การกลายพันธุ์ในบริเวณที่เกี่ยวข้องกับการดื้อยากลุ่มควิโนโลนของยีนเป้าหมายของควิโนโลน
ในเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ที่แยกได้จากปลาน้ำจืดในประเทศไทย

นายวันนิวัฒน์ มะทะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

MUTATION IN QUINOLONE RESISTANCE DETERMINING REGIONS OF QUINOLONE TARGET GENES IN *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM FRESHWATER FISH IN THAILAND

Mr. Wanniwat Mata

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	MUTATION IN QUINOLONE RESISTANCE DETERMINING REGIONS OF QUINOLONE TARGET GENES IN *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM FRESHWATER FISH IN THAILAND
By	Mr. Wanniwat Mata
Field of Study	Veterinary Pathobiology
Thesis Advisor	Assistant Professor Dr. Channarong Rodkhum

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science
(Professor Dr. Roongroje Thanawongnuwech)

THESIS COMMITTEE

.....Chairman
(Associate Professor Dr. Nuvee Prapasarakul)

.....Thesis Advisor
(Assistant Professor Dr. Channarong Rodkhum)

.....Examiner
(Associate Professor Dr. Rungtip Chuanchuen)

.....Examiner
(Associate Professor Dr. Nopadon Pirarat)

.....External Examiner
(Associate Professor Dr. Chanwit Tribuddharat)

วันนิวัฒน์ มะทะ : การกลายพันธุ์ในบริเวณที่เกี่ยวข้องกับการดื้อยากลุ่มควิโนโลนของยีนเป้าหมายของควิโนโลนในเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ที่แยกได้จากปลาน้ำจืดในประเทศไทย (MUTATION IN QUINOLONE RESISTANCE DETERMINING REGIONS OF QUINOLONE TARGET GENES IN *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM FRESHWATER FISH IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชาญณรงค์ รอดคำ, 75 หน้า.

ฟลาโวแบคทีเรียม คอลัมแนร์ หรือ เอฟ คอลัมแนร์ เป็นแบคทีเรียที่ก่อโรคคอลัมนาริส (columnaris disease) ซึ่งมีผลกระทบอย่างรุนแรงต่อปลาน้ำจืดหลายๆชนิดทั่วโลก ยาปฏิชีวนะหลายชนิดถูกนำมาใช้รักษาโรคคอลัมนาริสในฟาร์มปลาในประเทศไทยโดยเฉพาะยากลุ่มควิโนโลน ฉะนั้นการดื้อต่อควิโนโลน (quinolone resistance) ในเอฟ คอลัมแนร์ ควรมีการเฝ้าระวัง จุดประสงค์ของการศึกษาครั้งนี้เพื่อทดสอบความไวรับต่อยาปฏิชีวนะ และตรวจหาการกลาย (mutation) ในส่วนของยีนเป้าหมายของควิโนโลนของเอฟ คอลัมแนร์ จากทั้งหมด 50 ไอโซเลท (isolates) จากงานวิจัยของ (Dong et al., 2014) ผลความไวรับพบว่า เกือบทุกไอโซเลทให้ผลไว (sensitive) ต่อยาทั่วไปที่ใช้ในการรักษาสัตว์น้ำ ยกเว้นบางไอโซเลทที่ดื้อ (resistant) ต่อกรดนาลิดิซิค (14%) และกรดออกโซลินิค (22%) ส่วนของผลของความเข้มข้นต่ำสุดที่จะระงับการเจริญของเชื้อ (minimum inhibitory concentration: MIC) ของกรดออกโซลินิคพบว่า 9 ไอโซเลทให้ผลกึ่งกลาง และ 16 ไอโซเลทให้ผลดื้อ จากนั้นส่วนคิวอาร์ดีอาร์ (quinolone resistance-determining regions: QRDRs) ของยีนไจร์เอ *(gyrA)* ไจร์บี *(gyrB)* พาร์ซี *(parC)* และพาร์อี (*parE)* ของเอฟ คอลัมแนร์จะถูกเพิ่มจำนวนโดยไพร์เมอร์จำเพาะที่ออกแบบในการศึกษาและส่งไปวิเคราะห์ลำดับพันธุกรรมต่อไป พบว่าไอโซเลทที่ให้ผลกึ่งกลางและดื้อต่อกรดออกโซลินิคจะพบการเกิดการกลายใหม่ 2 ยีนคือ ไจร์เอและพาร์ซี ในยีนไจร์เอตำแหน่งที่ 83 ตามระบบของเชื้อเอสเชอริเชีย โคไล (*Escherichia coli)* มีการแทนที่ของเซอรีน ด้วยฟีนิลอะลานีน หรือไทโรซีน ที่ค่า MIC=4 µg/ml หรืออะลานีน ที่ค่า MIC=8,16 µg/ml ในขณะที่ยีนพาร์ซีตำแหน่ง 87 พบการแทนที่ของฮิสติดีน ด้วยไทโรซีน แต่ไม่พบการกลายในยีนไจร์บีและพาร์อี จากผลการทดลองสรุปว่า การเกิดการกลายทั้งในยีนไจร์เอและพาร์ซีเป็นกลไกสำคัญของการดื้อต่อยากลุ่มควิโนโลนและเป็นเป้าหมายสำคัญของกรดออกโซลินิคในเอฟ คอลัมแนร์ด้วย นอกจากนี้การศึกษานี้เป็นการพบหนึ่งในกลไกลการดื้อต่อยากลุ่มควิโนโลนในเอฟ คอลัมแนร์เป็นครั้งแรก อย่างไรก็ตามกลไกอื่นที่เกี่ยวข้องควรมีการศึกษาต่อไป

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5575317231 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: FLAVOBACTERIUM COLUMNARE / MUTATION / QRDR / QUINOLONE / RESISTANCE

WANNIWAT MATA: MUTATION IN QUINOLONE RESISTANCE DETERMINING REGIONS OF QUINOLONE TARGET GENES IN *FLAVOBACTERIUM COLUMNARE* ISOLATED FROM FRESHWATER FISH IN THAILAND. ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, 75 pp.

Flavobacterium columnare is the causative agent of columnaris disease, which is seriously affected several freshwater fish species worldwide. Many kinds of antibiotics have been applied in fish farms for the treatment of columnaris disease in Thailand, especially quinolones. Thus, quinolone resistance (QR) in F. columnare should be monitored. The objectives of this study were to determine antimicrobial susceptibility and to detect the mutations in quinolone resistance-determining regions (QRDRs) of gyrA, gyrB, parC, and parE in F. columnare. Totally 50 F. columnare isolates from previous study (Dong et al., 2014) were examined. All isolates tested were sensitive to most routinely drugs used in aquaculture except 2 quinolones; nalidixic acid (NA) and oxolinic acid (OA), which performed the resistant results (14% for NA and 22% for OA). For minimum inhibitory concentration (MIC) of OA, out of 50 isolates, 9 were intermediate and 16 were resistant. The QRDRs of F. columnare were amplified by specific designed primers and sequenced. All OA-intermediate and -resistant isolates revealed novel double point mutations and amino acid substitutions in gyrA and parC: at position 83 in gyrA according to Escherichia coli system: Ser to Phe, Ser to Tyr (MIC=4 μg/ml), and Ser to Ala (MIC=8,16 μg/ml) while in parC at position 87: His to Tyr (MIC≥4 μg/ml). No mutation was detected in gyrB and parE. These results suggested that mutations in both gyrA and parC are the main QR mechanism and are considered as the major target of OA in F. columare. Morover, this is the first investigation of QR mechanism in F. columnare. However, other mechanisms should require further research.

Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
Academic Year: 2014

ACKNOWLEDGEMENTS

For this research, actually I would like express in deep feeling of my graduate to many people for their kinds and big helps. I am so thankful to Asst. Prof. Dr. Channarong Rodkhum, my dearest thesis advisor for giving me a good opportunity to continue study in the great institute and providing huge things as the guideline and very good comments, supports, inspirations, kindness, and encouragement to complete the thesis as well as master's degree learning process. Everything starts and finishes by his best supervising from him. Furthermore, I would like to thank, Ms. Suphap Kumlungpaet and Ms. Waree Niyomtham, both experienced microbiologist and scientists for providing many assistances and knowledge regarding laboratory works. I also like to special thanks to Dr. Pattanapon Kayansamruaj for his useful comments and memorable moments in laboratory and documentary works.

I would like to thank all staffs and graduate students at the department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University for sharing and helping the experiences and good techniques to fulfil my thesis work.

I am thankful for The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) Grant No. GCUGR1125572514A (102-2), The Overseas Academic Presentation Scholarship for Graduate Students and CU.Graduate School Thesis Grant for financial and research supports.

Finally, I am sincerely thanks my family for their love, support, understanding, and encouragement to reach my master degree.

CONTENTS

Page

Page

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAC	Acetyltransferase
ABOs	Alanine
Ala	Antibiotics
AMP	Ampicillin
AMR	Antimicrobial resistance
AO	Anacker and Ordal
AST	Antimicrobial susceptibility test
ATCC	American Type Culture Collection
CAMHB	Cation-Adjusted Mueller-Hinton broth
CFU	Colony-forming units
CIP	Ciprofloxacin
CLSI	The Clinical and Laboratory Standards Institute
DMHB	Diluted Mueller-Hilton Broth
DO	Doxycycline
E	Erythromycin
ENR	Enrofloxacin
FFC	Florfenicol
FQ	Fluoroquinolone
gyrA	DNA gyrase subunit A
gyrB	DNA gyrase subunit B
h	hour
His	Histidine
IZD	Inhibition zone diameter
KC	Koi carp
MHA	Mueller Hinton Agar
MIC	Minimal Inhibitory Concentration
min	minute
ml	milliliter
NA	Nalidixic acid

NOR	Norfloxacin
NT	Nile tilapia
OA	Oxolinic acid
OXT	Oxytetracycline
parC	Topoisomerase subunit A
parE	Topoisomerase subunit B
Q	Quinolone
QR	Quinolone resistance
QRDR	Quinolone Resistant-Determining Region
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
PMQR	Plasmid-mediate quinolones resistance
RT	Red tilapia
SCF	Striped catfish
Ser	Serine
SXT	Sulfamethoxazole/trimetroprime
Tyr	Tyrosine
UV	Ultraviolet
µg	microgram
µm	micrometer

CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Flavobacterium columnare is one of the important Gram-negative bacterial pathogens that cause columnaris disease in both cultured- and wild- freshwater fish worldwide. Columnaris disease or gill and skin disease is an infectious disease with caused mortality up to 100% within less than 1 week. The disease plays important role of severe economic losses in either fish farms or industries throughout the world (Shoemaker et al., 2008).

In Thailand, several kinds of fish have been cultured for human consumption. Among various fish species, Nile tilapia, red tilapia and channel catfish are popular and valuable freshwater fish for Thai consumers (DOF, 2010). When the columnaris disease occurred in fish farms or industries in Thailand, the farmers often used many types of antimicrobial agents for treatment and control this disease, e.g., flumequine, enrofloxaxin, oxolinic acid, and oxytetracycline (FDA, 2012). Depth study of columnaris diseases in Thailand is still limited, currently, improper usage of antibiotics (ABOs) usually applies in farms, e.g., overdose, lowerdose, extralebelled, misuse, inappropriate use, illegal use, etc. Therefore, these improperly use of ABOs might become the important problem because it's residue by chemical pollution from antibiotics to the environment. Moreover, residual ABOs may accelerate and develop the antimicrobial resistant of the bacteria which possibly cause emergence of drug-resistant strains of the aquatic bacteria including *F. columnare* (Young, 1993). This bacterium has been reported as fish pathogen in many fish worldwide (Marks et al., 1980). Nevertheless, the effective method for treating columnaris disease is still limited to ABOs, while the effective-commercial vaccine is still not yet available (Tusevljak et al., 2013). Despite excessive use of ABOs for the treatment of this disease, but there is lack of recent information and publication of the antimicrobial susceptibility test (AST) and the occurrence of antimicrobial resistance (AMR) of *F. columnare* in Thailand. Thus, the monitoring of AMR by AST regarding antimicrobial use (AMU) in aquaculture of *F. columnare* should be considered.

Several kinds of ABOs have been permitted by Ministry of Public Health in Thailand to use for the treatment and control the infectious diseases including

columnaris disease, for example, oxolinic acid, oxytetracycline, tetracycline, florfenicol, flumequine and enrofloxacin (FDA, 2012). However, quinolones and tetracyclines are the majority usage in aquaculture for treating the infectious diseases especially columnaris disease (Smith, 2008; Tusevljak et al., 2013). Oxolinic acid is one of licensed quinolone drugs which often use in aquaculture worldwide (Smith, 2008). Nowadays, quinolones have been widely applied in aquaculture during the disease outbreaks. However, quinolones were not approved for aquaculture farming in some countries since their residue in aquatic animal products were reported and may cause quinolone resistance in human (WHO, 1999); (Tusevljak et al., 2013). Thus, quinolone usage in aquaculture should be concerned in order to monitor the problems of antimicrobial resistance and the transmission of their resistance especially quinolone resistance (QR) from aquatic animals to human.

Since the increased use of quinolones in aquaculture treatment for treating fish diseases worldwide led to the increasing of QR in aquatic bacteria. From update publication, the major of *F. columnare* collected from 17 fish species were resistant to enrofloxacin, flumequine and oxolinic acid up to 42% and their resistances were closely related with quinolone using in aquaculture farming, but they did not perform the study of mechanism of resistance (Declercq et al., 2013a). In addition, one of the most common mechanisms which bacteria acquire resistance to quinolone is by the spontaneous mutation in the specific region named quinolone resistance-determining regions (QRDR) in chromosomal genes, *gyrA, gyrB, parC* and *parE* that altered the target enzymes, DNA gyrase and topoisomerase IV (Ruiz, 2003). QRDR mutations responsible for amino acid substitutions are the main causes of QR in almost Gram-negative bacteria including *F. columnare*. Although bacteria in genus *Flavobacterium* are often reported as an important bacterial pathogen in aquatic animals with the high frequency of quinolones usage for treating the disease, but only one specie in this genus has been published, *F. psychrophilum.* The report of the mutation in QRDR of *gyrA* encoded amino acid substitutions and related to the resistance of oxolinic acid of *F. psychrophilum* have been published (Izumi and Aranishi, 2004; Shah et al., 2012). Recently, evidences of QR due to the mutations in QRDR of several organisms have been reported, but no report of the study about the QRDR mutation associated with QR of *F. columnare* has published yet. However, various kind of methods have been used to detect the alteration of targets enzymes related to QR especially molecular techniques, polymerase chain reaction (PCR) and

DNA sequencing are the standard methods which most of researches used for studying in molecular mechanisms of antimicrobial resistance (Fluit et al., 2001). Therefore, the purpose of this study was to survey and monitor the QR caused by the mutation in QRDR of chromosomal genes that altered the target enzymes of quinolone in *F. columnare* by PCR amplification and analysis of DNA sequences. Moreover, the AST for monitoring AMR of *F. columnare* to the routinely AMU in aquaculture including drugs in group of fluoroquinolones and quinolones by disk diffusion test were performed. The determination of MICs by broth microdilution of *F. columnare* to only fluoroquinolones and quinolones was also carried out. Furthermore, the QRDR sequences of *gyrA, gyrB*, *parC* and *parE* were amplified and sequenced in order to determine the QRDRs mutation of each quinolone target gene refer to the QR mechanisms of *F. columnare*.

1.2 Research questions

1. What is the antimicrobial susceptibility pattern and the occurrence of QR among isolates of *F. columnare* recovered from freshwater fish?
2. What is association between the mutation in QRDRs of *gyrA, gyrB, parC* and *parE* associated with QR mechanisms and their quinolone susceptibility of *F. columnare* isolates recovered from freshwater fish?

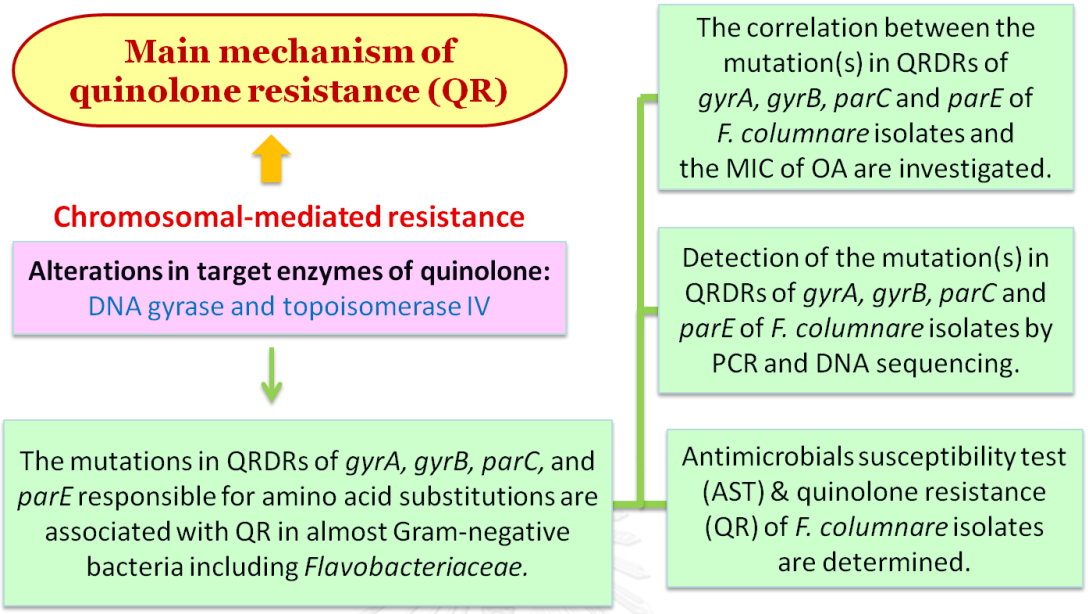
1.3 Objectives of Study

1. To investigate the antimicrobial susceptibility pattern and the occurrence of antimicrobial resistance especially QR among *F. columnare* isolates recovered from freshwater fish.
2. To detect the QRDR mutations of *gryA*, *gryB parC*, and *parE* of *F. columnare* isolates recovered from freshwater fish.

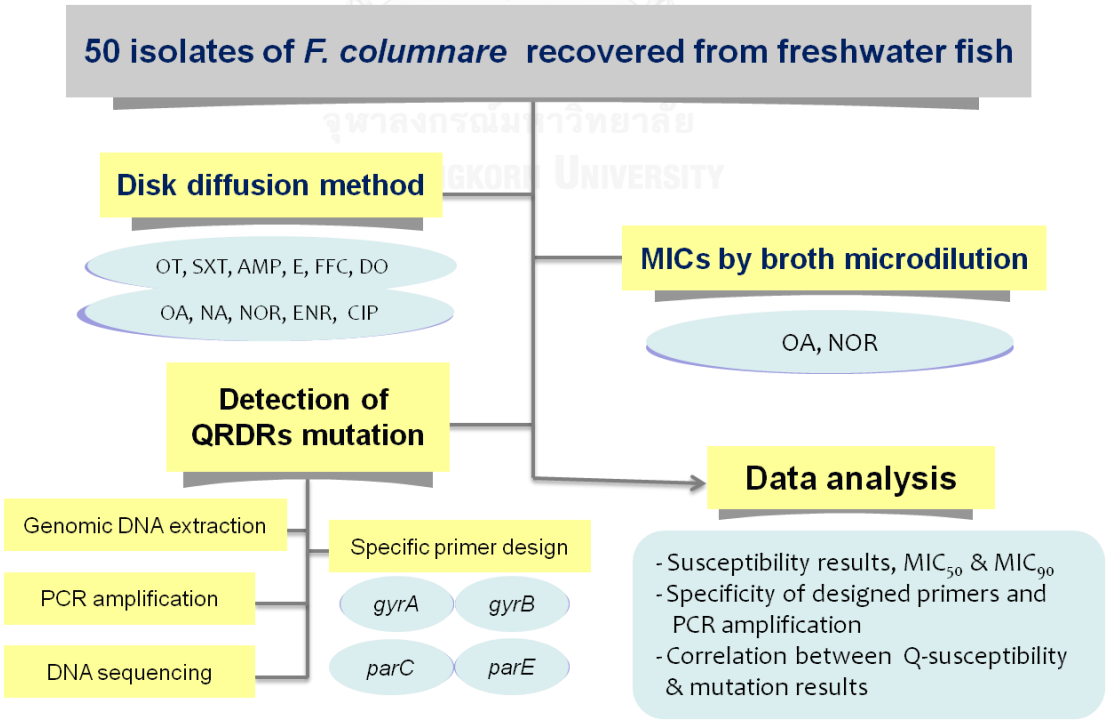
1.4 Hypothesis

The mutations in QRDRs of *gyrA*, *gryB*, *par*C, or *parE* are associated with QR mechanisms of *F. columnare* isolates recovered from freshwater fish.

