การติดเชื้อแบคทีเรียแบบผสมผสานและการจำแนกคุณลักษณะของเชื้อแบคทีเรียจากปลาที่เป็นโรคคอลัมนาริส

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CONCURRENT BACTERIAL INFECTIONS AND BACTERIAL CHARACTERIZATION FROM COLUMNARIS DISEASED FISH

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Pathobiology
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Thesis Title	CONCURRENT BACTERIAL INFECTIONS AND BACTERIAL CHARACTERIZATION FROM COLUMNARIS DISEASED FISH
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ฮา ทานห์ ดง : การติดเชื้อแบคทีเรียแบบผสมผสานและการจำแนกคุณลักษณะของเชื้อแบคทีเรียจากปลาที่เป็นโรคคอลัมนาริส (CONCURRENT BACTERIAL INFECTIONS AND BACTERIAL CHARACTERIZATION FROM COLUMNARIS DISEASED FISH) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: เดชฤทธิ์ นิลอุบล, 123 หน้า.

โรคคอลัมนาริสเป็นโรคติดเชื้อแบคทีเรียที่มีสาเหตุมาจากเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ (*Flavobacterium columnare*) ซึ่งมีรายงานการเกิดโรคในปลาน้ำจืดหลายชนิดทั่วโลก การระบาดของโรคมักทำให้ปลาเลี้ยงมีอัตราการตายที่สูง และทำให้ธุรกิจการเพาะเลี้ยงสัตว์น้ำเกิดความสูญเสียทางเศรษฐกิจเป็นอย่างมาก ในระบบการเพาะเลี้ยงสัตว์น้ำตามธรรมชาติปลามีโอกาสสัมผัสกับเชื้อโรคต่างๆ หลากหลายชนิด ดังนั้นในสภาวะการเลี้ยงจริงปลามักป่วยและตายจากการติดเชื้อโรคมากกว่า 2 ชนิดหรือหลายชนิดพร้อม ๆ กัน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการติดเชื้อแบคทีเรียแบบผสมผสานในปลาที่แสดงอาการของโรคคอลัมนาริสและนำเอาเชื้อแบคทีเรียที่แยกได้จากปลามาทำการศึกษาคุณลักษณะต่าง ๆ โดยมุ่งเน้นศึกษาในปลานิลและปลาสวาย จากการระบาดของโรคคอลัมนาริส 2 ครั้งในฟาร์มปลานิล 2 แห่งในประเทศไทยโดยปลาแต่ละตัวแสดงอาการภายนอกของโรคคอลัมนาริสและตรวจพบการติดเชื้อแบคทีเรีย 2-4 ชนิด จากเชื้อแบคทีเรียที่ถูกพิสูจน์ได้ทั้ง 5 ชนิด ซึ่งประกอบด้วย *F. columnare*, *Aeromonas veronii, Streptococcus agalactiae, Plesiomonas shigeloides* และ *Vibrio cholera* โดยในการทดสอบการติดเชื้อร่วมกันของเชื้อแบคทีเรียชนิดต่าง ๆ ในห้องปฏิบัติการ พบว่าเชื้อ *A. veronii* และ *F. columnare* ให้ผลการทดสอบการติดเชื้อร่วมโดยปลามีรอยโรคและอาการทั้งภายในและภายนอกคล้ายคลึงกับการติดเชื้อตามธรรมชาติมากที่สุด ผลการทดลองนี้แสดงให้เห็นว่า *A. veronii* และ *F. columnare* คือเชื้อก่อโรคหลักที่ร่วมเป็นสาเหตุการตายของปลาจากเหตุการณ์ระบาดของโรคดังกล่าวข้างต้น ในขณะที่เชื้อก่อโรคอื่นๆ เป็นเชื้อก่อโรคฉกฉวยโอกาส การติดเชื้อร่วมตามธรรมชาติของ *F. columnare* และ *Edwardsiella ictaluri* ในปลาสวาย (striped catfish) ที่แสดงอาการของโรคคอลัมนาริส ได้ถูกรายงานเป็นกรณีแรกในการศึกษานี้ การทดสอบการติดเชื้อร่วมของ *F. columnare* และ *Edwardsiella ictaluri* ในปลาสวายโดยการฉีดเข้าช่องท้องและการฉีดเข้ากล้ามเนื้อทำให้ปลาแสดงอาการและรอยโรคทางจุลพยาธิวิทยาเหมือนกับการติดเชื้อร่วมตามธรรมชาติซึ่งยืนยันได้โดยการเติมเต็มตามทฤษฎีของคอค (Koch's postulates) ความหลากหลายทางพันธุกรรม ของ *F. columnare* แสดงให้เห็นว่าสายพันธุ์ของ *F. columnare* ที่แยกได้จากปลานิลและปลาสวายมีความแตกต่างกันในวิวัฒนาการทางพันธุกรรมเมื่อทดสอบจากยีน 16S rRNA นอกจากนี้ยังพบว่าส่วนใหญ่ของเชื้อ *F. columnare* ที่แยกได้จากปลานิลในประเทศไทยถูกจัดอยู่ในจีโนโมวา II (Genomovar II) และมีโคโลนีแบบไรซอย (rhizoid colonies morphotype) บนอาหารเลี้ยงเชื้อ AOA ความรุนแรงของเชื้อ *F. columnare* ที่มีโคโลนีทั้งแบบไรซอยและไม่ใช่ไรซอยถูกทดสอบในปลานิลแดงในระยะตัวอ่อน ผลการทดสอบแสดงให้เห็นว่าสายพันธุ์ที่มีโคโลนีแบบไรซอย ; CUVET1214 แสดงความรุนแรงสูงสุดในขณะที่สายพันธุ์ที่มีโคโลนีแบบไม่ใช่ไรซอย ; CUVET1201 ไม่แสดงความรุนแรง การเกาะติด (adherence) และการคงอยู่ (persistence) ของ *F. columnare* ทีมีโคโลนีทั้ง 2 แบบถูกทดสอบกับปลานิลในระยะตัวอ่อนด้วยการแช่ปลาทั้งตัวกับเชื้อแบคทีเรีย ผลการทดสอบแสดงให้เห็นว่าเชื้อที่มีโคโลนีแบบไม่ใช่ไรซอยไม่มีความสามารถในการคงอยู่ในปลานิลและเชื้อดังกล่าวไม่มีความรุนแรงในการก่อโรค นอกจากนี้ได้มีการศึกษาคุณลักษณะที่หลากหลายของเชื้ออื่นๆ ที่ไม่ใช่เชื้อ *F. columnare* (non-*F. columnare* bacteria) แต่เกี่ยวข้องกับการเกิดโรคคอลัมนาริสในปลา ซึ่งได้ถูกพิสูจน์ว่าเป็นเชื้อฉกฉวยโอกาสที่ร่วมในก่อโรคกับเชื้อก่อโรคหลักอื่นๆ ในการศึกษานี้

