29-35.

Holt RA 1987. *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. Oregon State University.

Jeffrey S and Christian M 1996. The quinolone antibiotics. Infectious disease update. 3(3): 87-92.

Kimura M 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16(2): 111-120.

Kumar S, Stecher G and Tamura K 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 33(7): 1870-1874.

Kungvankij P TL, Pudadera BJ, and Potesta IO 1985. Training manual biology and culture of sea bass (*Lates calcarifer*). In: Network of Aquaculture Centres in Asia, Bangkok, Thailand.

LaFrentz BR, Garcia JC, Dong HT, Waldbieser GC, Rodkhum C, Wong FS and Chang SF 2017. Optimized reverse primer for 16S-RFLP analysis and genomovar assignment of *Flavobacterium columnare*. J Fish Dis. 40(8): 1103-1108.

LaFrentz BR, Garcia JC, Waldbieser GC, Evenhuis JP, Loch TP, Liles MR, Wong FS and Chang SF 2018. Identification of Four Distinct Phylogenetic Groups in *Flavobacterium columnare* With Fish Host Associations. Front Microbiol. 9: 452.

Mata W, Putita C, Dong HT, Kayansamruaj P, Senapin S and Rodkhum C 2018. Quinolone-resistant phenotype of *Flavobacterium columnare* isolates harbouring point mutations both in gyrA and parC but not in *gyr*B or *par*E. J Glob Antimicrob Resist. 15: 55-60.

McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJ, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T and Wright GD 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother. 57(7): 3348-3357.

Minarini LA and Darini AL 2012. Mutations in the quinolone resistance-determining regions of *gyr*A and *par*C in *Enterobacteriaceae* isolates from Brazil. Braz J Microbiol. 43(4): 1309-1314.

Nakano M, Deguchi T, Kawamura T, Yasuda M, Kimura M, Okano Y and Kawada Y 1997. Mutations in the *gyr*A and *par*C genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 41(10): 2289-2291.

Nawaz M, Sung K, Kweon O, Khan S, Nawaz S and Steele R 2015. Characterisation of novel mutations involved in quinolone resistance in *Escherichia coli* isolated from imported shrimp. Int J Antimicrob Agents. 45(5): 471-476.

Nei M and Kumar S 2000. Molecular evolution and phylogenetics. In: Oxford University Press, Oxford ; New York. xiv, 333 p.

Nikaido H 2009. Multidrug resistance in bacteria. Annu Rev Biochem. 78: 119-146.

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F and Stevens R 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 42(Database issue): D206-214.

Pacha RE and Ordal EJ 1967. Histopathology of experimental columnaris disease in young salmon. Journal of Comparative Pathology. 77(4): 419-423.

Pan XS, Yague G and Fisher LM 2001. Quinolone resistance mutations in *Streptococcus pneumoniae* GyrA and ParC proteins: mechanistic insights into quinolone action from enzymatic analysis, intracellular levels, and phenotypes of wild-type and mutant proteins. Antimicrob Agents Chemother. 45(11): 3140-3147.

Reller LB, Weinstein M, Jorgensen JH and Ferraro MJ 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. Clin Infec Dis. 49(11): 1749-1755.

Rico A, Knopperts F, Khatikarn J, Satapornvanit K and J Van den Brink P 2012. Ecological risk assessment of antibiotics used in tilapia cage aquaculture: A case study in the Tha Chin River (Central Thailand). AQUA 2012, Prague, Czech Republic.

Riviere JE and Papich MG 2009. Veterinary Pharmacology and Therapeutics. In: Wiley.

Salma R, Dabboussi F, Kassaa I, Khudary R and Hamze M 2013. *gyr*A and *par*C mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* from Nini Hospital in north Lebanon. J Infect Chemother. 19(1): 77-81.

Santos L and Ramos F 2018. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int J Antimicrob Agents. 52(2): 135-143.

Shoemaker CA, Olivares-Fuster O, Arias CR and Klesius PH 2008. *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*). Vet Microbiol. 127(3-4): 353-359.

Suomalainen LR, Kunttu H, Valtonen ET, Hirvela-Koski V and Tiirola M 2006. Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland. Dis Aquat Organ. 70(1-2): 55-61.

Tekedar HC, Karsi A, Reddy JS, Nho SW, Kalindamar S and Lawrence ML 2017. Comparative Genomics and Transcriptional Analysis of *Flavobacterium columnare* Strain ATCC 49512. Front Microbiol. 8: 588.

Tusevljak N, Dutil L, Rajic A, Uhland FC, McClure C, St-Hilaire S, Reid-Smith RJ and McEwen SA 2013. Antimicrobial use and resistance in aquaculture: findings of a globally administered survey of aquaculture-allied professionals. Zoonoses Public Health. 60(6): 426-436.

Wang Y-T, Lee M-F and Peng C-F 2014. Mutations in the quinolone resistance-determining regions associated with ciprofloxacin resistance in *Pseudomonas aeruginosa* isolates from Southern Taiwan. Biomarkers and Genomic Medicine. 6(2): 79-83.

Watts JEM, Schreier HJ, Lanska L and Hale MS 2017. The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions. Mar Drugs. 15(6).

WHO 2006. Report of a joint FAO/OIE/WHO expert consultation on antimicrobial use in aquaculture and antimicrobial resistance : Seoul, Republic of Korea, 13-16 June 2006.