1.5 Conceptual framework



1.6 Research plan



CHAPTER II

Literature Review

2.1 Overview of *Flavabacterium columnare* and columnaris disease

F. columnare is a dermotropic bacterium, which belongs to the family Flavobacteriaceae, genus Flavobacterium. "Flavobacteria" is the synonymous name of the group of bacteria in family Flavobacteriaceae. Flavobacteria have free-living, saprophytic, or parasitic life style and one of pathogenic bacteria in aquatic animals (Bernadet and Bowman, 2006). Members of bacteria in the family comprise at least 10 important genera, e.g., *Flavobacterium* spp., *Chryseobacterium* spp., *Capnocytophaga* spp., *Myroides* spp., *Bergeyella* spp., *Empedobacter* spp., *Ornithobacterium* spp., etc (Jooste and Hugo, 1999). Almost species of bacteria in genus Flavobacterium are ubiquitous bacteria which distributed in a variety of environments such as soil, plants, water, particularly in freshwater. However, the interested genera that often cause the diseases in aquatic animals are Chryseobacterium and Flavobacterium. Recently, they have been 2 published species of bacteria in genus Flavobacterium become the most important bacterial pathogens in aquaculture worldwide, *F. columnare* and *F. phychrophilum*.

Regarding to bacterial characteristics, *F. columnare* is a very long Gram-negative rod (0.3 - 0.7 µm wide and 3 - 10 µm long) and yellow pigmented bacterium. This bacterium exhibit gliding and motility activity on agar and can grow on low nutrient media and produce yellow colonies by production of flexirubin pigments (Jooste and Hugo, 1999). Colonies of the bacteria are flat, sticky attach to the surface of selective media like Anacker and Ordal's (AO) or Cytophaga medium (Anacker and Ordal, 1955) with the typical colony shape like rhizoids colonies. The optimum temperature for the growth of *F. columnare* is 25-28°c with strictly aerobic condition (Woodland, 2004). In addition, *F. columnare* which is normally found in the aquatic environment is also an etiological agent of columnaris disease as opportunistic pathogen in several kinds of fish, warm and cold, ornamental, wild and cultured fish especially freshwater fish. Many reports found this bacterial pathogen was able to isolated from diseased fish such as Nile tilapia, channel catfish, Koi carp, Rainbow trout, etc. (Amin et al., 1988; Decostere et al., 2002; Kubilay et al., 2008). The synonyms name of this disease are Myxobacterial disease, Fin rot, Saddleback,

Cotton wool disease, Black patch necrosis. Channel catfish is the susceptible host which commonly infected with this pathogen and cause severe disease. The water temperature, age of fish and the season have an effect on the severity of columnaris disease (Klesius et al., 2008). The mortality rate is depends on species of fish and water and environmental temperature (Pilarski et al., 2008). The important clinical signs of this disease were yellow to brown lesions on gills, skins, fins on fish and that cause skin lesions, gill necrosis, fin erosion and lead to the high mortality of fish later (Declercq et al., 2013b). The recent report of columnaris disease in Thailand has been reported in 2012, the pathogenic *F. columnare* were isolated from skin and gill lesions and brains of tilapia (Ha et al., 2013; Tohmee and Deemagarn, 2013).

Several routes to control and prevent the columnaris disease are suggested from many authors. Management is commonly used for prevention of this disease, e.g., reduction of the fish density. Some researchers supported that ozone; salt, acid and nitrite were effective used for treatment the disease. Furthermore, antimicrobial agents and chemical agents were often used for treatment. Firstly, chemical agents or disinfectants such as potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2), and copper sulfate ($CuSO_4$) can be adapted to prevent as effective treatment (Declercq et al., 2013b). Besides the chemical agents, the another approaches were development to use as more effective treatment, e.g., vaccination by bacterin, formalin-killed bacteria, strains containing sialic acid and probiotic by commensal bacteria that can against the pathogen (Declercq et al., 2013a). For the treatment by using antimicrobial agents will be described next part.

2.2 Antimicrobial Used (AMU) in aquaculture

The routinely usage of antimicrobial agents, e.g., amoxicillin, tetracycline, erythromycin, chlortetracycline, oxytetracycline, florfenicol, oxolinic acid, ormetoprim/sulfamethoxine, trimethoprim/sulfamethoxazole, enrofloxacin, flumequine have been widely used worldwide for treatment of several diseases in aquaculture (Smith, 2008). Since columnaris disease is often occurred as external infection or epidermal surfaces before it develop to advance or septicemia stage, thus the external treatment is still effective for this disease. They have been several studies about AMU for the treatment of this disease in many of fish especially salmonid fish. Bath therapies are one of the efficient approaches which have been used with the effective drugs for treatment such as chloramphenicol, nifurpirinol, nifurprazine and oxolinic acid. Oxytetracycline was given by oral administration in

salmon during the outbreaks of columnaris disease occurred. Other drugs for orally used were sulfonamide, nitrofuran and florfenicol but the less success information of drugs using still have been reported (Declercq et al., 2013b).

There have been widely used of chemical and antimicrobial agents in aquaculture field in Thailand. Twelve of antimicrobial agents are licensed for usage in aquaculture, enrofloxacin, sarafloxacin, oxolinic acid, oxytetracycline, sulfadimethoxin-ormethoprim, sulfadimethoxin-trimethoprim, sulfadimethoxin, sulfamonomethoxin, sulfadiazine, trimethoprim, ormethoprim and toltrazuril (FDA, Thailand; (Smith, 2008). Tetracyclines and Quinolones were reported as the often used for aquaculture treatment in several countries. Tetracyclines are the most frequently used for many kinds of aquatic animals, e.g., salmon, tilapia, catfish, trout and shrimp worldwide. Amazingly, quinolones which are restricted or prohibited use in aquaculture was still reported as routinely usage in Europe and Asia (Tusevljak et al., 2013).

2.3 Antimicrobial susceptibility testing (AST) and antimicrobial resistance (AMR) of *F. columnare*

The susceptibility test of bacteria to antimicrobial agents is the method that used for evaluating the empirical usage or the therapeutic value of antimicrobials in clinical practicing. This method is important step to choose the empirical antimicrobials and also to detect the resistance of bacteria to antimicrobials. Three standard methods according to The Clinical and Laboratory Standards Institute (CLSI) are recommended for the testing; agar disk diffusion, agar dilution and broth dilution. Furthermore, there are two tests for determining the antimicrobial susceptibility, disk diffusion test as a qualitative test and Minimum Inhibitory Concentration (MIC) test as a quantitative test (Jorgensen and Ferraro, 1998).

For the investigation of susceptibility test of bacteria in aquaculture field or fish pathogenic bacteria, there have published approved standard protocols that recommended the standard reference methods for aquatic pathogen including *F. columnare.* CLSI 2006 proposed the two documents, M42-A and M49-A for disk diffusion test and MIC tests by broth dilution respectively as the control methods for bacteria isolated from aquatic animal particularly gliding bacteria (group 3) (CLSI, 2006b); (Alderman and Smith, 2001; Smith, 2001). There have been some reports that represented the antimicrobial susceptibility of Flavobacteria in several kinds of AMU

in aquaculture treatment by agar dilution and disk diffusion (Aber et al., 1978; Chang et al., 1997). Updated information of antimicrobial susceptibility of *F. columnare* from 17 fish species worldwide was published in 2012 by broth microdilution technique (Henriquez-Nunez et al., 2012). However, according to CLSI guidelines, Mueller-Hinton agar (MHA) and cation-adjusted Mueller-Hinton broth (CAMHB) are used for disk diffusion and MIC respectively (CLSI 2006;(Dalsgaard, 2001). These two media are not appropriate for the growth of *F. columnare*, the improvement or modification of the method were developed based on the broth dilution by using the diluted Mueller-Hilton Broth (DMHB) at 1:5 with using *Escherichia coli* ATCC 25922 as a quality control (Darwish et al., 2008) and *Aeromonas salmonicida subsp. salmonicida* reference strain ATCC 33658 in different temperature; 22 °c and 28 °c for determining the QC ranges (Miller et al., 2003). These modified methods are applicable to perform the susceptibility of *F. columnare* to antimicrobials agents.

There have been widely reports of antimicrobial resistance to several aquatic bacteria, e.g., *Vibrio* spp., *Aeromonas* spp., *Streptococus* spp., and *Flavobacterium* spp. (Jorgensen and Ferraro, 2009), especially quinolones and tetracyclines, the frequently AMU for the treatment in common aquatic diseases. The *in vitro* antimicrobial susceptibility tests of *F. columnare* to several AMU in aquaculture were performed. *F. columnare* was natural resistant to polymyxin B and neomycin. Two ABOs were used by adding into Cytophaga agar (Fijan, 1969) as selective media. Afterwards, Decostere and colleagues found that tobramycin were able to use for selective supplement to Shieh medium because of the resistance of *F. columare* to tobramycin and inhibition of the other bacteria. This medium can also improve the growth of this bacteria (Decostere et al., 1997). *F. columnare* were resistant to oxytetracycline, neomycin, polymycin B and tobramyxin by using disk diffusion (Fijan, 1969). The currently information about the AMR have been published in 2012 by using broth microdultion to determine MICs of *F. columnare* collected worldwide to many kinds of ABOs. They reported the first time acquired resistance of *F. columnare* to chloramphenicol, nitrofuran, oxytetracycline, enrofloxacin, flumequine and oxolinic acid. This is the first report of quinolone resistance in *F. columnare* isolated from many kinds of fish species worldwide and performed the prudent use of ABOs is necessary and important for the appropriate selection of

approval ABOs for the treatment in aquatic diseases to reduce the high risk of resistance (Declercq et al., 2013a). The increasing use of quinolones has been rapidly developed the resistance as quinolone resistance (QR) to aquatic bacteria.

2.4 Overview of quinolone drugs

Quinolones are synthetic broad-spectrum antimicrobial drug and commonly used in both clinical for human and veterinary medicine for animals. The use of quinolone is under the critically important licensable antimicrobials agents for human health (WHO, 1999) such as oxolinic acid, nalidixic acid and flumequine which have been widely applied in aquaculture treatment (Smith, 2008). Oxolinic acid and nalidixic acid are the first-generation of quinolone, while flumequine is a first-generation of fluoroquinolones. Fluoroquinolones is one subset of the major quinolones used in clinical which have been developed for more potency by changing a fluorine atom in their structures. Quinolones are in recommended list of antimicrobial agents for the treatment of several infectious diseases in aquaculture. One fluoroquinolone; enrofloxacin and three quinolones; oxolinic acid, nalidixic acid and flumequine can be effectively employed for the treatment of columnaris disease (Declercq et al., 2013b).

2.5 Reviews on quinolone resistance (QR) mechanisms in Gram-negative bacteria

2.5.1 Mechanisms of action of quinolone

First of all, the mechanisms of action of quinolone should be briefly explained. Quinolones act by inhibiting the action of two essential type II topoisomerases as target enzymes of quinololne, DNA gyrase and topoisomerase IV (Topo IV) (Fabrega et al., 2009). Both enzymes are important for cell survival in processes of bacterial DNA replication, transcription, recombination and repair of DNA (Hooper, 2001). In general knowledge of Gram-negative bacteria, quinolone binds to the primary targets genes, DNA gyrase and block the activity by forming the drug-enzyme-DNA complex as resulting in the inhibition of DNA synthesis, finally the bacteria cell dead is occur. For another target gene, Topo IV which is the primary target of quinolone in most Gram-positive bacteria, quinolone also bind Topo IV and disrupt the separation of two replicated DNA and resulting in bacterial cell dead. Both mechanisms of quinolone and fluororquinolone resistance (FQ-R) are considered as the same mechanisms.

2.5.2 Mechanisms of quinolone resistance

Quinolones resistance (QR) in Gram-negative bacteria involved two main mechanisms; alteration in the quinolone target enzymes and alteration that decrease drug accumulation and/or the overexpression of efflux pump systems (Ruiz, 2003; Jacoby, 2005). Both mechanisms are chromosomal-mediated QR. However, the updated information revealed that mobile element is also considered as plasmid-mediated QR by carrying resistant gene, which might important for the transfer of the QR (Poirel et al., 2012).

2.5.2.1 Alteration in the target enzymes

Main mechanisms of QR are the chromosome-encoded resistance or chromosomally mediated, which is alteration in two quinolone target enzymes; DNA gyrase and topoisomerase IV by mutations in their encoded genes. DNA gyrase is composed of two types of each subunit; GyrA and GyrB subunits encoded by *gyrA* and *gyrB* genes, respectively. DNA gyrase is mainly important for DNA replication by catalyzing negative supercoils to DNA, removing positive and negative supercoils, and catenating and decatenating the circular DNA both initiation and elongation phases. Additionally, DNA gyrase is also involved the transcription, DNA recombination, repair, and transposition. For another target enzyme, topoisomerase IV is compose of two subunits homologues to DNA gyrase; ParC is encoded by *parC* gene and ParE is also encoded by *parE* gene. These *parC* and *parE* have highly homologous to *gyrA* and *gyrB*, respectively. Topoisomerase IV is essential for removing the interlinking of daughter chromosome while processing in DNA replication.

In Gram-negative bacteria, most of studies were refer the QR from bacteria in family *Enterobacteriaceae,* particularly well known pathogen; *E. coli.* DNA gyrase seems to be more susceptible for the inhibition by quinolone than topoisomerase IV and is the primary target of QR in most Gram-negative bacteria, whereas topoisomerase IV which is less sensitive to quinolone but is the primary target of QR in Gram-positive bacteria instead (Hooper, 1999). From literatures and previous reports showed that the mutations leading to amino acid substitutions in the specific regions of each target gene; *gyrA, gyrB, parC* and *parE* which were often detected in the so-call "quinolone resistance-determining regions" (QRDR), especially the mutation in the hot spots of specific 40 amino acids between positions 67 and 106 within the N-terminal domain in *gyrA* of *E. coli* that have been reported and involved

with the development of QR ((Yoshida et al., 1990); (Yoshida et al., 1991). This QRDR region is supposed to be the putative active site for the interaction between quinolone and DNA gyrase (Nakamura et al., 1989). The target genes which are most frequently found the chromosomal mutations that result in amino acid substitutions in almost Gram-negative bacteria, are *gyrA* and *parC* genes (Hooper, 2001). Double point mutations in QRDRs of *gyrA* (S83I) and *parC* (S85L) were usually associated with the high resistance of first-generation quinolones oxolinic acid and often performed low level of resistance of *V. anguillarum* to some fluoroquinolones such as enrofloxacin (Colquhoun et al., 2007; Rodkhum et al., 2008). Single mutations associated with high MICs of quinolone and/or fluoroquinolone in Gram-negative bacteria are mainly due to the alteration in DNA gyrase, especially *gyrA,* which is primarily changed within the hot spots and usually be the initial altered refer to QR. The first report of point mutation causing amino acid substitution in QRDRs of *gyrA* have been reported in *E. coil* related to the high level of quinolone nalidixic acid resistance (Yoshida et al., 1990). Additionally, amino acid changes at positions 83 and 87 (Ser83 and Asp87) in *gyrA* are called the hot spots for the mutation in *E. coli* and most often mutated in many QR-isolate bacteria (Hooper, 1999). The most frequently amino acid positions found the mutation causing QR as hot spots in *gyrA* in *E. coli* are out of considered QRDR by the mutation in codon 51 that reduce the susceptibility to quinolone even only the mutated in mutants by *in vitro* (Friedman et al., 2001). In addition, model used for the description of QR mechanisms as the representative of Gram-negative bacteria by the mutations that altered target enzymes associated with QR is *E. coli.* Several studies have been reported that mutations in many *E. coli* strains from various samples are often detected in both mutations in one target genes, e.g., *gyrA, gyrB, parE* (Ruiz, 2003) and double or more mutations found in target genes, e.g., *gyrA* and *parC (Saenz et al., 2003), gyrA* and *gyrB* (Nakamura et al., 1989) related to the decrease of quinolone susceptibility led to QR. In *parC* gene is also most commonly found the mutations resulting in QR, e.g., amino acid changes at positions 80 and 84 of *parC* in *E. coli,* which showed the high MIC of ciprofloxacin (Vila et al., 1996).

For another target genes; *gyrB* and *parE* are usually involved with QR especially in Gram-positive bacteria (van Hoek et al., 2011). Amino acid substitutions resulting in QR at codons 426 and 447 in *gyrB* gene of *E. coli* have been reported confer the level of resistance to quinolone nalidixic acid (Yoshida et al., 1991) but in

difference effects to the susceptibility of 7-position fluorinated quinolone as fluoroquinolone (FQ) such as norfloxacin, enrofloxacin with low-level of QR (D426N) and increased susceptibility of FQ (K447E) (Nakamura et al., 1989). Mutation in *gyrB* is rarely found in both Gram-positive and -negative bacteria associated with QR. The most bacteria which often found of *gyrB* mutation associated with QR are *E. coli, Salmonella* spp., *Pseudomonas* spp. but with low prevalence of mutations and cases found because two subunits of DNA gyrase, *gyrA* is more sensitive for quinolone affinity than *gyrB*. Due to *gyrB* and *parE* are highly homologous subunits with their conserved QRDRs, both target genes are proposed that the second target of quinolone and development of QR and rarely found the mutations in both target genes as single and double mutation in most bacteria.

Recently, some study regarding other pathogenic bacteria with the frequently used or exposed with quinolone for the treatments are often found the relations between mutations resulting in amino acid changes and the increased of quinolone susceptibility causuing QR in all QRDRs of the target genes such as *Enterococcus faecalis* (Oyamada et al., 2006), *Proteus mirabilis* (Weigel et al., 2002), *Sallmonella enterica* (Hirose et al., 2002; Eaves et al., 2004), *Pseudomonas aeruginosa* (Mouneimne et al., 1999) usually associated with the high-MICs and resistant results to their quinololone and/or fluoroquinolone tested and other evidences, e.g. efflux system, plasmid carry resistant genes. The development of QR in *parE* of Gram-negative bacteria seems to be secondary cause of resistant mechanism by mutations in QRDR and amino acid changes which less affected to the quinolone susceptibility. For example only one amino acid substitution in *parE of E. coli* (L445H) have been reported by *in vitro* experiment and seems to be related with *gyrA* mutation resulting in high-MIC of quinolone and resistant presence (Breines et al., 1997). ParE mutation is often found in Gram-positive bacteria with the QR. Additionally, in some study has been described that substitutions in *parE* is rarely happened and may not associated with QR mechanisms. However, mutations and amino acid changes in *parE* are one of evidences found associated with the higher susceptibility of quinolone in many pathogenic bacteria coincidently found with another mechanism. In summary, the mutations in target genes encode DNA gyrase and topoisomerase IV have decreased the quinolone susceptibility by possible mechanism that quinolone reduced itself for

binding and stabilizing drug-enzyme-DNA complex, finally also reduced the drug affinity and resulting in the development of QR.

2.5.2.2 Alteration that decrease drug accumulation and/or the overexpression of efflux pump systems

Both mechanisms are associated the entry and pump out of quinolone in bacterial cell. For Gram-negative bacteria, decreased drug accumulation by altering outer membrane protein affected to expression of porins is considered as one of QR mechanisms. Before quinolone bind the target enzyme inside the bacterial cell in cytoplasm, quinolone must cross the cell wall and cytoplasmic membrane by one of two ways is though the specific porins. In *E. coli,* alteration in outer membrane protein such as OmpA, OmpC, and OmpF may reduce the expression of porins causing membrane impermeability and resulting in resistance to some quinolones and also decrease the susceptibility of other drugs such as chloramphenicol, tetracycline (Van Bambeke et al., 2000). The decreased of drug accumulation are associated with the decrease of porins protein expression causing membrane permeability. In addition, overexpression of efflux pump system is also associated with QR. Due to many efflux pump systems were different in bacterial species and related with the ability to pump out the quinolone of the cell, e.g., AcrAB, EmrAB, Mdfa, in *E. coli;* MexXY, Oprm in *P. aeruginosa,* etc., the detail of various efflux pump system weren't provided. In *E. coli,* mutations in some genes which act as regulation of efflux pump system are influenced with the increase of pump activity such as *soxS, acrR, marR,* etc. and lead to overexpression of the efflux pump. Mutations in MarRAB and SoxRS are also affected to the overepression of Omp and some efflux pump such as AcrAB. However, alteration of target enzymes and efflux pump system are usually concomitant found in associated with QR in *E. coli.* The increase activity of AcrAB efflux pump system was found in resistant *E. coli* isolates in addition to the double mutations detected in *gyrA* and *parC* that resulting in high MIC of some fluoroquinolones (Morgan-Linnell et al., 2009). Two alterations seem to be exist together and cause the QR as one of mechanisms of resistance to quinolone in Gram-negative bacteria.