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	.....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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HA THANH DONG: CONCURRENT BACTERIAL INFECTIONS AND BACTERIAL CHARACTERIZATION FROM COLUMNARIS DISEASED FISH. ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, CO-ADVISOR: ASST. PROF. DR. DACHRIT NILUBOL, 123 pp.

Columnaris disease, a bacterial infection caused by *Flavobacterium columnare,* has been reported in various freshwater fish species worldwide. Disease outbreaks usually result in mass mortality of cultured fish and severe financial losses for aquaculture producers. In aquaculture system, where the fish naturally exposed to various potential pathogens, the reality of dead-loss due to dual or multiple infections is highly predictable and probably outweighs single infection. This study aims to investigate concurrent bacterial infections and pathogen characterization from columnaris diseased fish with emphasis on tilapia and striped catfish. From two outbreaks in Nile tilapia farms in Thailand where fish exhibiting external clinical signs of columnaris disease, each naturally diseased fish was found to be concurrently infected by 2 to 4 out of the 5 identified pathogens including *F. columnare*, *Aeromonas veronii, Streptococcus agalactiae, Plesiomonas shigeloides* and *Vibrio cholera.* Upon experimental challenge tests, fish exposed to *A. veronii* or *F. columnare* mimicked major internal and external clinical signs of naturally infected fish, respectively. This suggested that *A. veronii* and *F. columnare* are two main pathogens co-responsible for the dead-loss of the outbreak cases reported in the present study, whereas remaining pathogens might serve as opportunistic agents in the disease outbreaks. Similarly, natural co-infections of *F. columnare* and *Edwardsiella ictaluri* were first reported in the striped catfish exhibiting external clinical signs of columnaris disease. Co-infection challenge by both intraperitoneal and intramuscular routes successfully mimicked typical signs and histopathological manifestations of natural co-infections thus fulfilling Koch's postulates. Genetic diversity of *F. columnare* revealed that isolates originated from tilapia and striped catfish were phylogenetically different based on 16S rRNA. It was also found that the majority of *F. columnare* isolates from Thailand belongs to Genomovar II and formed rhizoid morphotype on AOA medium. The virulence of two morphotypes (rhizoid and non-rhizoid colonies) of *F. columnare* from tilapia collection was determined *in vivo*. The typical rhizoid isolate (CUVET1214) was a highly virulent isolate whereas the non-rhizoid isolate (CUVET1201) was avirulent to red tilapia fry. Adherence and persistence of both *F. columnare* morphotypes to tilapia fry were determined by whole fish bacterial loads. The results suggested that an inability of the non-rhizoid morphotype to persist in tilapia fry may explain lack of virulence. Additionally, diversity of non-*F. columnare* bacteria associated with columnaris diseased fish was characterized and proven to be merely opportunistic bacteria in this study.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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CONTENTS