WHO 2007. Critically important antimicrobials for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use. *in* Report of the Second WHO Expert Meeting. Copenhagen.

Xavier BB, Das AJ, Cochrane G, De Ganck S, Kumar-Singh S, Aarestrup FM, Goossens H and Malhotra-Kumar S 2016. Consolidating and Exploring Antibiotic Resistance Gene Data Resources. J Clin Microbiol. 54(4): 851-859.

Yoshida H, Bogaki M, Nakamura M and Nakamura S 1990. Quinolone resistance-determining region in the DNA gyrase *gyr*A gene of *Escherichia coli*. Antimicrob Agents Chemother. 34(6): 1271-1272.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM and Larsen MV 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 67(11): 2640-2644.

Zhang Y, Nie P and Lin L 2016. Complete Genome Sequence of the Fish Pathogen *Flavobacterium columnare* Pf1. Genome Announc. 4(5).

Zhang Y, Zhao L, Chen W, Huang Y, Yang L, Sarathbabu V, Wu Z, Li J, Nie P and Lin L 2017. Complete genome sequence analysis of the fish pathogen *Flavobacterium columnare* provides insights into antibiotic resistance and pathogenicity related genes. Microb Pathog. 111: 203-211.

APPENDIX A

Table A1 All sequences used for phylogenetic analysis

Isolate	Origin	Host	Year	Genetic group	NCBI accession no.		Reference
					16s rRNA	*dna*K	
90-106	USA (Mississippi)	Channel catfish	1990	3	MG516967	MG16301	LaFrentz et al. (2018)
AL-02-36	USA (Alabama)	Largemouth bass	2002	2	MG516972	MG516310	LaFrentz et al. (2018)
ALG-00-530	USA (Alabama)	Channel catfish	2000	2	MG516971	MG516309	LaFrentz et al. (2018)
ARS-1	USA (Alabama)	Channel catfish	1996	3	MG516969	MG516303	LaFrentz et al. (2018)
ATCC 49512	France	Brown trout	1987	1	FCOL_RS00670	FCOL_RS07335	Tekedar et al. (2012)
BZ-1-02	Brazil	Nile tilapia	2002	4	MG516975	MG516299	LaFrentz et al. (2018)
CC1351	Indonesia	Common carp	unknown	2	MG516954	MG516322	LaFrentz et al. (2018)
CC1802	Thailand (Chachoengsao)	Asain sea bass	2018	4	-	-	This study
CC1803	Thailand (Chachoengsao)	Asain sea bass	2018	4	-	-	This study
CC1805	Thailand (Chachoengsao)	Asain sea bass	2018	4	-	-	This study
CC1808	Thailand (Chachoengsao)	Asain sea bass	2018	4	-	-	This study
CF1	Thailand	Striped catfish	2014	2	BWK60_13005	BWK60_RS11795	Kayansamruaj et al. (2017)
CUVET1214	Thailand (Phetchaburi)	Red tilapia	2012	4	BWK62_15295	BWK62_RS07285	Kayansamruaj et al. (2017)
CUVET1215	Thailand (Phetchaburi)	Red tilapia	2012	3	BWK59_13545	BWK59_RS13620	Kayansamruaj et al. (2017)
CUVET1345	Thailand (Chachoengsao)	Red tilapia	2013	4	KF774289	-	Dong et al. (2015)

CUVET1346	Thailand (Chachoengsao)	Red tilapia	2013	4	KF774290	-	Dong et al. (2015)
CUVET1362	Thailand (Kanchanaburi)	Red tilapia	2013	2	BWK63_13940	BWK63_RS10080	Kayansamruaj et al. (2017)
F4-HK	USA (Indiana)	Yellow perch	2012	1	MG516965	MG516300	LaFrentz et al. (2018)
FBCC-CC-12K	USA (Florida)	Channel catfish	2013	2	MG516974	MG516304	LaFrentz et al. (2018)
GA-02-14	USA (Georgia)	Rainbow trout	2002	3	MG516968	MG516302	LaFrentz et al. (2018)
IA-S-4	USA (Iowa)	Walleye	2011	1	MG516966	MG516308	LaFrentz et al. (2018)
Israel	Israel	Common carp	unknown	1	MG516963	MG516311	LaFrentz et al. (2018)
MS-02-475	USA (Mississippi)	Channel catfish	2002	2	MG516973	MG516306	LaFrentz et al. (2018)
NK01	Thailand	Nile tilapia	2014	4	BWG23_14835	BWG23_RS03235	Kayansamruaj et al. (2017)
PT-14-00-151	USA (Mississippi)	Channel catfish	2000	2	MG516970	MG516305	LaFrentz et al. (2018)
SP1802	Thailand (Samutprakarn)	Asain sea bass	2018	2	-	-	This study
SP1803	Thailand (Samutprakarn)	Asain sea bass	2018	2	-	-	This study
SP1805	Thailand (Samutprakarn)	Asain sea bass	2018	2	-	-	This study
SP1809	Thailand (Samutprakarn)	Asain sea bass	2018	2	-	-	This study
TI2063	Africa	Tilapia	unknown	3	MG516959	MG516333	LaFrentz et al. (2018)
F. psychrophilum JIP02/86	France	Rainbow trout	1986	-	NC_009613	NC_009613	Duchaud et al. (2007)
F. johnsoniae UW101	England	Soil	unknown	-	NC_009441	NC_009441	McBride et al. (2009)