2.5.2.3 Plasmid mediated-QR

Not only the mutations in target genes and alteration that decreased drug accumulation resulting in QR as the chromosomal mediated-QR, but the mobile

element can be associated with QR by carrying and transferring resistant gene call plasmid-mediate quinolones resistance (PMQR) is also associated with QR. The first report of PMQR has been published in *Klebsiella pneumonia* in 1987 with the transfer of low-level QR to *E. coli (Martinez-Martinez et al., 1998)*. However, previously study already reported that the presence of PMQR associated with quinolone nalidixic acid resistance to *Shigella* spp., (Munshi et al., 1987). Plasmid is a mobile genetic element which involves the transfer of gene-associated with drug resistance. Previous studies demonstrated that plasmid can carry the gene named "*qnr*" encoded Qnr protein. Qnr act as the protection of DNA gyrase and topoisomerase IV from quinolone inhibition (Martinez-Martinez et al., 1998). The plasmid in *Klebsiella pneumonia* has been found and contained *qnr* which encode qnr protein is able to transfer to other organisms. Qnr act as quinolone target protection in some Gram-negative bacteria, e.g., *E. coli.* Nowadays, several Qnr proteins such as QnrA, QnrB, QnrC, QnrD, QnrS and QnrVS have been reported in bacteria in *Enterobacteriaceae*, e.g., *E. coli, Salmonella* spp., *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp. (Ruiz et al., 2012). In addition, another gene may play important roles by mechanisms of plasmid associated with QR in many kinds of bacteria for example *cr* variant of aminoglycoside acetyltransferase (AAC) that associated with ciprofloxacin resistance and *qepA,* a plasmid-mediated efflux pump in QR (Martinez-Martinez et al., 1998; van Hoek et al., 2011). Resistant genes encoding protein on plasmid can provided only low-level of QR of resistant isolates (*E. coli* and *K. pneumoniae*) (Poirel et al., 2012). However, no evidence for the occurrence of plasmid in *F. columnare* was found (Tekedar et al., 2012). Thus, plasmid may not associate with QR in *F. columnare.*

CHAPTER III

Materials and Methods

3.1 Bacterial isolates and growth conditions

Totally 50 isolates of *F. columnare* recovered from freshwater fish in Thailand were used in this study (Table 1). The name of *F. columnare* isolates were annotated as CUVET (CU: Chulalongkorn University, VET: Faculty of Veterinary Science). All isolates were the cultured collections of CU VET MICRO from the previous study, identified by biochemical tests, and confirmed the identification by molecular characteristics (Dong et al., 2014). One Vietnamese isolate recovered from diseased fish; CUVET1232/M1W was also included as an internal control in this study. All bacteria were cultured and incubated at 28°c for 48 h on Anacker and Ordal's (AO) or Cytophaga medium (Anacker and Ordal, 1955). Stock cultures were kept in AO broth supplemented with 20% glycerol and 30% bovine serum at in -80 °c until need. Bacterial colonies and morphologies including Gram staining were observed after obtaining from frozen and complete incubation.

Table 1. *Flavobacterium columnare* isolates used in the study.

	Bacteria	Host fish	Organs	Geno-movar	Isolation locality	Isolation year
1	CUVET1201	RT	Gill	II	Ratchaburi, Thailand	2012
2	CUVET1202	RT	Gill	II	Ratchaburi, Thailand	2012
3	CUVET1203	RT	Gill	II	Ratchaburi, Thailand	2012
4	CUVET1204	RT	Tail	II	Ratchaburi, Thailand	2012
5	CUVET1212	RT	Tail	II	Petchaburi, Thailand	2012
6	CUVET1213	RT	Kidney	II	Petchaburi, Thailand	2012
7	CUVET1214	RT	Gill	II	Petchaburi, Thailand	2012
8	CUVET1215	RT	Kidney	I	Petchaburi, Thailand	2012
9	CUVET1336	RT	Skin	II	Kanchanaburi, Thailand	2013
10	CUVET1338	RT	Gill	II	Kanchanaburi, Thailand	2013
11	CUVET1340	RT	Liver	II	Kanchanaburi, Thailand	2013
12	CUVET1341	RT	Gill	II	Kanchanaburi, Thailand	2013
13	CUVET1343	RT	Skin	II	Kanchanaburi, Thailand	2013

	Bacteria	Host fish	Organs	Geno-movar	Isolation locality	Isolation year
14	CUVET1344	RT	Gill	II	Kanchanaburi, Thailand	2013
15	CUVET1221	KC	Skin/Ulcer	II	Bangkok, Thailand	2013
16	CUVET1337	RT	Gill	II	Kanchanaburi, Thailand	2013
17	CUVET1339	RT	Gill	II	Kanchanaburi, Thailand	2013
18	CUVET1342	RT	Gill	II	Kanchanaburi, Thailand	2013
19	CUVET1345	RT	Gill	II	Chachoengsao, Thailand	2013
20	CUVET1346	RT	Gill	II	Chachoengsao, Thailand	2013
21	CUVET1347	RT	Gill	II	Chachoengsao, Thailand	2013
22	CUVET1348	RT	Gill	II	Chachoengsao, Thailand	2013
23	CUVET1349	RT	Gill	II	Chachoengsao, Thailand	2013
24	CUVET1350	RT	Skin	II	Chachoengsao, Thailand	2013
25	CUVET1351	RT	Skin	II	Chachoengsao, Thailand	2013
26	CUVET1352	RT	Gill	II	Chachoengsao, Thailand	2013
27	CUVET1353	RT	Gill	II	Chachoengsao, Thailand	2013
28	CUVET1354	RT	Skin	II	Chachoengsao, Thailand	2013
29	CUVET1355	RT	Skin	II	Chachoengsao, Thailand	2013
30	CUVET1356	RT	Skin	II	Chachoengsao, Thailand	2013
31	CUVET1357	RT	Skin	II	Chachoengsao, Thailand	2013
32	CUVET1358	RT	Skin	II	Chachoengsao, Thailand	2013
33	CUVET1359	RT	Gill	II	Kanchanaburi, Thailand	2013
34	CUVET1360	RT	Gill	II	Kanchanaburi, Thailand	2013
35	CUVET1361	RT	Kidney	II	Kanchanaburi, Thailand	2013
36	CUVET1362	RT	Gill	II	Ayutthaya, Thailand	2013
37	CUVET1363	RT	Gill	II	Ayutthaya, Thailand	2013
38	CUVET1364	RT	Gill	II	Ayutthaya, Thailand	2013
39	CUVET1365	RT	Gill	II	Ayutthaya, Thailand	2013
40	CUVET1367	RT	Kidney	II	Ayutthaya, Thailand	2013
41	CUVET1368	RT	Skin	II	Ayutthaya, Thailand	2013
42	CUVET1369	RT	Gill	II	Ayutthaya, Thailand	2013
43	CUVET1370	RT	Kidney	II	Ayutthaya, Thailand	2013
44	CUVET1374	RT	Gill	II	Ayutthaya, Thailand	2013
45	CUVET1375	RT	Gill	II	Ayutthaya, Thailand	2013
46	CUVET1376	RT	Skin	II	Ayutthaya, Thailand	2013
47	CUVET1377	RT	Gill	II	Ayutthaya, Thailand	2013

	Bacteria	Host fish	Organs	Geno-movar	Isolation locality	Isolation year
48	CUVET1378	RT	Gill	II	Ayutthaya, Thailand	2013
49	CUVET1379	RT	Skin	II	Ayutthaya, Thailand	2013
50	CUVET-BU1	NT	Skin	II	Chachoengsao, Thailand	2013
51*	CUVET1232 /M1W	SCF	Unknown	II	Cantho, Vietnam	Unknown

RT: Red tilapia *(Oreochromis* sp.*)*, KC: Koi carp *(Cyprinus carpio koi)*, SCF: Striped catfish *(Pangasius hypophthalmus)*, NT: Nile tilapia *(Oreochromis niloticus)*, (*): Internal control.

3.2 Antimicrobial susceptibility test

Two standard methods were used in this study, Disk diffusion method by Kirby-Bauer Disk Diffusion Susceptibility Test Protocol as a qualitative testing and broth microdilution for determining the minimum inhibitory concentrations (MICs) as a quantitative testing.

3.2.1 Antimicrobial agents

The following 6 antimicrobial agents that routinely used for the treatment of columnaris disease and other bacterial diseases in fish farming were used for disk diffusion testing; oxytetracycline, sulfamethoxazole/trimetroprime, ampicillin, erythromycin, florfenicol, and doxycycline (Oxoid, UK). Moreover, two antimicrobial agents in Quinolone groups; oxolinic acid, and nalidixic acid (Sigma, Italy) and three antimicrobial agents in Fluoroquinolone groups; enrofloxacin, norfloxacin and ciprofloxacin (Sigma, Italy) were also included.

3.2.2 Disk diffusion method

Antibiotic susceptibility of all *F. columnare* isolates were determined by disk diffusion method on 1:5 diluted Mueller-Hilton agar; DMHA (Oxoid, UK) (4g/L) with adding agar (Darwish et al., 2008) as suggested by the specific guideline document M42-A for gliding bacteria (Group 3) (CLSI, 2006b). Discs containing oxytetracycline 30µg, sulfamethoxazole/ trimetroprime 25µg, ampicillin 10µg, erythromycin 15µg, florfenicol 30µg, and doxycycline 30µg, oxolinic acid 10µg, nalidixic acid 30µg (quinolone group) including enrofloxacin 5µg, norfloxacin 10µg, and ciprofloxacin 5µg (fluoroquinolone group) (Oxoid, UK) were used in this study. All *F. columare* were

cultured and incubated at 28°c for 48 h on Anacker and Ordal agar (AOA) (Anacker and Ordal, 1955). Then pure colonies were swabbed and suspended in 3 ml of 0.85% normal saline by using sterile cotton swabs for adjusting the bacterial concentration to 1.5 x 10^8 colony-forming units (CFU) per ml (0.5 McFarland standard). Bacterial suspension were spread in 6-way cross streak on the DMHA's surface. The antimicrobial disks were placed on the dried plates for 3-4 discs per plate. All plates were incubated at 28 °C for 48 h. After incubation, the diameters of each inhibition zone were measured and recorded as the millimeter for interpretation. *E. coli* ATCC 25922 was included as reference strain for quality control.

3.2.3 Minimum inhibitory concentration (MIC) testing

The MICs of all *F. columnare* were determined by broth microdilution method according to the specific guideline document M49-A for gliding bacteria (Group 3) (CLSI, 2006a). All bacteria after subculture and incubating reach to the log phase of the growth (48 h) were inoculated on 1:5 Diluted Mueller-Hilton Broth (DMHB; 4g/L) with pH = 7.2. This medium was slightly modified for fastidious organisms including *F. columnare* (Darwish et al., 2008). All *F. columnare* were cultured in AO's broth at 28°c for 48 h before testing. The bacterial density was adjusted by swabbing the bacterial colonies into 3 ml of 0.85% normal saline by sterile cotton swabs. The turbidity of bacterial suspensions were adjusted to 0.08-0.10 at 625 nm equal to 0.5 McFarland standard suspension (1.5 x 10^8 CFU/ml) (Darwish, Farmer et al. 2008; CLSI 2006) by using Spectophotometer machine. The dilution of bacterial density as 1:100 to adjust the concentration of bacteria before inoculating into the wells, 300 µl of bacterial suspensions were transferred separately into 9.7 ml of 1:5 DMHB. The density of bacterial suspensions after dilution equal to 1.5 x 10^6 CFU/ml were added into the wells 1:1 with each antimicrobial comcentration. The final concentration of bacteria in each well were provided as standardized inocula to approximately 1.5 x 10^5 CFU/ml (Hesami et al., 2010).

Oxolinic acid and norfloxacin were prepared as stock solutions in concentrations of 10240 µg/ml by dissolving with 0.1 N NaOH. Stocks solutions were adjusted by sterile distilled water to 25 ml as the appropriate volume for stocking. The solutions were stored at -20°C before using. Then, stock solutions were diluted in two-fold dilution into 96-microtiter plates with 1:5 DMHB according to CLSI document

M49-A for 10 concentrations from 0.125 to 64 µg/ml for all ABOs (CLSI, 2006). Each well contained the ratio of bacterial suspensions and antimicrobial dilutions as 1:1, 100 µl of bacterial suspension and 100 µl of ABOs by using the multichannel pipette. The bacterial inoculums without ABOs and only DMHB without bacterial inoculums were included in each plate as positive and negative control, respectively. All plates were incubated at 28 °C for 48 h. The MIC was determined by recording the lowest concentration of antimicrobial agent with no visible growth of the bacteria in each well for the interpretation. *E. coli* ATCC 25922 was also included as reference strain for quality control. The tests were carried out in duplicate.

3.3 Detection of QRDR mutations

3.3.1 Genomic DNA preparation

Total genomic DNA of all *F. columnare* isolates were prepared by modified *boiled-extraction method (Bader et al., 2003; Figueiredo et al., 2005; Shah et al., 2012)*. Briefly, pure colonies of each isolate were cultured in 5 ml of AO's broth at 28 °C for 48 h. After incubation, the bacterial suspensions were centrifuged for 5 min at 10,000 rpm. The supernatant was discarded, and then the bacterial pellet was collected followed by dissolving in approximate 200 µl in sterile distilled water in microcentrifuge tube. The solution of bacteria was heated in water bath at 90 °C for 10 min. After heating, the bacterial mixture was rapidly cooled on ice for 5 min. Finally, all tubes were centrifuged for 5 min at 10,000 rpm. The supernatant was directly used as DNA template without further purification for PCR amplification. All DNA samples were stored in microcentrifuge tube at -20 °C until use.

3.3.2 Specific primer design

The target-specific primer pairs were designed in this study by manual design method according to manual supplement (Dieffenbach et al., 1993) base on complete genome sequence of type strain *F. columnare* ATCC 49512 (accession no. CP003222, version CP003222.2) (Tekedar et al., 2012) (Fig. 1). New PCR primers were designed as forward and reverse primers to amplify the partial sequence, which is putative QRDRs of each quinolone target gene; *gyrA, gyrB, parC* and *parE* of *F. colymnare* by using Alignment Explorer/CLUSTAL method of the Molecular

Evolutionary Genetics Analysis software version 5.2 (MEGA 5.2 program) package. The position of the predicted region as putative QRDR regions of were determined by following the prior sequences of other bacteria from previous studies, which have been published and submitted to GenBank database (Table 2). The expected sizes of each gene fragment and products on target templates were determined by NCBI/Primer-BLAST.

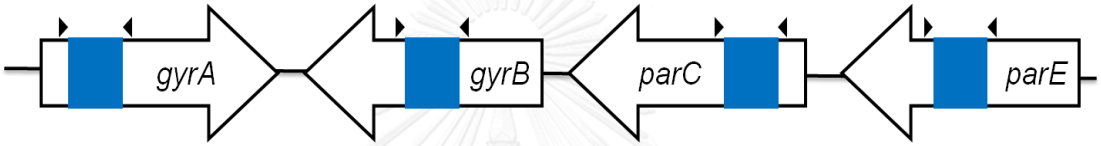


Figure 1. Each target gene (arrow-shaped boxes), flanking sequences (straight lines), putative QRDRs (blue boxed), and designed primers (black arrowheads) of type strain *F. columnare* ATCC 49512.

Table 2. GenBank accession numbers of QRDR sequences of *gyrA, gyrB, parC* and *parE* in other bacterial sequences from previous publications for specific primers design in this study.

QTGs	Bacterial species	Accession number	References
gyrA	*Flavobacterium.*	AB158102, AB158113, AB158121	(Izumi and Aranishi, 2004)
	psychrophilum	HQ113164, HQ113170, HQ113175	(Shah et al., 2012)
	Aeromonas sp.	AY027899, AY027900	(Goni-Urriza et al., 2002)
	Salmonella Enterica	GU190966, GU190967	(Hamidian et al., 2011)
gyrB	*F. psychrophilum*	HQ113195, HQ113200, HQ113203	(Izumi and Aranishi, 2004)
	Aeromonas sp.	AY027900, AY027902	(Goni-Urriza et al., 2002)
	S. Typhimurium	U30842	(Gensberg et al., 1995)
	S. Enterica	AB072393, AB072396	(Hirose et al., 2002)
parC	*F. psychrophilum*	HQ113183, HQ113184, HQ113180	(Izumi and Aranishi, 2004)
	Aeromonas sp.	JF343817	(Shakir et al., 2012)
	S. Enterica	AF435418	(Goni-Urriza et al., 2002)
parE	*F. psychrophilum*	FJ222661	(Dimitrov et al., 2009)
	Aeromonas sp.	HQ283333, HQ283334, HQ283343	(Izumi and Aranishi, 2004)
	S. Enterica	AF435421, AF435422	(Goni-Urriza et al., 2002)
		AB072701	(Hirose et al., 2002)
		KF649757	(Wasyl et al., 2014)

QTGs: quinolone target genes

3.3.3 Polymerase chain reaction (PCR) amplification and DNA sequencing

PCR programs used for QRDR amplifications were carried out according to manufacturer's instructions and slightly modified from previous public protocols (Izumi and Aranishi, 2004; Shah et al., 2012). The reaction mixtures and conditions were initially adjusted in this study. Initial denaturation at 95°C for 1-2 min; 30 cycles of denaturation at 90°C for 30 s, annealing for 30 s, extension at 72°C for 1 min, Final extension at 72°C for 3 min and final soak at 4°C. Gradient PCR was used in order to determine the optimum annealing temperature (Ta) for each new primer. The temperature used were calculated by ±10 values from calculated melting temperatures (Tm) on Primer-BLAST results, which were divided into 6 sets of temperature ranged from 44-56°C by using the thermal cycler (TC-96/G/H(b), BIOER). One *F. columnare* isolate, CUVET1232/M1W was used as a positive control according

to previous study (Ha, 2014). Negative control reaction with 3 another Gram-negative bacterial DNA from the CUVET MICRO laboratory cultured; *Escherichia coli* ATCC 29522*, Pseudomonas aeroginosa* ATCC 27853*,* and *Salmonella* Enteritidis VMCU11 were conducted for checking the specificity of the primers. PCR amplifications were performed in a total reaction of 50 µl in thin-wall tube with a thermal cycler. The reaction mixture contained 6 µl of DNA template (100-500 ng genomic DNA), 2 µl of 10 µM Forward primer, 2 µl of 10 µM Reverse primer, 2.5 mM $MgCl_2$, 15 µl of Nuclease-Free H_2O and 2 units of Taq polymerase. Negative controls without adding DNA template were included. All PCR products were checked on 1% agarose-TBE gel by staining with RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology, Korea) with M25 DNA Ladder (SibEnzyme, Russia) via electrophoresis (100 V, 30 min). The results of PCR amplification were visualized under UV light of the gel documentation system (VilberLourmat, France). The PCR products were purified for DNA sequencing by using Nucleospin® Extract II Kit (Macherey-Nagel, Germany). Finally all products were sent for sequencing by 1st BASE DNA Sequencing Services (Malaysia). Some of *F. columnare* isolates were selected for sequencing according to MIC of OA to 3 groups as OA-sensitive isolate, OA-intermediate isolate, and OA-resistant isolate.

3.3.4 Analysis of DNA sequences

The results of QRDR sequences of *gyrA, gyrB, parC* and *parE* from *F. columnare* isolates were assembled by using BioEdit Program and blasted by using Nucleotide BLAST program (National Center for Biotechnology Information; NCBI). The results of mutation and amino acid substitutions of all selected sequences were determined by using Alignment Explorer/CLUSTAL method and Translate Protein Sequences of MEGA 5.2 program, respectively. The sequence of one OA-sensitive *F. columnare* isolate with lowest OA-MIC was used to compare the putative mutation including results of amino acid substitutions with another *F. columnare* isolates. The reference strain *E. coli* K-12 MG1655 (accession no. X57174) was also used for determining the amino acid positions according to *E. coli* numbering system.

3.4 Data analysis

The antimicrobial susceptibility pattern by disk diffusion and the MIC determinations by broth microdilution of *F. columnare* and *E. coli* ATCC 25922 were interpreted following the CLSI document (CLSI, 2012) and previous studies. The occurrence of QR in *F. columare* was calculated from the recorded data of resistant isolates number divided by total isolates number as the percentage (%) followed both results of disk diffusion (OA and NA) and MIC (OA). The Q-susceptibility of *F. columnare* was classified the level of QR according to MIC of OA as low-resistance (4-8 µg/ml) and high-resistance (>8 µg/ml) (Turnidge and Paterson, 2007; Henriquez-Nunez et al., 2012). WHONET software was used for the analysis and management of MIC of OA as MIC_{50} and MIC_{90}. Specificity of the designed primers were evaluated by using NCBI/Primer-BLAST (Ye et al., 2012), the results from PCR amplification (Chuang et al., 2013) and the DNA sequencing results. The correlation between MIC of OA and the mutations including amino acid substitution results from QRDR sequences of each gene were determined by MEGA 5.2 program (Stelling and OBrien, 1997). The nucleotide sequence of one OA-sensitive isolate in this study was used as basis to compare the mutation to all isolates.