Page

Page

Page

CHAPTER I
General introduction

1.1 Outline of the thesis

The thesis was divided into six chapters. The first chapter is an overview of the study, which provides background and rationale, objectives, scopes and advantages of the study. The second chapter emphasizes on characterization of naturally concurrent infections of bacterial pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. The third chapter characterizes concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish (*Pangasianodon hypophthalmus*) both naturally and experimentally. Chapter IV focuses on diversity of non-*Flavobacterium columnare* isolated from columnaris-like diseased fish. Chapter V investigates virulence, adherence and persistence of rhizoid and non-rhizoid morphotypes of *Flavobacterium columnare in vivo*. The last chapter characterizes genetic diversity of *F. columnare* isolated from three different fish species including Nile tilapia, red tilapia and striped catfish.

1.2 Background and rationale

Aquaculture is becoming the most important food animal-producing sector. Intensive culture forms, therefore, are highly developed corresponding to the leading position. Tilapia (*Oreochromis* spp.) and striped catfish (*Pangasianodon hypophthalmus*) has been commercially produced in most Asian countries (Nguyen and Dang, 2009; Fitzsimmons et al., 2011). Tilapia, a native cichlid fish in Africa and Middle East, was introduced to Thailand in 1965 and has become the second most important aquaculture freshwater fish (DOF, 2010; Bhujel, 2011). While striped catfish is native to Southeast Asia region, including Cambodia, Laos, Viet Nam and Thailand (Robert and Vidthayanon, 1991; Poulsen et al., 2004). If tilapia has been considered as an “aquatic chicken”, the recent boom in striped catfish culture and significant export levels in Vietnam has brought global attention to the striped catfish culture industry (Nguyen and Dang, 2009). Intensive culture, where fish are cultivated at high density in large scale commercial fish farms, infectious diseases are easily outbreak

and resulted in serious economic loss. Infectious diseases caused by bacteria are major threats in both tilapia and striped catfish farms (Crumlish et al., 2002; Abdel-Fattah and El-Sayed., 2006; Crumlish et al., 2010; Tien et al., 2012; Kayansamruaj et al., 2014a; Kayansamruaj et al., 2014b).

Single infection of individual bacterium in tilapia farms have been reported in Thailand, including *S. agalactiae* (Rodkhum et al., 2011; Kayansamruaj et al., 2014b), *F. columnare* (Dong et al., 2015), *Francisella noatunensis* subsp. *orientalis* (Nguyen et al., 2015). Most laboratory studies in tilapia diseases are typically aimed at characterization of a single bacterial pathogen but lack an understanding of concurrent infections. The reality of disease outbreaks in cultured farms may in all probability be caused by multiple pathogen infections. Few recent reports revealed that experimental co-infections of ectoparasite *Gyrodactylus niloticus* or *Ichthyophthirius multifiliis* with *S. iniae* induced significant higher mortality in Nile tilapia to compare with single infection (Xu et al., 2007; Xu et al., 2009). A case of skin and subcutaneous mycoses in tilapia caused by natural co-infections between a fungi *Fusarium oxysporumin* and *A. hydrophila* was recently reported (Cutuli et al., 2015). However, concurrent infections of multiple pathogens in naturally diseases fish remain poor understood and need further investigations.