CHAPTER IV

Results

4.1 Antimicrobial susceptibility test

4.1.1 Disk diffusion method

The antimicrobial susceptibility by disk diffusion method of all *F. columnare* isolates to different antimicrobial agents was performed in Table 3. All isolates were sensitive to oxytetracycline (OT), Sulfamethoxazole/trimetroprime (SXT), florfenicol (FFC), and doxycycline (DO), which are routine drug use out of quinolones and fluoroquinolones in aquaculture treatment. Few isolates performed intermediate to only 2 from 6 antimicrobial agents, ampicilin (AMP) and erythromycin (E), respectively. No resistant isolates found in first group of 6 antimicrobial agents tested. Interestingly, for the drug tested in quinolone group. Oxolinic acid (OA), which is more often quinolone drug use in aquaculture than nalidixic acid (NA) performed higher percentage of both intermediate and resistant in all isolates, 10% and 22%, respectively. Moreover, the minority were also intermediate and resistant to all quinolones tested. For the fluoroquinolones tested, all isolates were sensitive to only ciprofloxacin (CIP), which is rather using in human clinic than aquaculture whereas, norfloxacin (NOR) and ENR showed only intermediate results: 8% and 4%, respectively. No fluoroquinolones-resistant isolates found in this study. The inhibition zone diameters of *E. coli* ATCC 29522 to each antimicrobial agent in this study were in acceptable range according to the CLSI document and previous study. Size of Inhibition zone diameter of *F. columnare* isolates to various kind of antimicrobial compounds were distribute and different. OT showed the largest size of the average inhibition zone diameter, whilst both NA and OA (quinolones) showed the lowest size related to the intermediate and resistant results. For another antimicrobial compounds, the size of zones performed median sizes vary from 29-39 mm. The pattern of antimicrobial susceptibility of all isolates tested was shown in Table 4. Nine and four isolates were resistant to only OA and NA, respectively, whilst three isolates: CUVET1344, 1349, and 1350 were resistant to both OA and NA. According to antimicrobial susceptibility by agar disk diffusion, only quinolones and

fluoroquinolones showed the high percent isolates of intermediate and resistant results on *F. columnare* isolates compare with another antimicrobial agents in routine drug use. Thus, the occurrence of QR was 14% from NA-resistant and 22% from OA-resistant. The MICs of quinolones and fluoroquinolones by broth microdilution were carried out in the next part.

Table 3. The antimicrobial susceptibility of 50 *F. columnare* isolates by disk diffusion method.

Name of antibiotics	Disk content	Average IZD	Results from agar disk diffusion test					
			Sensitive		Intermediate		Resistant	
			n	%	n	%	n	%
Oxytetracycline (OT)	30 µg	40.9	50	100	0	0	0	0
Sulfamethoxazole/ trimetroprime (SXT)	25 µg	29.3	50	100	0	0	0	0
Ampicillin (AMP)	10 µg	31.7	45	90	5	10	0	0
Erythromycin (E)	15 µg	32.3	49	98	1	2	0	0
Florfenicol (FFC)	30 µg	35.8	50	100	0	0	0	0
Doxycycline (DO)	30 µg	39.7	50	100	0	0	0	0
Nalidixic acid[a] (NA)	10 µg	23.8	40	80	4	8	7	14
Oxolinic acid[a] (OA)	10 µg	23.6	34	68	5	10	11	22
Norfloxacin[b] (NOR)	10 µg	27.6	46	92	4	8	0	0
Enrofloxacin[b] (ENR)	5 µg	38.3	48	96	2	4	0	0
Ciprofloxacin[b] (CIP)	5 µg	34.8	49	98	1	2	0	0

IZD: Inhibition zone diameter (millimeter); n: number of isolates; %: percentage isolates; [a] quinolone group; [b] fluoroquinolone group

Table 4. The antimicrobial susceptibility pattern of *F. columnare* isolates.

Isolate number (CUVET)	n	Types of pattern		
		Sensitive	Intermediate	Resistant
1201,1202,1215	3	OT,SXT,E,FFC,DO	AMP	-
1203	1	OT,SXT,E,FFC,DO	AMP, NOR	OA
1204	1	OT,SXT,E,FFC,DO	AMP, NOR,NA	OA
1343	1	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP	NA	OA
1344,1349,1350	3	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP	-	NA,OA
1346,1347	2	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP	OA	NA
1348	1	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP,NA	OA	-
1339,1355,1374,1376,1379	5	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP,NA	-	OA
1360	1	OT,SXT,AMP,E,FFC,DO, NOR,ENR,CIP,OA	NA	-
1361	1	OT,SXT,AMP,E,FFC,DO,ENR,CIP,NA	NOR,OA	-
1362	1	OT,SXT,AMP,E,FFC,DO,ENR,CIP,NA,OA	NOR	-
1375	1	OT,SXT,AMP,FFC,DO, NOR,ENR,OA	E,CIP,NA	OA
1377,1378	2	OT,SXT,AMP,E,FFC,DO, NOR,CIP,NA,OA	ENR	NA
1212,1213,1214,1336,1338,1340,1341,1221,1337, 1342,1345,1351,1352,1353,1354,1356,1357,1358, 1359,1363,1364,1365,1367,1368,1369,1370,-BU1	27	OT,SXT,AMP,E,FFC,DO,NOR,ENR,CIP,NA,OA	-	-

OT: oxytetracycline, SXT: sulfamethoxazole/trimetroprime, AMP: ampicillin, E: erythromycin, FFC: florfenicol, DO: doxycycline, ENR: enrofloxacin, CIP: ciprofloxacin, NA: nalidixic acid, OA: oxolinic acid, NOR: norfloxacin, n: number of isolates.

4.1.2 Minimum inhibitory concentrations

The MIC values of oxolinic acid and norfloxacin of reference strain *E. coli* ATCC 25922 were both sensitive and fell into acceptable ranges according to CLSI document and previous study (0.25 µg/ml and 0.25 µg/ml, respectively). The MIC determinations of 50 *F. columnare* isolates to oxolinic acid and norfloxacin were shown in Table 5. The MIC values of OA was varied from <0.125 µg/ml (1 isolate, CUVET 1213) to 16 µg/ml, which were wider than MIC values of NOR. The lowest and highest MIC of NOR were 0.125 and 8 µg/ml, respectively. MIC distributions of OA and NOR of all *F. columnare* isolates was performed in Fig 2. Each concentration performed different percent isolates between 2 antimicrobial agents. For NOR, over a half of MIC value were less than or equal to 1 µg/ml (68%) whilst 70% of MIC value of OA were between 1-4 µg/ml, which was higher both percentage and the range of value than NOR. The highest percent isolates of OA and NOR were the same MIC value equal 1 µg/ml, but percent of OA was higher than NOR as 32% and 22%, respectively. All MIC values were analyzed by WHONET software and summarized in Table 6. MIC_{50} of OA and NOR were equal 1 µg/ml, however, MIC_{90} of OA was higher than NOR as 8 and 4 µg/ml, respectively. The geometrical mean or average and percent resistant 95% confidence interval of MIC of OA was higher than NOR. Moreover, out of 50 isolates, 16 were classified the level of quinolone resistance by MIC of OA into 2 groups as low group and high group. 14 isolates were belonged to low group with MIC = 4-8 µg/ml and 2 isolates were belonged to high group with MIC = 16 µg/ml. For overall quinolone resistance determination according MIC results, percent of resistant and intermediate to OA were 3 and 2 folds times higher than NOR, respectively (Fig. 3). The highest percent of interpretations of all group was sensitive to both OA and NOR. The occurrence of QR calculated from MIC of OA was 32%.

Table 5. Minimum inhibitory concentrations (MICs) of oxolinic acid and norfloxacin on 50 *F. columnare* isolates.

Isolate no.	MIC (µg/ml)		Isolate no.	MIC (µg/ml)	
	OA	NOR		OA	NOR
CUVET 1213	<0.125	0.5	CUVET 1369	2	0.5
CUVET 1360	0.125	0.25	CUVET 1201	2	1
CUVET 1359	0.25	0.25	CUVET 1202	2	1
CUVET 1363	0.25	0.5	CUVET 1338	2	0.125
CUVET 1364	0.25	0.5	CUVET 1340	2	0.125
CUVET 1365	0.25	0.25	CUVET 1341	2	0.25
CUVET 1367	0.25	0.25	CUVET 1342	2	0.25
CUVET 1336	0.5	0.25	CUVET 1214	2	0.5
CUVET 1354	0.5	0.5	CUVET 1243	2	0.25
CUVET 1212	1	0.125	CUVET 1361	4	4
CUVET 1215	1	0.125	CUVET 1337	4	0.125
CUVET 1221	1	1	CUVET 1346	4	4
CUVET 1345	1	1	CUVET 1347	4	4
CUVET 1351	1	2	CUVET 1348	4	8
CUVET 1352	1	0.5	CUVET 1349	4	4
CUVET 1353	1	1	CUVET 1350	4	4
CUVET 1356	1	1	CUVET 1355	4	4
CUVET 1357	1	0.5	CUVET 1203	4	8
CUVET 1358	1	0.5	CUVET 1204	4	8
CUVET 1362	1	4	CUVET 1374	8	2
CUVET 1368	1	0.25	CUVET 1375	8	2
CUVET 1370	1	1	CUVET 1376	8	1
CUVET 1377	1	2	CUVET 1379	8	1
CUVET 1378	1	1	CUVET 1339	16	2
CUVET-BU1	1	1	CUVET 1344	16	4

OA: oxolinic acid, NOR: norfloxacin, µg: microgram, ml: milliliter, no: number.

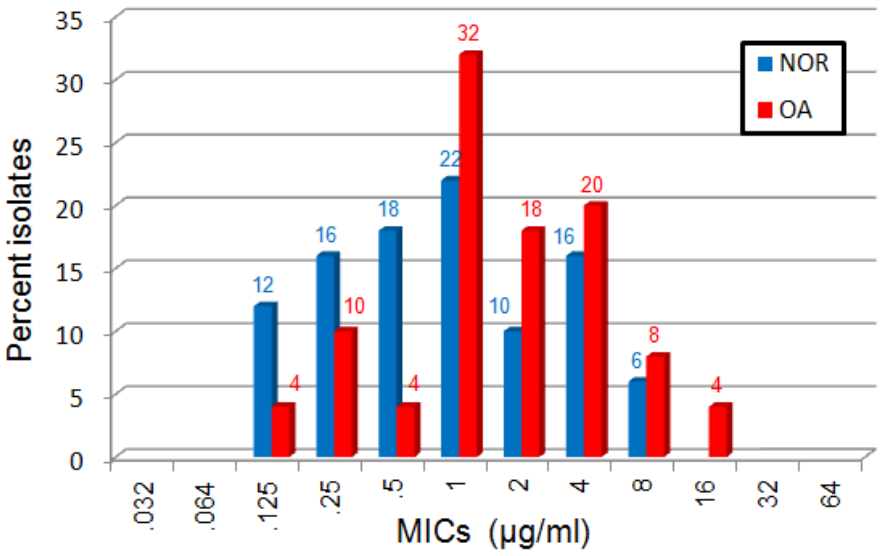


Figure 2. MIC distributions of oxolinic acid (OA) and norfloxacin (NOR) on 50 *F. columnare* isolates

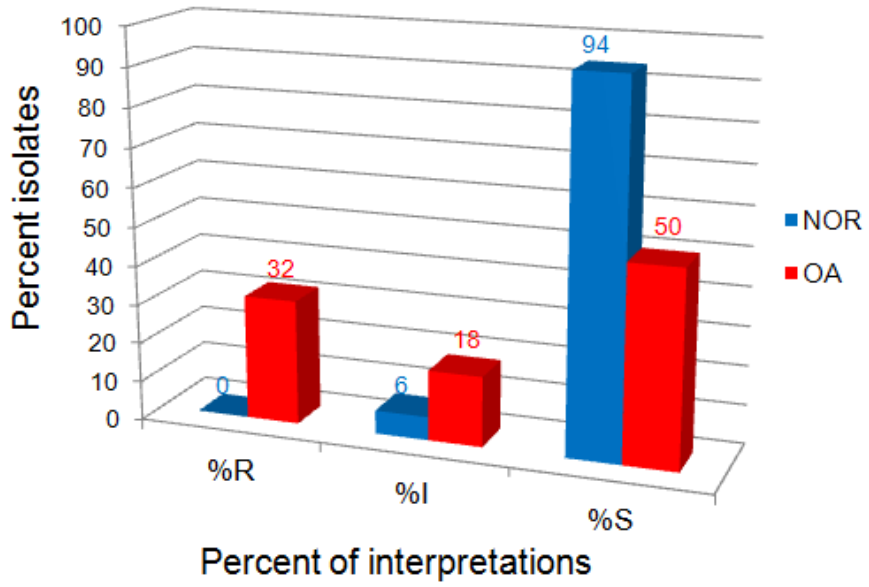


Figure 3. Percent of resistant-intermediate-sensitive (%RIS) by following MIC of oxolinic acid and norfloxacin on 50 *F. columnare* isolates.

Table 6. Analysis results base on MIC values (μg/ml) of 50 *F. columnare* isolates by WHONET software and level of quinolone resistance classified by MIC of OA.

Antimicrobial agent	Break-point	MIC_{50}	MIC_{90}	Geom. Mean	MIC Range	No. of isolates classified by level of OA-resistant	
						Low-QR*	High-QR**
OA	S ≤ 1	1	8	1.516	0.125	14	2
	R ≥ 4				– 16		
NOR	S ≤ 4	1	4	0.847	0.125	-	-
	R ≥16				– 8		

R: Resistant, C.I.: Confidence Intervals, MIC = minimum inhibitory concentration, Geom.: Geometric, OA: oxolinic acid, NOR: norfloxacin, S: sensitive, R: Resistance, *4-8 μg/ml, **(>8 μg/ml).

4.2 Specificity of designed primers by Primer-BLAST

The designed PCR primers used in this study were listed in Table 7. The specificity of each primer was firstly estimated by Primer-BLAST. In Primer-BLAST web interface, nr database were set with the limited organism to *Flavobacterium* sp. All primer pairs for amplification of *gyrA, gyrB, parC,* and *parE* expected regions were correspond to quinolone target genes (DNA gyrase and topoisomerase IV) of the type strain *F. columnare* ATCC 49512 on GenBank database under accession number CP003222; DNA gyrase subunit A, DNA gyrase subunit B, DNA topoisomerase IV subunit A, and DNA topoisomerase IV subunit B, respectively (Fig. 4).

Table 7. The designed primers used for QRDR amplifications of *gyrA, gyrB, parC* and *parE* from *F. columnare* isolates in this study.

Primers	Primer sequence (5'→3')	Nucleotide positions*	Size of amplicon (bp)**	Annealing Temperature (°C)***
GyrA FC_F	GAGCGTTACCAGATGTTAGAG			
GyrA FC_R	TGGCCATACCAACGGCAATA	92 - 535	444	55
GyrB FC_F	CGATCTCGTCCTTGTTTGGC			
GyrB FC_R	ATACGCACGAAGGAGGAACA	599 - 1094	493	48
ParC FC_F	TCGCAAGCTGTGAAGGCATA			
ParC FC_R	CCTGATGCTACGATTACCAAG	1697 - 2581	884	48
ParE FC_F	GCATTACGTCGTTCCTCATCAC			
ParE FC_R	TACAAGCAGAACGCGAACGT	245 - 786	541	55

* Putative QRDRs base on type strain *F. columnare* ATCC 49512 position (included primers)

** Predicted sizes of PCR product by Primer-BLAST *** The optimum temperatures were chosen from Gradient PCR

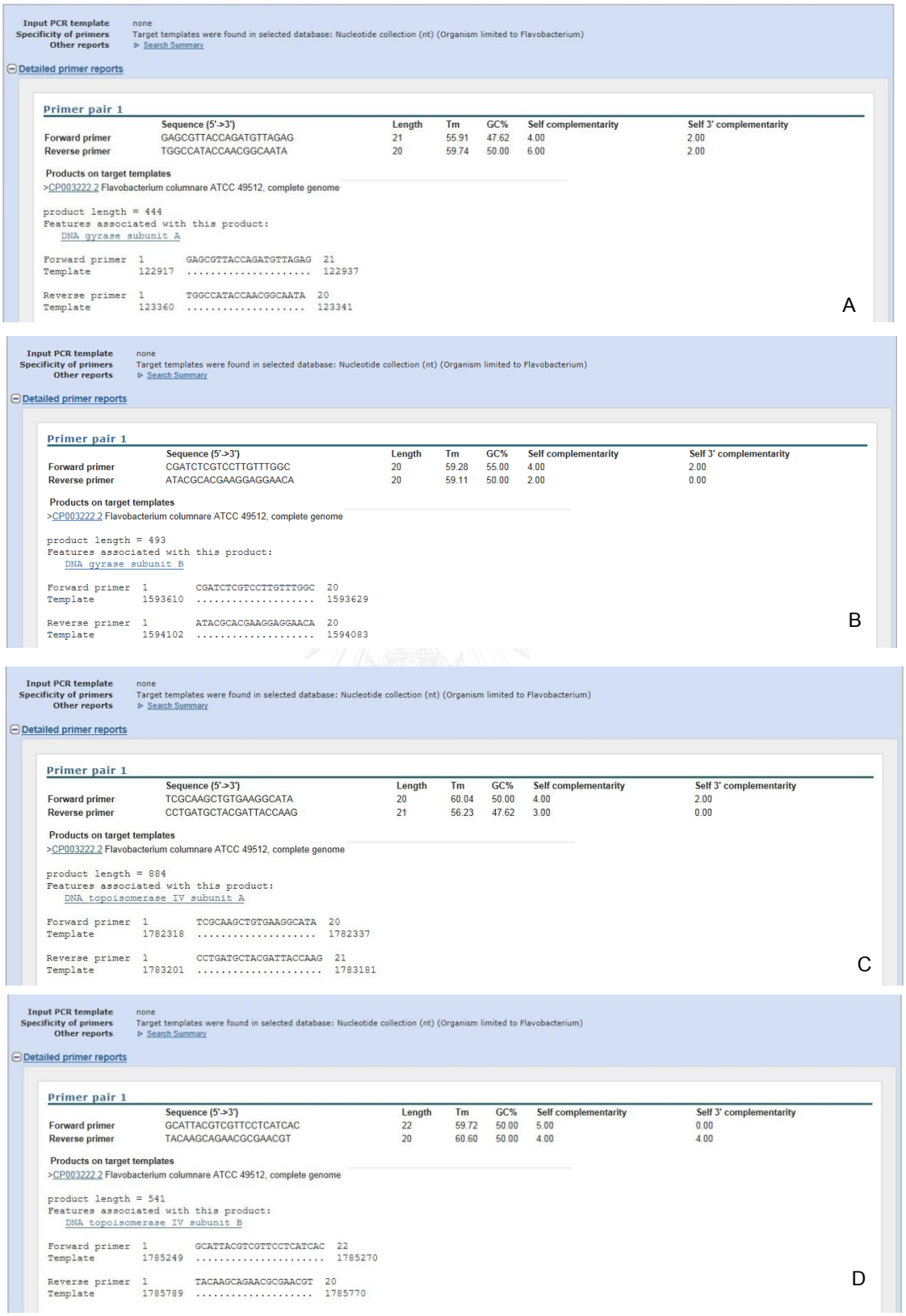


Figure 4. Specificity of designed primers evaluated by Primer-BLAST: Primers pairs for QRDR amplifications of *gyrA* (A), *gyrB* (B), *parC* (C), and *parE* (D).

The necessary design considerations of primers were evaluated for the appropriate condition before developing the process of PCR amplification. For the primers' detail, the important parameters such as primer length, %GC content, melting temperature (Tm), and self complementary from Primer-BLAST result were in acceptable values followed the standard considerations (Chuang et al., 2013). The expected product lengths of 4 amplicons were performed to measure the predicted size on target DNA in PCR steps. Other specifications of primer pairs as PCR amplification and DNA sequencing results were described in next part.

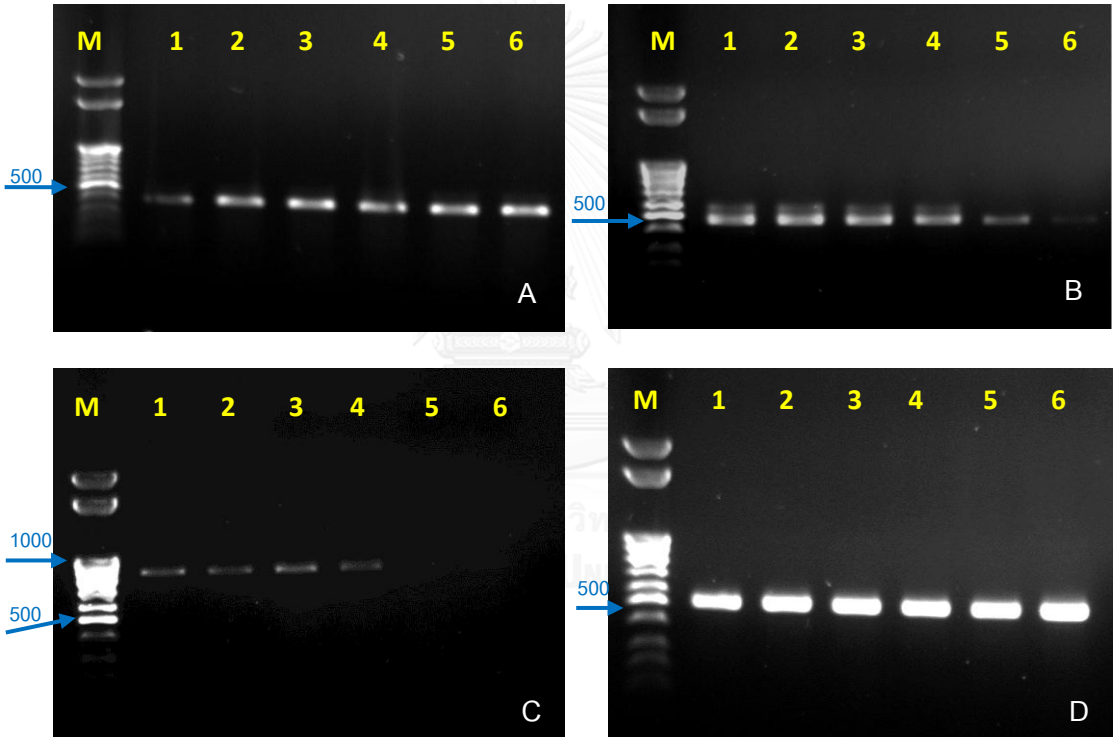


Figure 5. Gradient PCR by designed primers for QRDR amplifications of *F. columnare* positive isolate, CUVET 1232/M1W generated one amplified band by different 6 annealing temperatures used. Approximately 440 bp of *gyrA* (A), 880 bp of *parC* (C), and 540 bp of *parE* (D) amplicons, Lane M: marker; Lane 1-6: 48°C, 49°C, 51°C, 53°C, 55°C, and 56°C, respectively; Approximately 490 bp of *gyrB* (B) amplicons, Lane M: Marker; Lane 1-6: 43°C, 44°C, 45°C, 47°C, 48°C, and 50°c, respectively

4.3 PCR amplification

In order to find the optimum annealing temperature (Ta) of each new designed primer, gradient PCR were performed in Fig 5. One amplified band was presented in the amplification of each primer used without unspecific band. The designed primers for amplification of QRDRs of *gyrA, gyrB, parC,* and *parE* were successfully amplified the expected products of the genes. Approximately 450 bp of *gyrA* and 490 bp of *gyrB* fragments were amplified by designed primers for *gyrA* QRDR and *gyrB* QRDR amplifications; GyrA FC_F, GyrA FC_R, GyrB FC_F, and GyrB FC_R, respectively. In addition, designed primers for amplifications of *parC* QRDR and *parE* QRDR; ParC FC_F, ParC FC_R, ParE FC_F, and ParE FC_R could amplify the target gene fragments approximately 880 bp of *parC* and 540 bp of *parE*, respectively. All amplified bands of each target gene showed the different intensities vary from low to high temperature range used. The strong amplified band presented in low temperature range as 48-53 °C in the amplification of *gyrB* QRDR and *parC* QRDR. On the contrary, the high-intensity band presented in high temperature range as 53-56°C of *gyrA* QRDR amplification, whilst primers for amplifying *parE* QRDR generated strong band in all temperature range used. Thus, the optimum Ta of 4 primer pairs which were chosen to amplify to QRDR sequences of each quinolone target gene from all *F. columare* isolates, were listed in Table 7 according to the presence of amplified band from each temperature used with highest yield and the absent of unspecific band.

The specificity of each designed primer was also considered by single PCR amplifications. The QRDR sequences of quinolone target genes from all *F. columare* isolates were obtained by following the determination of the optimum Ta. DNA fragments approximately 440 bp, 490 bp, 880 bp, and 540 bp of *gyrA, gyrB, parC,* and *parE*, respectively from all *F. columnare* isolates were amplified with the product sizes (Fig. 6) correlated with their results from Primer-BLAST (Fig. 4). Three bacterial DNAs from other Gram-nagative bacteria used in this study; *E. coli, P. aeruginosa*, and *S.* Enteritidis were not amplified by designed primers of each target gene (Fig. 7).

4.4 DNA sequencing

The QRDRs of *gyrA, gyrB, parC,* and *parE* of *F. columnare* isolates were amplified by single PCR by designed primers. Totally 27 isolates of *F. columnare* were selected for sequencing according to MIC of OA as 8 OA-sensitive isolates (one isolate with the lowest MIC of OA <0.125 µg/ml, CUVET1213 was used as quinolone-susceptible isolate for comparison of the mutation), 4 OA-intermediate isolates, and 15 OA-resistant isolates. The visible yield of amplicons approximately 440 bp, 490 bp, 880 bp, and 540 bp, which covered QRDRs of *gyrA, gyrB, parC,* and *parE* , respectively of *F. columnare* isolates were determined the nucleotide sequences by capillary electrophoresis method.

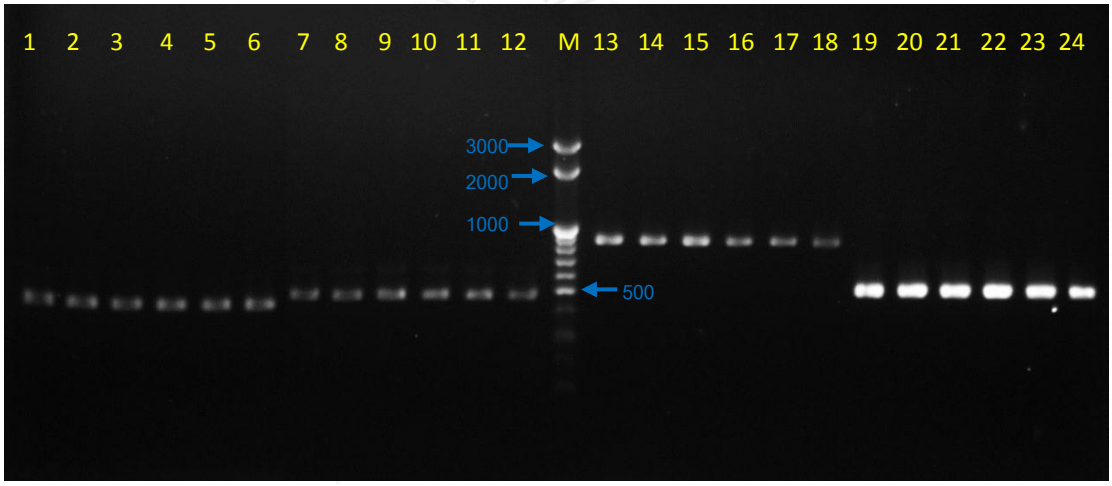


Figure 6. PCR amplification of QRDR amplifications of *F. columnare* isolates, Approximately 440 bp of *gyrA,* 490 bp of *gyrB*, 880 bp of *parC*, and 540 bp of *parE* amplicons were generated by designed primers. Lane M: marker, Lane 1,7,13,19: CUVET 1203, Lane 2,8,14,20: CUVET 1204, Lane 3,9,15,21: CUVET 1212, Lane 4,10,16,22: CUVET 1221, Lane 5,11,17,23: CUVET 1337, and Lane 6,12,18,24: CUVET 1350, respectively.

.

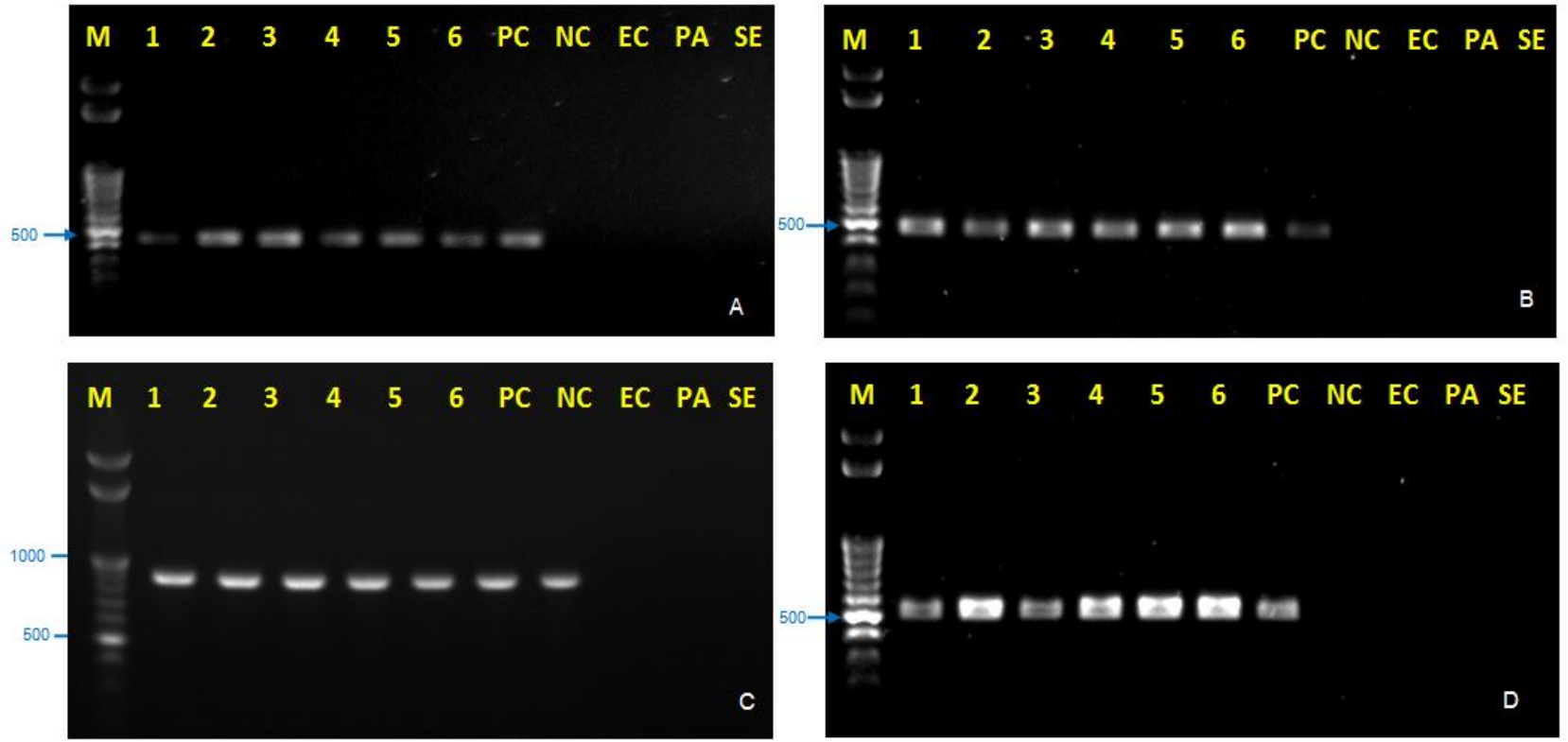


Figure 7. Specificity of designed primers by PCR amplification of QRDR sequences of *gyrA* (A), *gyrB* (B), *parC* (C), and *parE* (D) from *F. columnare* isolates. Lane M: Marker; Lane 1-6: CUVET 1213, CUVET 1221, CUVET 1237, CUVET 1361, CUVET 1204, and CUVET 1339, respectively; Lane PC: Positive control, CUVET 1232/M1W ; Lane NC: Negative control (distilled water); Lane EC: *E. coli* ATCC 29522; Lane PA: *P. aeruginosa* ATCC 27853; Lane SE: *S.* Enteritidis VMCU11.

4.5 DNA and amino acid sequences analysis

The nucleotide sequences of *gyrA, gyrB, parC, and parE* QRDRs of *F. columnare* isolates were separately checked the quality and assembled using ContigExpress. Each gene sequence was inserted, aligned, and translated to amino acid using MEGA 5.2 program for the interpretation of mutation associated with quinolone resistance.

For the partial sequences analysis of DNA gyrase subunits, the gene fragments of *gyrA* and *gyrB* amplified from *F. columnare* isolates were corresponded to nucleotide positions 116 to 518 and 874 to 1297, respectively. According to *E. coli* numbering system, *gyrA* QRDR encoded 135 amino acids between positions 39 to 173, while *gyrB* QRDR encoded 150 amino acids between positions 291 to 441 (excluding primer sequences). The nucleotide sequences of *gyrA* and *gyrB* from sensitive isolates were identical to type strain, *F. columnare* ATCC 49512 (CP003222.2) with identities at 93% of *gyrA* fragment and 88% of *gyrB* fragment, respectively. The deduced amino acid sequence of *gyrA* was 99% identical with DNA gyrase subunit A (WP_014164241.1), while for *gyrB* was 99% identical with DNA gyrase subunit B (BAG16316.1) of *F. columnare* ATCC 49512.

In part of partial sequences analysis of topoisomerase IV subunits, the gene fragments of *parC* and *parE* amplified from *F. columnare* isolates were corresponded to nucleotide positions 148 to 990 and 1099 to 1597, respectively. The nucleotide sequences of *parC* QRDR had 84% identity with the corresponding *parC* fragment of type strain, *F. columnare* ATCC 49512 (CP003222.2), however, *parE* QRDR had 84% identity with the corresponding *parE* fragment of *F. branchiophilum* FL-15 (FQ859183.1). QRDR sequences of *parC* and *parE* encoded 281 amino acids between positions 50 to 330 and 167 amino acids between positions 367 to 533 in *E. coli* numbering system, respectively (excluding primer sequences). The deduced amino acid sequence of *parC* was 95% identical with DNA topoisomerase subunit A (WP_014165635.1), while for *parE* was 99% identical with DNA topoisomerase subunit B (WP_014165638.1) of *F. columnare* ATCC 49512.

Table 8. Susceptibility to oxolinic acid and mutations detected responsible for amino acid substitution in QRDRs of 4 quinolone target genes from selected 27 *F. columnare* isolates for sequencing analysis.

F. columnare isolates	MIC		Mutation in QRDRs [a]							
	OA (µg/ml)	Level	*gyrA*		*gyrB*		*parC*		*parE*	
			Base change	Aa change with position	Base change	Aa change with position	Base change	Aa change with position	Base change	Aa change with position
CUVET 1360	0.125	S	-	-	-	-	-	-	-	-
CUVET 1336	0.5	S	-	-	-	-	-	-	-	-
CUVET 1212	1	S	-	-	-	-	-	-	-	-
CUVET 1221	1	S	-	-	-	-	-	-	-	-
CUVET 1362	1	S	-	-	-	-	-	-	-	-
CUVET 1268	1	S	-	-	-	-	-	-	-	-
CUVET-BU1	1	S	-	-	-	-	-	-	-	-
CUVET 1338	2	I	-	-	-	-	-	-	-	-
CUVET 1214	2	I	-	-	-	-	-	-	-	-
CUVET 1237	2	I	-	-	-	-	-	-	-	-
CUVET 1361	2	I	-	-	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1343	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1346	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1347	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-

F. columnare isolates	MIC		Mutation in QRDRs [a]							
	OA (μg/ml)	Level	*gyrA*		*gyrB*		*parC*		*parE*	
			Base change	Aa change with position	Base change	Aa change with position	Base change	Aa change with position	Base change	Aa change with position
CUVET 1348	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1349	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1350	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1355	4	L-QR	TCT→TTT	Ser83-Phe	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1203	4	L-QR	TCT→TAT	Ser83-Tyr	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1204	4	L-QR	TCT→TAT	Ser83-Tyr	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1374	8	L-QR	TCT→GCT	Ser83-Ala	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1375	8	L-QR	TCT→GCT	Ser83-Ala	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1376	8	L-QR	TCT→GCT	Ser83-Ala	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1379	8	L-QR	TCT→GCT	Ser83-Ala	-	-	CAC→TAC	His87-Tyr	-	-
CUVET 1339	16	H-QR	TCT→GCT	Ser83-Ala	-	-	CAC→CAT	-	-	-
CUVET 1344	16	H-QR	TCT→GCT	Ser83-Ala	-	-	CAC→CAT	-	-	-

MIC: minimum inhibitory concentration, Aa: amino acid, L-QR: Low quinolone-resistant, H-QR: High quinolone-resistant, OA: oxolinic acid, Ser: Serine, Phe: Phenylalanine, Tyr: Tyrosine, Ala: Alanine, His: Histidine

[a] Dashes indicate that the DNA and amino acid sequences were the same as those of one OA-sensitive ***F. columnare*** **isolate CUVET 1213 (OA-MIC < 0.125 μg/ml)** in this study.

The MIC of OA with levels of resistance and the mutations detected as well as amino acid substitutions were summarized in Table 8. One OA-sensitive *F. columnare* isolate, CUVET 1213 used as basis for the comparison of mutations to other selected isolates was not shown in the table. The mutations in QRDRs of quinolone target genes in *F. columnare* isolates were correlated with MIC values of OA. For eight OA-susceptible isolates including isolate number CUVET 1213, no mutations were found in all QRDRs including three OA-intermediate isolates tested. Interestingly, one of four quinolone-intermediate isolates, CUVET 1361 carried one point mutation responsible for amino acid substitution in codon 87 of *parC* QRDR; His-87 to Tyr (CAT instead of CAC), while no mutation detected in another QRDRs of this isolate (Fig 8).

Moreover, the mutations in the QRDRs of *gyrA* and *parC* of quinolone-resistant isolates were related to the level of resistance classified by MIC of OA. Thirteen OA-resistant isolates revealed double point mutations resulting in amino acid substitutions in the QRDRs of *gyrA* at position 83 and *parC* at position 87 except for 2 high-resistant isolates (MIC of OA = 16 µg/ml), which had only *gyrA* mutation without changing in *parC*. The derived amino acids of *parC* from thirteen low-resistant isolates (OA-MIC = 4-8 µg/ml) performed the same substitutions in codon 87 as one quinolone-intermediate isolate, otherwise the deduced amino acid sequences in codon 83 of *gyrA* represented the difference of amino acid substitutions related the MIC values of OA. Out of 13 low-resistant isolates, 7 (MIC = 4 µg/ml) had a Ser-83 → Phe mutation (TTT instead of TCT), 2 (MIC = 4 µg/ml) had a Ser-83 →Tyr mutation (TAT instead of TCT), and 4 (MIC = 8 µg/ml) had a Ser-83 → Ala mutation (GCT instead of TCT as well as 2 high-resistant isolates (MIC = 16 µg/ml) (Fig 10). Furthermore, double silent mutations were detected in *gyrA* QRDR of 2 high-resistant isolates; CUVET 1339 and CUVET 1344 resulting in base substitutions of C for T at bp 240 and bp 264 without changing in amino acid sequences, whereas a Ser-83 → Ala mutation was also detected. No mutations in were detected in the QRDRs of *gyrB* and *parE* in all isolates tested.

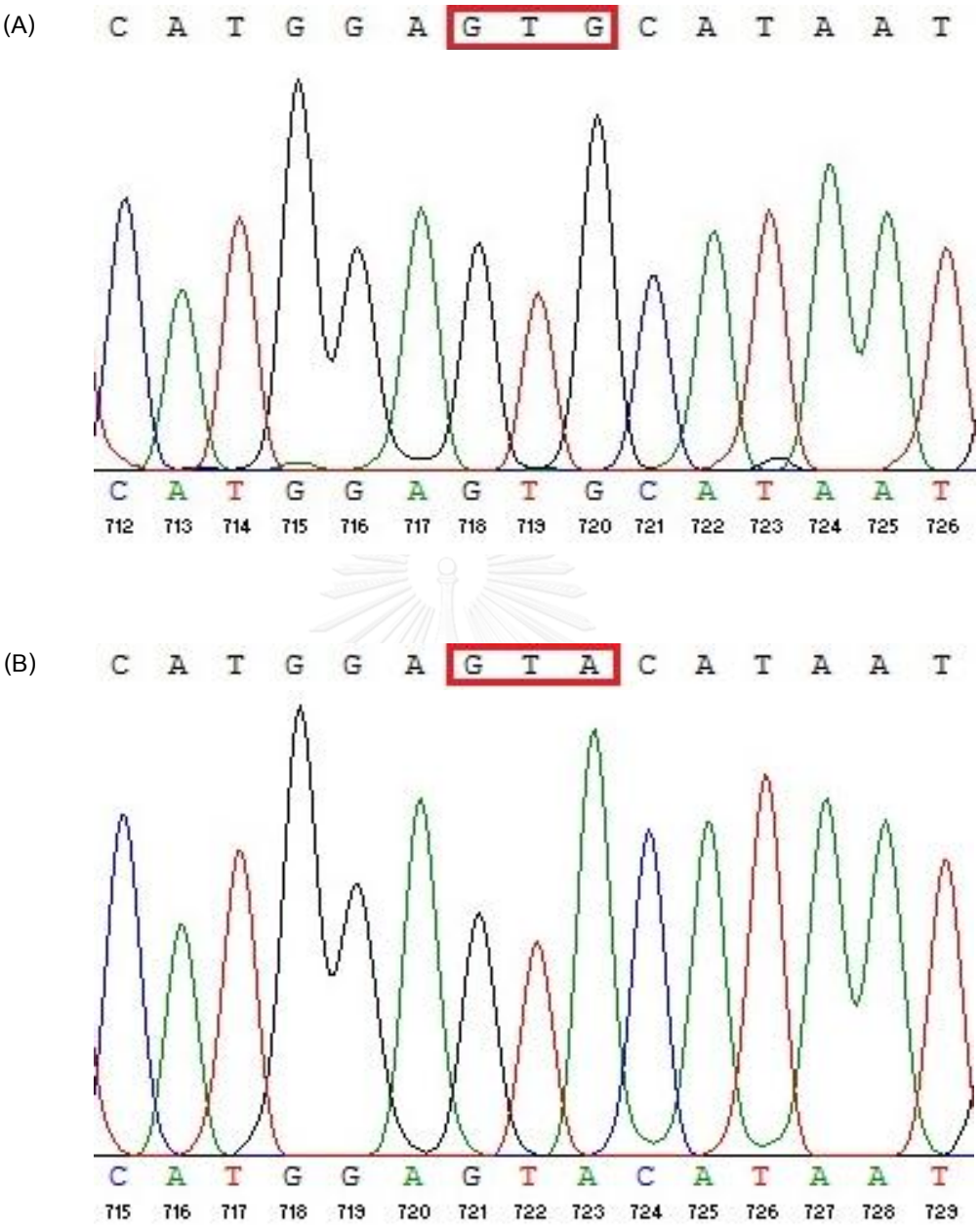


Figure 8. The chromatogram from ContigExpress showed the difference of *parC* QRDR sequences without reverse transcription process of one oxolinic acid-sensitive *F. columnare* isolate CUVET1213 (A), and low- and high-resistant isolate (B) at position 87. (GTG → GTA)

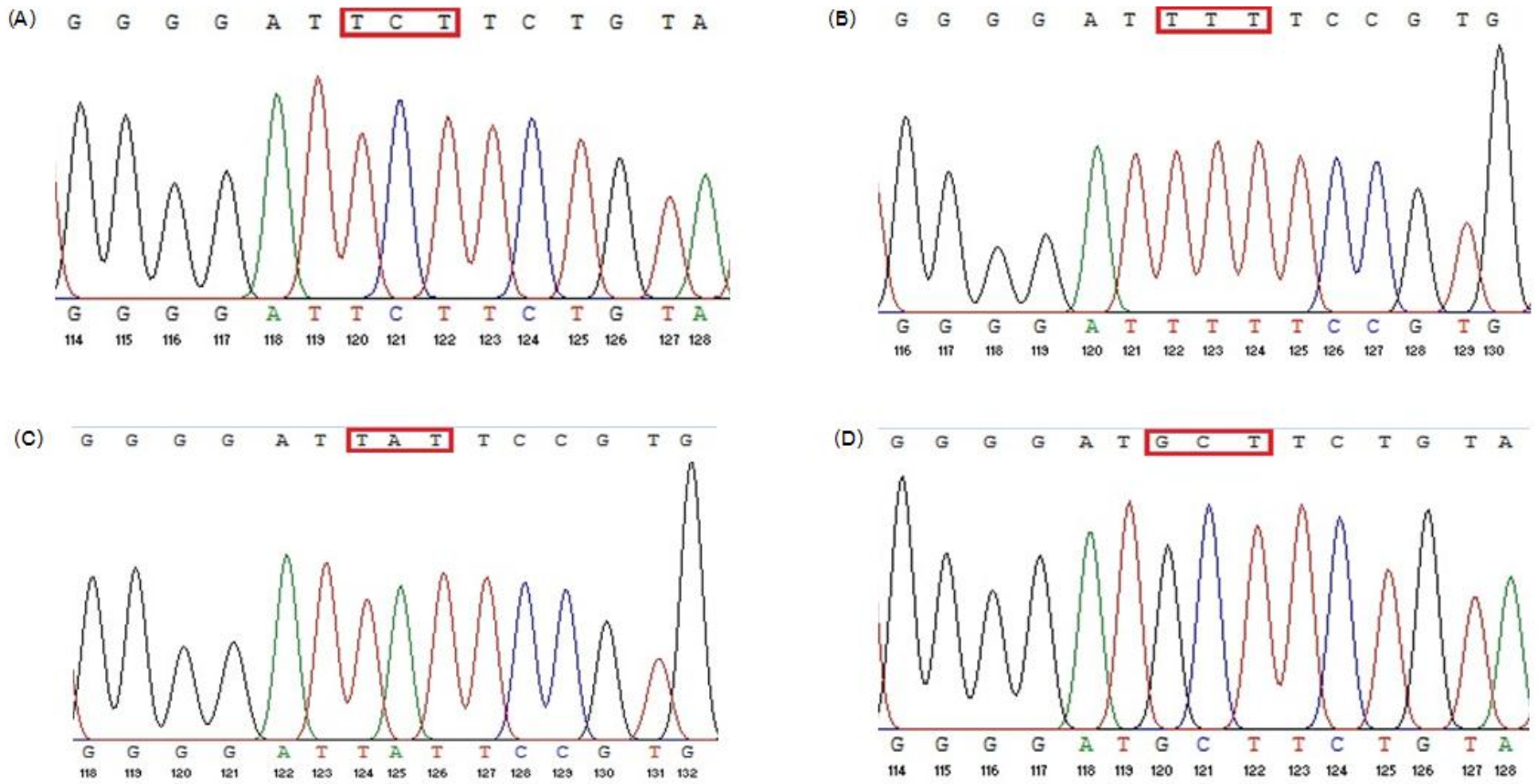


Figure 9. The chromatogram from ContigExpress showed the difference of *gyrA* QRDR between one *F. columnare* oxolinic acid-susceptible isolate, CUVET1213 (A), low-resistant isolates (B,C) and high-resistant isolate (D) at position 83. (TCT→TTT, TCT→TAT, and TCT→GCT)

CHAPTER V

DISCUSSION

Columnaris disease is one of the bacterial infections caused by *F. columnare,* which is considerable for the high mortality rate in several kinds of fish especially freshwater fish and affected economic losses worldwide (Declercq et al., 2013b). Almost valuable freshwater fish in Thailand, e.g., Nile tilapia, Red tilapia have been cultured in freshwater area together with the often use of antimicrobial agents as a curative approach while the outbreak occurred in the fish farm. Firstly, the AST of *F. columnare* to routine drugs used in aquaculture in Thailand is the important data. Our study was fulfilled this information for monitoring the situation of AMU in Thailand for the treatment of bacterial diseases including columnaris disease by AST determination.

In this study, the AST of *F. columnare* isolates performed by disk diffusion method on DMHA as recommended in M42-A document showed that all isolates were well grown on agar and produced less rhizoid colony than culturing on CA, which was used for antibiogram of *F. columnare* after first success isolation followed NCCLS guidelines (Kubilay et al., 2008). The inhibition zones of isolates on DMHA were easier for measuring the zone diameters. Quarter from isolates were still produced rhizoid colony in DMHA, which minor interfered their interpretations, anyway, the border of inhibition zones were still fell in acceptable range for evaluating the zone diameters. The quality control; *E. coli* ATCC 29522 was also grown on DMHA and produced the inhibition zone to some antibiotic disks tested into acceptable ranges followed CLSI document and previous studies (CLSI, 2006b; Gieseker et al., 2012), although neither quality control ranges of some ABOs to *E. coli* nor *F. columnare* weren't exist yet. In addition, diameter of inhibition zones for the interpretation of *F. columnare* to some ABOs weren't available for aquatic bacteria. Thus, general CLSI document (CLSI, 2012) and previous study of *F. psychrophilum* (Kum et al., 2008) were adapted to perform the interpretative criteria instead.

Regarding disk diffusion test results, almost antimicrobial agents used in aquaculture referred to be effective for the treatment of columnaris disease in fish farms in Thailand. Over 90% of isolates were sensitive to 9 ABOs tested except 2 ABOs; nalidixic acid and oxilinic acid, which were drugs in Q even there haven't

general used last decade since the development of FQs, e.g., norfloxacin, and enrofloxacin, which have more efficacies in antimicrobial properties. Some isolates showed double resistance to nalidixic acid and oxilinic acid, but none resistant to all any FQs tested. Interestingly, many kinds of drugs have been applied in fish farms, but only quinolones nalidixic acid and oxilinic acid found the resistant results. These results were related to the former information, oxolinic acid and oxytetracycline were often used for the treatment of flavobacteriosis, which caused by *Flavobacterium* spp., anyway, enrofloxacin, flumequine, and chloramphenical were also additionally used for the treatment of columnaris disease in Thailand (FDA, 2012). Chloramphenicol was banned for the use in aquaculture for few years ago (DOF, 2010). However, FQs and Qs are usually applied by misuse or overdose use in aquaculture, which can lead to development of resistance due to their residue in the environment. The antibiogram of *F. columnare* by disk diffusion test in this study was consistent to previous reports (Aber et al., 1978; Dinesh Kumar, 2012; Tohmee and Deemagarn, 2013). Some reports found the multiple drug resistance (MDR) of *F. columnare*, but the detail of information weren't available (Dinesh Kumar, 2012; Hyun Mi Jin, unpublished). In addition, almost previously AST results has performed by disk diffusion technique for Flavobacteria instead of each separately genus or specie result in the past. The antibiograms were reported in Genus level as well as other bacteria in family *Flavobacteriaceae*. After the modification of taxonomy, the separately AST of *F. columnare* has been exhibited later. In part of method, other agar methods have also been conducted in order to re-examine the methods for reliable the susceptibility test of *Flavobacterium* species, e.g., agar dilution, E-test (Fraser and Jorgensen, 1997) and determine the zone diameter breakpoint by agar dilution and disk diffusion (Chang et al., 1997). From many reports, disk diffusion method is the simple and popular method to determine the AST as a qualitative testing for many bacterial species including bacteria isolated from aquatic animals (Aber et al., 1978). *F. columnare* is the member of gliding bacteria, the interpretative inhibition zone of this bacterium is hardly defined and measured due to the rhizoid colony produced on agar surface. CA was used for antibiogram of *F. columnare* after first success isolation followed NCCLS guidelines (Kubilay et al., 2008). However, disk

diffusion tests for gliding bacteria including *F. columnare* are not suitable for the interpretation due to the rhizoid colony and the delayed growth. Therefore, CLSI proposed another method for the determination of AST of bacteria isolated from aquatic animals including *F. columnare* by broth microdilution in 1:7 diluted form of standard MHB for group 3 gliding bacteria (CLSI, 2006a). By the way, since *F. columnare* is one of the fastidious organisms, diluted 1:7 DMHB wasn't support the growth of this bacterium. (Darwish et al., 2008) developed the methods by using diluted 1:5 DMHB followed the CLSI guideline (CLSI, 2006a) and previous study (Farmer, 2004). Thus, direct measurement of MIC is rather than use of disk diffusion method (Chang et al., 1997).

In part of MIC determinations of 2 ABOs tested, both oxolinic acid and norfloxacin were chosen by following the AST results by disk diffusion test. Oxolinic acid is the first generation of Q antibiotic, which performed the resistant results to some *F. columnare* isolates and still widely and effectively use for the treatment of columnaris disease whilst norfloxacin is the first generation of FQ antibiotic. Although norfloxacin isn't the general drug use in aquaculture, anyway, in order to monitor the situation of QR in *F. columnare*, norfloxacin was also selected for the experiment rather another FQs as enrofloxacin in spite of the widely use in aquaculture. However, the CLSI released the updated performance standard for the susceptibility of bacteria isolated from aquatic animal, anyway, no cut-offs or breakpoints for OA and NOR in *F. columnare* are available as well as disk diffusion test (Shah et al., 2012; Declercq et al., 2013a). The breakpoint of OA in this study was adapted from previous study regarding aquatic bacteria as *Aeromonas* spp. (Alcaide et al., 2010), whilst for NOR's breakpoint was followed general CLSI document in group of fastidious organisms (CLSI, 2012; Gieseker et al., 2012). However, the definitely interpreted results of MIC values of OA and NOR were different due to their different breakpoints for the evaluation: ≥ 4 µg/ml of OA was considered to have acquired resistance, anyway, the resistance to NOR was interpreted as ≥ 16 µg/ml (fourth-fold higher). MIC values of NOR ≥ 4 µg/ml might be interpreted as resistance instead of intermediate in this study. Therefore, the breakpoints for evaluation of AST in aquatic organisms should be discussed and re-examined.

From CLSI document used, other reference type strain; *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 wasn't available in our laboratory, thus,

E. coli ATCC 29522 was used for quality control instead followed previous studies (Miller et al., 2003; Darwish et al., 2008; Gieseker et al., 2012). All *F. columnare* isolates and reference strain were well grown on diluted 1:5 DHMB and performed the MIC values in acceptable ranges for both ABOs tested (Darwish et al., 2008). The MIC of OA in this study was similar to the previous publications that performed the MIC values to many antimicrobial agents tested including OA, which were considered to have acquired resistance, anyway, MIC of OA wasn't interpreted as definitely results as resistant, intermediate, or susceptible due to the lack of available breakpoints from CLSI for interpretation (A.M. Declercq, 2011; Declercq et al., 2013a). For another *Flavobacterium* species, (Henriquez-Nunez et al., 2012) also reported the AST of *F. psychrophilum* isolates by both disk diffusion test and MIC to ABOs including OA. They found that *F. psychrophilum* performed high MIC of OA values with the cut-off calculated from data themselves by normalized resistance interpretation (NRI) equal ≥ 26.8 μg/ml, which was quite higher than acceptable breakpoint for *F. columnare.*

The MIC results and the AST by disk diffusion test of all isolates were quite accordant. For example, 16 isolates which performed intermediate and resistant results by disk diffusion were also have MIC values as similar interpretative criterions as intermediate (9 isolates) and resistant (16 isolates). Some isolates have differ results from 2 methods regarding OA tested, e.g., 5 isolates; CUVET 1346, 1347, 1348, 1361, and 1376 showed the intermediate to OA by disk diffusion test, anyway, these isolates have MIC of OA ≥ 4 μg/ml as OA-resistant results. The difference between the occurrence of QR calculated from AST by disk diffusion test (36 %) and MIC of OA (16%) was come from the different source for calculation, although, the isolates tested were also showed the resistant result to NA by disk diffusion test, but the use of NA in aquaculture was rarely and less effective. Thus, the occurrence of QR in this study was focus on the result of OA as well as the prevalence of OA. According to MIC of OA, 16 isolates were classified into low and high resistance in order to find the relation between the OA-resistant results and mutation detected in four QRDRs of quinolone targets genes.

Regarding the QR in aquatic bacteria, since the use of quinolones including fluoroquinolones in aquaculture, e.g., oxolinic acid, and enrofloxacin for the treatment of columnaris disease are increasing, the occurrence of QR in bacteria in

Genus *Flavobacterium* have been reported (Henriquez-Nunez et al., 2012; Declercq et al., 2013a). In addition, some quinolones which are prohibited use in aquaculture due to their resistant development still have been widely used for the treatment in fish farms worldwide, including Thailand. Thus, QR of *F. columanare* to some Qs and FQs, e.g., OA and ENR were frequently reported during the last decade. In general basis, the alteration in quinolone target enzymes by mutations in QRDRs in *gyrA* and *gyrB* of DNA gyrase and in *parC* and *parE* of topoisomerase IV are the major mechanisms of QR in both Gram –positive and –negative bacteria (Ruiz, 2003). However, no report of the association between QR and evidences of their resistance in *F. columnare* was published. Therefore, this study firstly reported the major QR mechanisms in *F. columnare* isolates in Thailand.

The detection of mutation in molecular level followed the major mechanism of resistance to quinolone by PCR and DNA sequencing are the most studies used with the successfully outcome in many publications (Walsh, 2000). The primer used for amplification of *gyrA* QRDR in *F. psychrophilum* were published by (Izumi and Aranishi, 2004). Then, *gyrB, parC,* and *parE* primers for amplification of their QRDRs in *F. psychrophilum* were described by (Shah et al., 2012). However, these 4 primer pairs were firstly tested the specificity by Primer-Blast. The results showed only the sequences of *F. psychrophilum* were found without *F. columnare* sequences specified to these primers. Because no specific primers for amplifying the QRDR sequences of *F. columnare* were available. Thus, the oligonucleotide primers in this study were designed base on type strain, *F. columnare* ATCC 29512, which has been published and deposited 1 complete sequence in GenBank database (Tekedar et al., 2012). That's why other QRDR sequences of other Gram-negative bacteria from previous studies were used to estimate the putative QRDR and conserve regions for the attachment of primers and PCR process in *F. columnare.* In addition, the specificity is one of the important criteria to evaluate for new primers designed. Thus, 3 procedures were used to evaluate the designed primers in this study; Primer-Blast, PCR amplification, and sequencing analysis. First, all designed primers were estimated by using online tool Primer-Blast, which compose of 2 main steps; primers design by Primer3 and Blast with a global alignment algorithm (Ye et al., 2012). Melting temperatures of each primer were useful for determination of Ta by Gradient PCR.

Second, fortunately, adjusted PCR mixtures and conditions followed manufacturers' instructor were success amplified all QRDRs of *F. columnare* positive isolates by each designed primer. For the determination of optimum Ta of primers, the temperature used which could generate the target product sizes were vary in temperature range, the optimum Ta of GyrA and ParE primers (55 °C) were higher than GryB and ParC primers (48 °C) resulting from gradient PCR. We attempted to adjust the same temperature used (48 °C) for amplification by all primer used in the same time as multiplex PCR developing. Unfortunately, non-specific products were presented in amplified *gyrA* and *parE* QRDRs. Generally, the optimum Ta of primer is approximately 55°C, anyway, the low Ta of GyrB and ParC primers (48 °C) might be caused by the mismatch of primers while in PCR process. The specificity of these primers might not enough due to the gene position used to amplify or design. Besides, 3 other Gram-negative bacterial DNA used in order to determine the specificity weren't amplified by all designed primers. We deduced from this result that all designed primers were specific for bacteria in Genus *Flavobacterium*, especially *F. columnare.* Third, the results from sequencing analysis also showed the specificity of designed primers that both nucleotide sequences and deduced amino acids from the amplifications had more than 80% identities to the target sequences of *F. columnare* in GenBank after Blast analysis except *parE* sequence. Previous studies have mentioned regarding the quinolone target genes used for the identification of some bacteria, e.g. *gyrA* (Borshchevskaya et al., 2013) and *gyrB* (Coenye and LiPuma, 2002). Therefore, these 3 procedures' result indicated that our designed primers were specific for the amplification of QRDR of *F. columnare* isolates in this study. However, the QRDRs of target genes were rarely used for bacterial identification.

For the sequencing analysis process in this study, one primer for sequencing could effectively generate the good quality sequences approximate 500-600 bp. For this reason, only one forward or reverse primer was enough to send for sequencing analysis of QRDRs; *gyrA, gyrB,* and *parE*, which have size of amplicons less than 600 bp, whilst for *parC* QRDR was sequenced by two primer pairs; forward and reverse because the amplicons size was approximate 900 bp. After contig, alignment, and protein translation processes of all sequences finished, each QRDR sequence were

corresponding to the correct amino acid positions according to QRDRs from literatures. The results of all QRDR sequences were responsible for the investigation of the association between the Q-susceptibility and the mutation in QRDRs of all quinolone target genes of *F. columnare* isolates.

The mutations resulting in amino acid substitutions in both quinolone target enzymes; DNA gyrase and topoisomerase IV involving QR in several organisms have been reported and described by previous studies (Ruiz, 2003; Jacoby, 2005). Several previously researches proposed that *gyrA* and *parC* are the most frequently found the mutations in QRDR of each gene, which are also related with their resistant results to Q and/or FQ and are considered as the mechanism of resistance in Gram-nagative bacteria (Hu et al., 2007). Mutation in *gyrA* is more frequency occurs in many QR-bacteria than another target gene. Additionally, the alterations at amino acids between position 67 and 106 in GyrA subunit of DNA gyrase and positions 80 and 84 in ParC subunit of topoisomerase IV of almost Gram-negative bacteria, e.g., *E. coli* are the most common substitutions found in Q and/or FQ resistant bacteria (Ruiz, 2003). Surprisingly, our results revealed the double point mutations in QRDRs of *gyrA* and *parC* involving QR considered from MIC of OA. The levels of QR were related to the mutations and amino acid substitutions. The novel mutations and amino acid substitutions detected in QRDRs of at position 83 of GyrA and position 87 of ParC from *F. columnare* isolates were associated with high-MIC of OA and level of QR. However, no mutation were detected in all QRDR of 1 OA-sensitive isolate CUVET 1261 including 7 OA-sensitive isolates and 3 OA-intermediate isolates. Interestingly, in one OA-intermediate isolate CUVET1261, the amino acid change in *parC* was initially detected whilst no mutation in *gyrA* was presented. This finding gives the idea that even *parC* is a secondary target of Q in Gram-negative bacteria (Vila et al., 1996), but in *F. columnare, parC* might be more sensitive for altered target by quinolone OA than *gyrA*. The amino acid changes in *gyrA* were relevant to *parC.* All selected isolates which were OA resistance (MIC≥4 µg/ml) exhibited amino acid changes in both *gyrA* and *parC* except 2 isolates with high-level of QR; CUVET1339, CUVET1344 (MIC≥16 µg/ml). In addition, silent mutations were detected at codon 80 (CAT→CAC) and 88 (GCC→GCT) in *gyrA* only, while no mutation in *parC* was found. This result

was presumptive that amino acid change and silent mutations in *gyrA* of high-level of QR isolates might be the prior cause of QR than in *parC.* Amino acid changes in *gyrA* were more variable than *parC.* In addition, all point mutations found in both *gyrA* and *parC* were missense mutation type or synonymous substitution but there were different in subtype; *parC* mutation His-87→Tyr is non-conservative mutation, which classified by the replacement of amino acid with the different biochemically structure from original, whilst *gyrA* mutations in 3 points at the position 83, Ser→Phe, Ser→Tyr, and Ser→Ala are conservative mutation, which the similarity amino acids structure as original. Although the mutation type in 2 target genes were different, but both of them were correlated with the MIC of OA. These results suggested that both *gyrA* and *parC* are the major targets of OA in *F. columnare.* Furthermore, in all L-QR isolates, only one type of amino acid substitution in *parC* by histidine residue replaced by tyrosine in position 87 was detected whilst three difference types of amino acid change were detected in *gyrA* by serine residue replaced by 3 amino acids different by MIC of OA; phenylalanine, tyrosine (MIC=4 μg/ml), and alanine (MIC=8,16 μg/ml) in position 83. These data might support the previous study that GyrA is the most important target for Q alteration in *F. columnare*. However, in order to verify the double mutation detected at position 83 and 87 in *gyrA* QRDR and *parC* QRDR, respectively of *F. columnare* were the targets of quinolone OA associated with QR, *in vitro* mutagenesis should be done.

In the present study, all 27 selected isolates for sequencing analysis were belong to genomovar II according to previously results (Dong et al., 2014), only one isolates named CUVE1215 was classified into genomovar I and performed sensitive to all quinolone drugs tested, e.g., OA, and NA. Unfortunately, this isolate wasn't selected for sequencing analysis. However, quinolone target genes might not related with the genomovar of the isolates. The mutation results were found from totally 16 OA-intermediate and -resistant *F. columnare* isolates which recovered from only four provinces in Thailand (Kanchanaburi, Ratchaburi, Ayutthaya, and Chachoengsao), related with the uses of OA, ENR (quinolones) and OXT (tetracyclines) including other unlabeled ABOs that often applied for the treatment of columnaris in fish farms by feeding and immersion. Thus, the prudent use of Q/FG should be concerned in tilapia farm in Thailand.

Regarding the QRDRs of *gyrB* and *parE* analyses. Although previous studies have confirmed that mutations and amino acid substitutions in both homologous subunits are associated with the mechanisms of QR in several bacteria, particularly Gram positive bacteria (Ruiz, 2003). Alterations of quinolone by mutations in *gyrB* and *parE* of some Gram-negative bacteria such as *E. coli* were considered for QR mechanisms (Nakamura et al., 1989; Yoshida et al., 1991). However, no mutations were detected in QRDRs of *gyrB* and *parE* of all selected isolates for sequencing. These findings support the previous reports that *gyrB* and *parE* aren't the main target of quinolone OA in *F. columnare* including other Gram negative bacteria. QRDRs of both *gyrB* and *parE* without mutation might be the conserved/housekeeping gene in *F. columnare* for the phylogenetic analysis to investigate the evolutionary study.

Mutations in only *gyrA* and *parC* associated with QR **of** *F. columnare* isolates (no *gyrB* and *parE* mutaions found) in this study were consistent with other aquatic bacteria from previous publications such as *Vibrio* spp. (Colquhoun et al., 2007; Rodkhum et al., 2008; Alcaide et al., 2010), *Aeromonas* spp. (Goni-Urriza et al., 2002), *Streptococcus agalactiae* (Kawamura et al., 2003), etc. However, the results of some isolates: 1 OA-intermediate CUVET1261 (*gyrA* mutation only), and 2 H-QR; CUVET 1339 and 1344 (*parC* mutation only) were different from other Gram-negative bacteria. The association between level of QR and alterations in *gyrA* and *parC* couldn't be concluded due to low number of intermediate and resistant isolates for sequencing and silent mutations found in *gyrA*. In *F. colunare, gyrA* and *parC* weren't presumed that which is the primary and secondary target of Q. Additionally, other chemical agents or other agents not only Q/FG might be able to alter QTGs and contribute the QR. This suggestions should be further investigated.

In *Flavobacterium* species, only *F. psychrophilum* has been published the mutations in *gyrA* with amino acid changed at position 83 and 87 substitutions from threonine to alanine and or isoleucine and from aspartic acid to tyrosine, respectively related with the MICs of NA and OA (Izumi and Aranishi, 2004). In addition, another study also reported *gyrA* mutation resulting in amino acid substitution at position 82: threonine residue replaced by arginine and silent mutations in another target genes; *gyrB, parC,* and *parE* among high MICs of OA and flumequine (Shah et al., 2012). However, the information of QR mechanisms from bacteria in family *Flavobacteriaceae* was limited. This study was the answer of major

part of QR mechanisms in *F. columnare* and might be useful for monitoring the QR in aquatic bacteria. Overall mechanisms of resistance to quinolone are not only cause by the major chromosomal-mediated mechanism by the alteration in quinolone target enzymes as mentioned above, but other mechanisms are also involved with the QR for example mobile elements carry resistant gene, post ABOs effect; quinolone uptake, efflux pump system, mutation in outer-membrane porin (Walsh, 2000; van Hoek et al., 2011). However, regarding the study of mobile genetic elements such as plasmid in *F. columnare*, no evidence of plasmid occurrence was determined from whole genome sequence data (Tekedar et al., 2012) and previous study (Suomalainen, 2005). Additionally, we also attempt to extract plasmid from one OA-resistant isolate by Alkaline Lysis with Sodium Dodecyl Sulfate (SDS) as conventional method according to (Birnboim and Doly, 1979; Kado, 1981; Chakroun et al., 1998) with slightly modification. *E. coli* PM109 in our laboratory which contained plasmid DNA was used as a plasmid marker. Nevertheless, no plasmid was found from our pilot study (data not shown). Other mechanisms associated with QR in *F. columnare* should require further researches such as multidrug efflux pump in *F. johnsoniae* that induced the multiple resistance to many kinds of antimicrobial agent (Clark et al., 2009).

From our investigation, the QR among *F. columnare* isolates was occurred to be related with double point mutations in the QRDRs of *gyrA* and *parC.* The licensable quinolones are used in only some countries in Europe and Asia including Thailand, but on the contrary, these two antibiotics are prohibit due to their residues may cause the antimicrobial resistance in USA and Canada (Cabello et al., 2013). However, limited numbers of antibiotics are licensed for the use in aquaculture, quinolones (such as oxolinic acid) and fluoroquinolones (such as enrofloxacin and flumequine) are still recommended for the use in most fish farms including in tilapia farms in Thailand, especially for the treatments of flavobacteriosis and columnaris disease by immersion or bath therapy, which is more convenient than drug mixing with foods and directly feeding (FDA, 2012). So long as quinolones are still widely applied in aquaculture, the occurrence of QR in bacteria in both aquatic and environment might be arising. The prudent use of Q/FQ should be concerned in aquaculture in Thailand. Additionally, another licensed drugs in lists, e.g.,

oxytetracycline, tetracycline, sulfamerazine might be rather applied in fish farms for the treatment of columnaris disease than quinolones and fluoroquinolones (DOF, 2010). The chemical agents should be considered as alternative use for the control, prevention and the treatment of the early stage of columnaris disease as external infection before the developing stage to septicemia (Declercq et al., 2013b). According to this study regarding QR in *F. columnare,* which was just the surveillance and monitoring some quinolones use, their resistance profile, and the effect of quinolone to one represent aquatic bacteria. Therefore, using quinolones and fluoroquinolones to the treatments of columnaris disease and other bacterial diseases in aquaculture should be considered to reduce or avoid in order to prevent the development of quinolone resistance, which might be the emergence of drug-resistant bacteria and transfer the resistant traits from bacteria in aquatic or environment to human.

5.1 Conclusion

The antimicrobial susceptibility pattern by disk diffusion test showed almost *F. columnare* isolates were sensitive to routinely drugs use in aquaculture except the occurrences of QR to OA (22%) and NA (14%) were presented. Since NA hasn't use in fish farms in Thailand, then, the MIC determinations of both first generation quinolone OA and fluoroquinolone NOR were determined. Only OA-resistant (32%) and -intermediate (16%) isolates were found with high and low level of QR classified by its MIC values, which were related with the double point mutations detected and amino acid changes at positions 83 in QRDRs of *gyrA* and 87 in *parC* whilst no mutation in *gyrB* and *parE*. These results strongly supported previous studies that both *gyrA* and *parC* are the major target of Q in most Gram-negative bacteria, whilst *gyrB* and *parE* mightn't associates with QR in *F. columnare*. Additionally, our designed primers in this study could successful amplify QRDRs of all QTGs in *F. columnare* with high specification. Almost isolates tested were recovered from red tilapia although OA weren't often applied in tilapia farms when compare with some more effective fluoroquinolones such as ENR. The use of OA in aquaculture should be prohibited and strictly controlled. Further studies are suggested to answer other machanisms of resistance to quinolones by other methods by following related previous reports and verify the hypothesis by *in vitro* induced mutagenesis to fulfill complete mechanisms.

5.2 Advantages of Study

1. Provide the information of antimicrobial susceptibility pattern especially the resistance to quinolone drugs and the occurrence of QR of *F. columnare* in Thailand.
2. This is the first investigation which provides novel information of the mutations in QRDRs of *gyrA* and *parC* associated with QR of *F. columnare* in Thailand.

.

REFERENCES

REFERENCES

A.M. Declercq FB, W. Van den Broeck, P. Bossier, F. Haesebrouck, A. Decostere. 2011. Antimicrobial susceptibility pattern of *Flavobacterium columnare*, the causative agent of columnaris disease 15 th International Conference on Diseases of Fish and Shellfish organized by the EAFP. P.

Aber RC, Wennersten C and Moellering RC, Jr. 1978. Antimicrobial susceptibility of flavobacteria. Antimicrob Agents Chemother. 14(3): 483-487.

Alcaide E, Blasco MD and Esteve C. 2010. Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. Res Microbiol. 161(1): 40-45.

Alderman DJ and Smith P. 2001. Introduction - Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. Aquaculture. 196(3-4): 211-243.

Amin NE, Abdallah IS, Faisal M, Easa Me-S, Alaway T and Alyan SA. 1988. Columnaris infection among cultured Nile tilapia *Oreochromis niloticus*. Antonie Van Leeuwenhoek. 54(6): 509-520.

Anacker RL and Ordal EJ. 1955. Study of a bacteriophage infecting the myxobacterium *Chondrococcus columnaris*. Journal of Bacteriology. 70: 738-741.

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.

Birnboim HC and Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. 7(6): 1513-1523.

Borshchevskaya LN, Kalinina AN and Sineokii SP. 2013. Design of a PCR test based on the *gyrA* gene sequence for the identification of closely related species of the *Bacillus subtilis* group. Applied Biochemistry and Microbiology. 49(7): 646-655.

Breines DM, Ouabdesselam S, Ng EY, Tankovic J, Shah S, Soussy CJ and Hooper DC. 1997. Quinolone resistance locus nfxD of *Escherichia coli* is a mutant allele of

the *parE* gene encoding a subunit of topoisomerase IV. Antimicrobial Agents and Chemotherapy. 41(1): 175-179.

Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dolz H, Millanao A and Buschmann AH. 2013. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol. 15(7): 1917-1942.

Chakroun C, Grimont F, Urdaci MC and Bernardet JF. 1998. Fingerprinting of *Flavobacterium psychrophilum* isolates by ribotyping and plasmid profiling. Diseases of Aquatic Organisms. 33(3): 167-177.

Chang JC, Hsueh PR, Wu JJ, Ho SW, Hsieh WC and Luh KT. 1997. Antimicrobial susceptibility of flavobacteria as determined by agar dilution and disk diffusion methods. Antimicrobial Agents and Chemotherapy. 41(6): 1301-1306.

Chuang LY, Cheng YH and Yang CH. 2013. Specific primer design for the polymerase chain reaction. Biotechnology Letters. 35(10): 1541-1549.

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

CLSI. 2006a. Clinical and Laboratory Standard Institute. Methods for Broth dilution Susceptibility Test of Bacteria Isolated from Aquatic Animals; Approved Guideline. 26(24): 1-60.

CLSI. 2006b. Clinical and Laboratory Standard Institute. Methods for Antimicrobial Disk Susceptibility Test of Bacteria Isolated from Aquatic Animals; Approved Guideline. 26 (23): 1-56.

CLSI. 2012. Clinical and Laboratory Standard Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. 32(3): 1-188.

Coenye T and LiPuma JJ. 2002. Use of the *gyrB* gene for the identification of *Pandoraea* species. Fems Microbiology Letters. 208(1): 15-19.

Colquhoun DJ, Aarflot L and Melvold CF. 2007. *gyrA* and *parC* mutations and associated quinolone resistance in *Vibrio anguillarum* serotype O2b strains isolated from farmed atlantic cod (*Gadus morhua*) in Norway. Antimicrobial Agents and Chemotherapy. 51(7): 2597-2599.

Dalsgaard I. 2001. Selection of media for antimicrobial susceptibility testing of fish pathogenic bacteria. Aquaculture. 196(3-4): 267-275.

Darwish AM, Farmer BD and Hawke JP. 2008. Improved Method for Determining Antibiotic Susceptibility of *Flavobacterium columnare* Isolates by Broth Microdilution. Journal of Aquatic Animal Health. 20(4): 185-191.

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(1): 27.

Decostere A, Ducatelle R and Haesebrouck F. 2002. Flavobacterium columnare (Flexibacter columnaris) associated with severe gill necrosis in koi carp (Cyprinus carpio L). Veterinary Record. 150(22): 694-695.

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

Dieffenbach CW, Lowe TMJ and Dveksler GS. 1993. General Concepts for Pcr Primer Design. Pcr-Methods and Applications. 3(3): S30-S37.

Dimitrov T, Dashti AA, Albaksami O, Udo EE, Jadaon MM and Albert MJ. 2009. Ciprofloxacin-Resistant *Salmonella enterica* Serovar Typhi from Kuwait with Novel Mutations in *gyrA* and *parC* Genes. Journal of Clinical Microbiology. 47(1): 208-211.

Dinesh Kumar YP, A. K. Singh and Abubakar Ansari. 2012. Columnaris disease and its drug resistance in cultured exotic African catfish *Clarias gariepinus* in India. Biochem. Cell. Arch. 12(2): 415-420.

DOF. 2010. Fisheries of statistics of Thailand 2008. Department of Fisheries Ministry of Agriculture and Cooperatives, ed, Bangkok, Thailand.

Dong HT, LaFrentz B, Pirarat N and Rodkhum C. 2014. Phenotypic characterization and genetic diversity of *Flavobacterium columnare* isolated from red tilapia, *Oreochromis* sp., in Thailand. J Fish Dis.

Eaves DJ, Randall L, Gray DT, Buckley A, Woodward MJ, White AP and Piddock LJV. 2004. Prevalence of mutations within the quinolone resistance-determining region of *gyrA, gyrB, parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrobial Agents and Chemotherapy. 48(10): 4012-4015.

Fabrega A, Madurga S, Giralt E and Vila J. 2009. Mechanism of action of and resistance to quinolones. Microbial Biotechnology. 2(1): 40-61.

Farmer B. 2004. Improved methods for the isolation and characterization of *Flavobacterium columnare*. Louisiana State University.

FDA. 2012. Drug control and Registration Food and Drug Administration Ministry of Public Health, Thailand, ed, Bangkok, Thailand.

Figueiredo HCP, Klesius PH, Arias CR, Evans J, Shoemaker CA, Pereira DJ and Peixoto MTD. 2005. Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. J Fish Dis. 28(4): 199-204.

Fijan NN. 1969. Antibiotic additives for the isolation of *Chondrococcus columnaris* from fish. Appl Microbiol. 17(2): 333-334.

Fluit AC, Visser MR and Schmitz FJ. 2001. Molecular detection of antimicrobial resistance. Clin Microbiol Rev. 14(4): 836-871, table of contents.

Fraser SL and Jorgensen JH. 1997. Reappraisal of the antimicrobial susceptibilities of *Chryseobacterium* and *Flavobacterium* species and methods for reliable susceptibility testing. Antimicrobial Agents and Chemotherapy. 41(12): 2738-2741.

Friedman SM, Lu T and Drlica K. 2001. Mutation in the DNA gyrase A gene of *Escherichia coli* that expands the quinolone resistance-determining region. Antimicrobial Agents and Chemotherapy. 45(8): 2378-2380.

Gensberg K, Jin YF and Piddock LJV. 1995. A Novel Gyrb Mutation in a Fluoroquinolone-Resistant Clinical Isolate of *Salmonella*-Typhimurium. Fems Microbiology Letters. 132(1-2): 57-60.

Gieseker CM, Mayer TD, Crosby TC, Carson J, Dalsgaard I, Darwish AM, Gaunt PS, Gao DX, Hsu HM, Lin TL, Oaks JL, Pyecroft M, Teitzel C, Somsiri T and Wu CC. 2012. Quality control ranges for testing broth microdilution susceptibility of *Flavobacterium columnare* and *F. psychrophilum* to nine antimicrobials. Diseases of Aquatic Organisms. 101(3): 207-215.

Goni-Urriza M, Arpin C, Capdepuy M, Dubois V, Caumette P and Quentin C. 2002. Type II topoisomerase quinolone resistance-determining regions of *Aeromonas caviae, A. hydrophila, and A. sobria* complexes and mutations associated with quinolone resistance. Antimicrob Agents Chemother. 46(2): 350-359.

Ha DT. 2014. Phenotypic and genotypic classification of *Flavobacterium columnare* isolated from Red tilapia (*Orechromis* sp.) in Thailand. Chulalongkorn University.

Ha DT, Pirarat N and Rodkhum C. 2013. Isolation and characterization of *Flavobacterium columnare* from Red Tilapia (*Oreochromis* sp.) in Thailand. ICVS, 2013. P.

Hamidian M, Tajbakhsh M, Tohidpour A, Rahbar M, Zali MR and Walther-Rasmussen J. 2011. Detection of novel *gyrA* mutations in nalidixic acid-resistant isolates of *Salmonella enterica* from patients with diarrhoea. International Journal of Antimicrobial Agents. 37(4): 360-364.

Henriquez-Nunez H, Evrard O, Kronvall G and Avendano-Herrera R. 2012. Antimicrobial susceptibility and plasmid profiles of *Flavobacterium psychrophilum* strains isolated in Chile. Aquaculture. 354: 38-44.

Hesami S, Parkman J, MacInnes JI, Gray JT, Gyles CL and Lumsden JS. 2010. Antimicrobial Susceptibility of *Flavobacterium psychrophilum* Isolates from Ontario. Journal of Aquatic Animal Health. 22(1): 39-49.

Hirose K, Hashimoto A, Tamura K, Kawamura Y, Ezaki T, Sagara H and Watanabe H. 2002. DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella enterica* serovar Typhi and serovar Paratyphi A. Antimicrobial Agents and Chemotherapy. 46(10): 3249-3252.

Hooper DC. 1999. Mechanisms of fluoroquinolone resistance. Drug Resistance Updates. 2(1): 38-55.

Hooper DC. 2001. Emerging mechanisms of fluoroquinolone resistance. Emerging Infectious Diseases. 7(2): 337-341.

Hu LF, Li JB, Ye Y and Li X. 2007. Mutations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in clinical strains of fluoroquinolone-resistant *Shigella* in Anhui, China. J Microbiol. 45(2): 168-170.

Hyun Mi Jin JYJ, and Che Ok Jeon. unpublished Antibiotic resistances of *Flavobacterium* species unpublished

Izumi S and Aranishi F. 2004. Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. Applied and Environmental Microbiology. 70(7): 3968-3972.

Jacoby GA. 2005. Mechanisms of resistance to quinolones. Clinical Infectious Diseases. 41 Suppl 2: S120-126.

Jooste PJ and Hugo CJ. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. International Journal of Food Microbiology. 53(2-3): 81-94.

Jorgensen JH and Ferraro MJ. 1998. Antimicrobial susceptibility testing: general principles and contemporary practices. Clinical Infectious Diseases. 26(4): 973-980.

Jorgensen JH and Ferraro MJ. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical Infectious Diseases. 49(11): 1749-1755.

Kado CI. 1981. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol. 145(3): 1365-1373.

Kawamura Y, Fujiwara H, Mishima N, Tanaka Y, Tanimoto A, Ikawa S, Itoh Y and Ezaki T. 2003. First *Streptococcus agalactiae* isolates highly resistant to quinolones, with point mutations in *gyrA* and *parC*. Antimicrob Agents Chemother. 47(11): 3605-3609.

Klesius PH, Shoemaker CA and Evans JJ. 2008. *Flavobacterium columnare* chemotaxis to channel catfish mucus. Fems Microbiology Letters. 288(2): 216-220.

Kubilay A, Altun S, Diler O and Ekici S. 2008. Isolation of *Flavobacterium columnare* from cultured rainbow trout (*Oncorhynchus mykiss*) fry in Turkey. Turkish Journal of Fisheries and Aquatic Sciences. 8(1): 165-169.

Kum C, Kirkan S, Sekkin S, Akar F and Boyacioglu M. 2008. Comparison of in vitro antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. Journal of Aquatic Animal Health. 20(4): 245-251.

Marks JE, Lewis DH and Trevino GS. 1980. Mixed infection in columnaris disease of fish. J Am Vet Med Assoc. 177(9): 811-814.

Martinez-Martinez L, Pascual A and Jacoby GA. 1998. Quinolone resistance from a transferable plasmid. Lancet. 351(9105): 797-799.

Miller RA, Walker RD, Baya A, Clemens K, Coles M, Hawke JP, Henricson BE, Hsu HM, Mathers JJ, Oaks JL, Papapetropoulou M and Reimschuessel R. 2003. Antimicrobial susceptibility testing of aquatic bacteria: Quality control disk diffusion ranges for *Escherichia coli* ATCC 25922 and A*eromonas salmonicida* subsp *salmonicida* ATCC 33658 at 22°C and 28°C. Journal of Clinical Microbiology. 41(9): 4318-4323.

Morgan-Linnell SK, Boyd LB, Steffen D and Zechiedrich L. 2009. Mechanisms Accounting for Fluoroquinolone Resistance in *Escherichia coli* Clinical Isolates. Antimicrobial Agents and Chemotherapy. 53(1): 235-241.

Mouneimne H, Robert J, Jarlier V and Cambau E. 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 43(1): 62-66.

Munshi MH, Sack DA, Haider K, Ahmed ZU, Rahaman MM and Morshed MG. 1987. Plasmid-mediated resistance to nalidixic acid in *Shigella dysenteriae* type 1. Lancet. 2(8556): 419-421.

Nakamura S, Nakamura M, Kojima T and Yoshida H. 1989. *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob Agents Chemother. 33(2): 254-255.

Oyamada Y, Ito H, Inoue M and Yamagishi J. 2006. Topoisomerase mutations and efflux are associated with fluoroquinolone resistance in *Enterococcus faecalis*. J Med Microbiol. 55(10): 1395-1401.

Pilarski F, Rossini AJ and Ceccarelli PS. 2008. Isolation and characterization of *Flavobacterium columnare* (Bernardet et al. 2002) from four tropical fish species in Brazil. Brazilian Journal of Biology. 68(2): 409-414.

Poirel L, Cattoir V and Nordmann P. 2012. Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. Front Microbiol. 3.

Rodkhum C, Maki T, Hirono I and Aoki T. 2008. *gyrA* and *parC* associated with quinolone resistance in *Vibrio anguillarum*. J Fish Dis. 31(5): 395-399.

Ruiz J. 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother. 51(5): 1109-1117.

Ruiz J, Pons MJ and Gomes C. 2012. Transferable mechanisms of quinolone resistance. International Journal of Antimicrobial Agents. 40(3): 196-203.

Saenz Y, Zarazaga M, Brinas L, Ruiz-Larrea F and Torres C. 2003. Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals. Journal of Antimicrobial Chemotherapy. 51(4): 1001-1005.

Shah SQ, Nilsen H, Bottolfsen K, Colquhoun DJ and Sorum H. 2012. DNA gyrase and topoisomerase IV mutations in quinolone-resistant *Flavobacterium psychrophilum* isolated from diseased salmonids in Norway. Microb Drug Resist. 18(2): 207-214.

Shakir Z, Khan S, Sung KD, Khare S, Khan A, Steele R and Nawaz M. 2012. Molecular Characterization of Fluoroquinolone-Resistant *Aeromonas* spp. Isolated from Imported Shrimp. Applied and Environmental Microbiology. 78(22): 8137-8141.

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

Smith P. 2001. Accuracy, precision and meaning of antimicrobial agent susceptibility testing of bacteria associated with fish diseases. Aquaculture. 196(3-4): 253-266.

Smith P. 2008. Antimicrobial resistance in aquaculture. Revue Scientifique Et Technique-Office International Des Epizooties. 27(1): 243-264.

Stelling JM and OBrien TF. 1997. Surveillance of antimicrobial resistance: The WHONET program. Clinical Infectious Diseases. 24: S157-S168.

Suomalainen L-R. 2005. *Flavobacterium columnare* in Finnish Fish Farming Characterisation and Putative Disease Management Strategies University of Jyvaskyla, University of Jyvaskyla.

Tekedar HC, Karsi A, Gillaspy AF, Dyer DW, Benton NR, Zaitshik J, Vamenta S, Banes MM, Gulsoy N, Aboko-Cole M, Waldbieser GC and Lawrence ML. 2012. Genome sequence of the fish pathogen *Flavobacterium columnare* ATCC 49512. J Bacteriol. 194(10): 2763-2764.

Tohmee N and Deemagarn T. 2013. A035-AQ008 *Flavobacterium columnare* isolated from brains of pond culture Nile tilapia in Thailand. ICVS, 2013. P.

Turnidge J and Paterson DL. 2007. Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev. 20(3): 391-408.

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.

Van Bambeke F, Balzi E and Tulkens PM. 2000. Antibiotic efflux pumps. Biochem Pharmacol. 60(4): 457-470.

van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP and Aarts HJ. 2011. Acquired antibiotic resistance genes: an overview. Front Microbiol. 2: 203.

Vila J, Ruiz J, Goni P and DeAnta MTJ. 1996. Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrobial Agents and Chemotherapy. 40(2): 491-493.

Walsh C. 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature. 406(6797): 775-781.

Wasyl D, Hoszowski A and Zajac M. 2014. Prevalence and characterisation of quinolone resistance mechanisms in *Salmonella* spp. Veterinary Microbiology. 171(3-4): 307-314.

Weigel LM, Anderson GJ and Tenover FC. 2002. DNA gyrase and topoisomerase IV mutations associated with fluoroquinolone resistance in *Proteus mirabilis*. Antimicrobial Agents and Chemotherapy. 46(8): 2582-2587.

WHO. 1999. Report of a Joint FAO/NACA/WHO Study Group on food safety issues associated with products from aquaculture WHO Technical Report Series. No. 883.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. Bmc Bioinformatics. 13.

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

Yoshida H, Bogaki M, Nakamura M, Yamanaka LM and Nakamura S. 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob Agents Chemother. 35(8): 1647-1650.

Young HK. 1993. Antimicrobial resistance spread in aquatic environments. J Antimicrob Chemother. 31(5): 627-635.

APPENDIX

APPENDIX A

Table 9. Diameter of inhibition zones with the interpretations on 50 *F. columnare* isolates to 6 routinely drugs used in aquaculture.

Bacterial isolates		Diameter of clear zone in millimeter unit (interpretations)					
		OT	SXT	AMP	E	FFC	DO
1	CUVET1201	45 (S)	35 (S)	15 (I)	30 (S)	32 (S)	45 (S)
2	CUVET1202	40 (S)	30 (S)	16 (I)	35 (S)	30 (S)	35 (S)
3	CUVET1203	45(S)	35 (S)	15 (I)	35 (S)	30 (S)	50 (S)
4	CUVET1204	50 (S)	40 (S)	15 (I)	38 (S)	28 (S)	50 (S)
5	CUVET1212	50 (S)	35 (S)	20 (S)	33 (S)	42 (S)	47 (S)
6	CUVET1213	50 (S)	36 (S)	18 (S)	30 (S)	40 (S)	44 (S)
7	CUVET1214	46 (S)	36 (S)	18 (S)	32 (S)	32 (S)	46 (S)
8	CUVET1215	50 (S)	38 (S)	15 (I)	38 (S)	32 (S)	50 (S)
9	CUVET1336	44 (S)	30 (S)	30 (S)	32 (S)	30 (S)	40 (S)
10	CUVET1338	44 (S)	26 (S)	42 (S)	28 (S)	38 (S)	44 (S)
11	CUVET1340	48 (S)	34 (S)	42 (S)	30 (S)	36 (S)	42 (S)
12	CUVET1341	48 (S)	28 (S)	30 (S)	32 (S)	36 (S)	44 (S)
13	CUVET1343	42 (S)	30 (S)	34 (S)	32 (S)	34 (S)	40 (S)
14	CUVET1344	46 (S)	24 (S)	44 (S)	29 (S)	30 (S)	44 (S)
15	CUVET1221	45 (S)	38 (S)	20 (S)	35 (S)	42 (S)	40 (S)
16	CUVET1337	50 (S)	24 (S)	40 (S)	32 (S)	40 (S)	50 (S)
17	CUVET1339	46 (S)	32 (S)	26 (S)	34 (S)	42 (S)	46 (S)
18	CUVET1342	46 (S)	30 (S)	36 (S)	34 (S)	38 (S)	46 (S)
19	CUVET1345	48 (S)	36 (S)	28 (S)	32 (S)	36 (S)	46 (S)
20	CUVET1346	40 (S)	32 (S)	26 (S)	30 (S)	36 (S)	40 (S)
21	CUVET1347	50 (S)	28 (S)	30 (S)	28 (S)	34 (S)	38 (S)
22	CUVET1348	40 (S)	30 (S)	40 (S)	36 (S)	30 (S)	40 (S)
23	CUVET1349	42 (S)	30 (S)	26 (S)	38 (S)	32 (S)	42 (S)
24	CUVET1350	38 (S)	38 (S)	28 (S)	36 (S)	38 (S)	48 (S)
25	CUVET1351	36 (S)	42 (S)	40 (S)	32 (S)	40 (S)	46 (S)

Bacterial isolates		Diameter of clear zone in millimeter unit (interpretations)					
		OT	SXT	AMP	E	FFC	DO
26	CUVET1352	43 (S)	42 (S)	38 (S)	40 (S)	36 (S)	40 (S)
27	CUVET1353	37 (S)	34 (S)	40 (S)	30 (S)	38 (S)	50 (S)
28	CUVET1354	42 (S)	38 (S)	32 (S)	28 (S)	30 (S)	42 (S)
29	CUVET1355	38 (S)	30 (S)	30 (S)	28 (S)	40 (S)	40 (S)
30	CUVET1356	50 (S)	29 (S)	30 (S)	30 (S)	34 (S)	36 (S)
31	CUVET1357	48 (S)	24 (S)	26 (S)	32 (S)	42 (S)	40 (S)
32	CUVET1358	44 (S)	30 (S)	38 (S)	40 (S)	32 (S)	48 (S)
33	CUVET1359	40 (S)	32 (S)	36 (S)	32 (S)	40 (S)	22 (S)
34	CUVET1360	36 (S)	20 (S)	40 (S)	33 (S)	38 (S)	32 (S)
35	CUVET1361	32 (S)	20 (S)	36 (S)	25 (S)	30 (S)	33 (S)
36	CUVET1362	36 (S)	22 (S)	36 (S)	31 (S)	40 (S)	35 (S)
37	CUVET1363	30 (S)	21 (S)	40 (S)	28 (S)	30 (S)	32 (S)
38	CUVET1364	42 (S)	21 (S)	32 (S)	30 (S)	32 (S)	32 (S)
39	CUVET1365	41 (S)	22 (S)	34 (S)	31 (S)	34 (S)	32 (S)
40	CUVET1367	44 (S)	21 (S)	32 (S)	28 (S)	36 (S)	32 (S)
41	CUVET1368	40 (S)	20 (S)	36 (S)	30 (S)	32 (S)	30 (S)
42	CUVET1369	37 (S)	18 (S)	36 (S)	30 (S)	40 (S)	36 (S)
43	CUVET1370	32 (S)	18 (S)	40 (S)	38 (S)	32 (S)	35 (S)
44	CUVET1374	30 (S)	24 (S)	46 (S)	40 (S)	48 (S)	44 (S)
45	CUVET1375	24 (S)	20 (S)	25 (S)	20 (I)	29 (S)	26 (S)
46	CUVET1376	31 (S)	34 (S)	44 (S)	34 (S)	44 (S)	41 (S)
47	CUVET1377	35 (S)	28 (S)	40 (S)	30 (S)	36 (S)	20 (S)
48	CUVET1378	30 (S)	24 (S)	36 (S)	36 (S)	30 (S)	22 (S)
49	CUVET1379	29 (S)	30 (S)	38 (S)	40 (S)	46 (S)	42 (S)
50	CUVET-BU1	27 (S)	27 (S)	30 (S)	28 (S)	44 (S)	38 (S)

S: Susceptible, I: Intermediate, R: Resistant, DO: doxycycline (30µg), AMP: ampicillin (10µg), E: erythromycin (15µg), OT: oxytetracycline (30µg), SXT: sulfamethoxazole/trimetroprime (25µg), FFC: florfenicol (30µg)

APPENDIX B

Table 10. Diameter of inhibition zones with the interpretations on 50*F. columnare* isolates to quinones and fluoroquinolones used in aquaculture.

	Bacteria	Diameter of clear zone in millimeter unit (interpretations)				
		FQ			Q	
		NOR	ENR	CIP	NA	OA
1	CUVET1201	22 (S)	36 (S)	42 (S)	24 (S)	26 (S)
2	CUVET1202	26 (S)	36 (S)	40 (S)	26 (S)	29 (S)
3	CUVET1203	18 (I)	44 (S)	38 (S)	22 (S)	17 (R)
4	CUVET1204	17 (I)	42 (S)	38 (S)	18 (I)	15- R)
5	CUVET1212	28 (S)	40 (S)	36 (S)	22 (S)	24 (S)
6	CUVET1213	26 (S)	40 (S)	36 (S)	22 (S)	22 (S)
7	CUVET1214	30 (S)	42 (S)	34 (S)	25 (S)	28 (S)
8	CUVET1215	26 (S)	38 (S)	42 (S)	28 (S)	22 (S)
9	CUVET1336	34 (S)	46 (S)	44 (S)	36 (S)	30 (S)
10	CUVET1338	40 (S)	40 (S)	32 (S)	20 (S)	20 (S)
11	CUVET1340	34 (S)	44 (S)	32 (S)	22 (S)	20 (S)
12	CUVET1341	30 (S)	42 (S)	30 (S)	30 (S)	26 (S)
13	CUVET1343	20 (S)	30 (S)	35 (S)	18 (I)	12 (R)
14	CUVET1344	32 (S)	40 (S)	36 (S)	14 (R)	17 (R)
15	CUVET1221	25 (S)	42 (S)	37 (S)	25 (S)	25 (S)
16	CUVET1337	30 (S)	48 (S)	40 (S)	40 (S)	26 (S)
17	CUVET1339	32 (S)	40 (S)	35 (S)	21 (S)	10 (R)
18	CUVET1342	40 (S)	50 (S)	36 (S)	40 (S)	30 (S)
19	CUVET1345	28 (S)	44 (S)	36 (S)	24 (S)	40 (S)
20	CUVET1346	36 (S)	40 (S)	32 (S)	14 (R)	19 (I)
21	CUVET1347	30 (S)	40 (S)	34 (S)	14 (R)	18 (I)
22	CUVET1348	30 (S)	50 (S)	35 (S)	19 (S)	19 (I)
23	CUVET1349	26 (S)	46 (S)	34 (S)	13 (R)	16 (R)
24	CUVET1350	30 (S)	44 (S)	38 (S)	14 (R)	16 (R)

	Bacteria	Diameter of clear zone in millimeter unit (interpretations)				
		FQ			Q	
		NOR	ENR	CIP	NA	OA
25	CUVET1351	32 (S)	40 (S)	36 (S)	22 (S)	30 (S)
26	CUVET1352	28 (S)	40 (S)	36 (S)	24 (S)	32 (S)
27	CUVET1353	21 (S)	41 (S)	34 (S)	22 (S)	25 (S)
28	CUVET1354	24 (S)	38 (S)	36 (S)	26 (S)	28 (S)
29	CUVET1355	22 (S)	44 (S)	40 (S)	20 (S)	14 (R)
30	CUVET1356	21 (S)	42 (S)	40 (S)	22 (S)	28 (S)
31	CUVET1357	22 (S)	48 (S)	37 (S)	22 (S)	28 (S)
32	CUVET1358	21 (S)	40 (S)	35 (S)	24 (S)	28 (S)
33	CUVET1359	25 (S)	34 (S)	40 (S)	32 (S)	24 (S)
34	CUVET1360	28 (S)	28 (S)	21 (S)	15 (I)	25 (S)
35	CUVET1361	16 (I)	32 (S)	32 (S)	31 (S)	18 (I)
36	CUVET1362	15 (I)	24 (S)	30 (S)	30 (S)	27 (S)
37	CUVET1363	25 (S)	40 (S)	34 (S)	32 (S)	26 (S)
38	CUVET1364	26 (S)	32 (S)	32 (S)	32 (S)	26 (S)
39	CUVET1365	28 (S)	38 (S)	32 (S)	32 (S)	28 (S)
40	CUVET1367	28 (S)	36 (S)	36 (S)	26 (S)	25 (S)
41	CUVET1368	30 (S)	34 (S)	28 (S)	30 (S)	30 (S)
42	CUVET1369	32 (S)	28 (S)	26 (S)	30 (S)	28 (S)
43	CUVET1370	30 (S)	33 (S)	34 (S)	32 (S)	26 (S)
44	CUVET1374	30 (S)	40 (S)	36 (S)	22 (S)	17(R)
45	CUVET1375	32 (S)	27 (S)	20 (I)	14 (I)	17 (R)
46	CUVET1376	26 (S)	40 (S)	34 (S)	24 (S)	19 (I)
47	CUVET1377	30 (S)	19 (I)	28 (S)	11 (R)	30 (S)
48	CUVET1378	32 (S)	17 (I)	40 (S)	12 (R)	32 (S)
49	CUVET1379	32 (S)	38 (S)	36 (S)	26 (S)	17 (R)
50	CUVET-BU1	34 (S)	38 (S)	36 (S)	28 (S)	27 (S)

S: Susceptible, I: Intermediate, R: Resistant, FQ: Fluoroquinolone, Q: Quinolone, ENR: enrofloxacin (5µg), CIP: ciprofloxacin (5µg), NA: nalidixic acid (10µg), OA: oxolinic acid (10µg), NOR: norfloxacin (10µg).

APPENDIX C

Media and reagent

Media for bacterial cultivation

1. Anacker and Ordal's Medium (Cytophaga Medium) (Anacker and Ordal, 1955).

Tryptone	0.5 g
Yeast extract	0.5 g
Beef extract	0.2 g
Sodium acetate	0.2 g
Agar	10 g
Distilled water	1 L

Adjust pH to 7.2 – 7.4 and autoclave 15 – 20 min at 121 °C

Media for AST

1. 1:5 Diluted Mueller-Hilton Agar (DMHA) (4g/L) (Darwish et al., 2008)

Mueller Hinton Agar with approximate formula per litre (Beef extract power 2.0 g, Acid Digest of Casein 17.5 g, Starch 1.5 g, Agar 17.0 g)	7.6 g
Agar	13.6 g
Distilled water	1 L

Adjust pH to 7.2 – 7.4 and autoclave 15 – 20 min at 121 °C

2. 1:5 Diluted Mueller-Hilton Broth (DMHB) (4g/L) (Darwish et al., 2008)

Mueller Hinton Agar with approximate formula per litre (Beef, infusion form 300 g, Bacto Casamino Acids 17.5 g, Bacto Soluble Starch 1.5 g)	4.2 g
Distilled water	1 L

Adjust pH to 7.2 – 7.4 and autoclave 15 – 20 min at 121 °C

Preservative formula

3. Preservative Stock Composition

Sterile glycerol 60%	100 mL
Fetal Bovine Serum	200 mL
Cultured bacteria in Cytophaga broth	
(after incubation reach to the log phage)	700 mL

Buffer solution

4. 10XTBE buffer

Tris base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 mL
DNase-free H_2O	1 L

Dilute with DNase-free H_2O 100 mL to 1 L (1X) to make the gel running electrophoresis and running buffer. Store at room temperature for 6 month to 1 year.

5. 0.85% Normal saline solution

Sodium chloride	0.85 g
Distilled water	1 L

VITA

AUTHOR Mr. Wanniwat Mata

DATE OF BIRTH 28 April1988

PLACE OF BIRTH Phrae, Thailand

EDUCATIONAL CREDENTIALS

Bachelor Science of Veterinary Technology (Hons)

(2007-2010)

Faculty of Veterinary Technology, Kasetsart University

ACADEMIC PRESENTATION

1. Poster presentation in the topic of "Mutations in gyrA and parC associated with quinolone resistance in Flavobacterium columnare isolated from freshwater fish in Thailand" at 9th Symposium on Diseases in Asian Aquaculture (DAA9): Advance Circular; 24-28 November 2014, Ho Chi Minh City, Vietnam

2. Poster presentation in the topic of "Susceptibility of Flavobacterium columnare to common drugs used in aquaculture and the mutation in quinolone target genes associated with quinolone resistance" in The 39th International Conference on Veterinary Science (ICVS 2014); 16-18 December 2014, Royal Jubilee Ballroom IMPACT Challenger Muang Thong Thani, Thailand