Table 1: Major infectious diseases affecting farmed tilapia.

Pathogen	**Disease**	**References**
Bacteria		
Aeromonas sorbia	Tail-rot disease	Li and Cai (2011)
Flavobacterium columnare	Columnaris	Figueiredo et al. (2005); Dong et al. (2015)
Streptococcus agalactiae *Streptococcus iniae* *Lactococcus garvieae*	Streptococcosis	Rodkhum et al. (2011); Anshary et al. (2014); Kayansamruaj et al. (2014b)
Francisella noatunensis subsp.	Francisellosis/systemic	Soto et al. (2009);

orientalis	visceral granulomas	Jeffery et al. (2010); Nguyen et al. (2015)
Edwardsiella ictaluri *Edwardsiella tarda*	Edwardsiellosis	Iregui et al. (2012); Soto et al. (2012)
Virus		
Iridovirus	N/A	Ariel and Owens (1997); McGrogan et al. (1998)
Betanodavirus	Viral nervous necrosis	Keawcharoen et al. (2015)
Herpes-like virus	Viral encephalitis of tilapia larvae	Sinyakov et al. (2011)
A novel RNA virus/tilapia lake virus (TiLV)	N/A	Eyngor et al. (2014)
Co-infections		
Fusarium oxysporumin & *A. hydrophila*	Skin and subcutaneous mycoses in tilapia	Cutuli et al. (2015)
Gyrodactylus niloticus & *S. iniae*	gyrodactylosis and streptococcosis	Xu et al. (2007)
Ichthyophthirius multifiliis & *S. iniae*	ichthyophthiriasis and streptococcosis	Xu et al. (2009)

Similarly, the high stock density of intensively cultured striped catfish farms also faced devastation through infectious pathogens such as channel catfish virus (Siti-Zahrah et al., 2013), parasistic monogenea *Thaparocleidus caecus* and *Thaparocleidus siamensis* (Šimková et al., 2013; Tripathi et al., 2014) or important bacteria *Edwarsiella ictaluri, E. tarda, F. columnare,* and *Aeromonas hydrophila* (Panangala et al., 2007; Crumlish et al., 2010; Shetty et al., 2014). *E. ictaluri* and *F. columnare* are recognized as the most highly pathogenic bacteria that cause enteric septicemia of catfish (ESC) and columnaris disease in freshwater fish respectively (Declercq et al., 2013; Hawke et al., 1981).

Among bacterial pathogens, *Flavobacterium columnare*, the causative agent of columnaris disease, is one of the most important threats for tilapia and striped

catfish industry (Crumlish et al., 2002; Figueiredo et al., 2005; Tien et al., 2012; Dong et al., 2015). Dong et al. (2015) reported columnaris disease in intensively cultured tilapia farms in Thailand. Numerous yellow-pigmented bacteria were recovered from diseased red tilapia and majority was identified as *F. columnare*. A novel genetic characterization of Thai isolates was also first described. However, genetic diversity of *F. columnare* from other fish host such as Nile tilapia (*O. niloticus*), striped catfish (*P. hypophthalmus*) as well as their pathogenicity remains undetermined. Moreover, we also observed that many non-*Flavobacterium columnare* bacteria were recovered from the fish showing clinical signs of columnaris disease. One of them was recently identified as *Francisella noatunensis* subsp. *orientalis* (Nguyen et al., 2015) and reported as causative agent of systemic visceral granulomas in tilapia. While remaining non-*F. columnare* bacteria remain unidentified. Typically, most laboratory studies in tilapia and striped catfish diseases are aimed at characterization of a single bacterial pathogen but lack an understanding of concurrent infections. The reality of disease outbreaks in cultured farms may in all probability be caused by multiple pathogen infections. Therefore, beside *F. columnare*, characterization of non-*Flavobacterium columnare* and their pathogenic potential should be uncovered. The objectives of this study are 1) to identify and characterize infectious pathogens concurrently infected cultured tilapia and striped catfish, which exhibited symptoms of columnaris disease, and their pathogenic potential; 2) to investigate genetic characterization of *F. columnare* isolated from different fish species (red tilapia, Nile tilapia and stripe catfish) and 3) to compare the virulence and ability of the rhizoid and non-rhizoid morphotypes of *F. columnare* to adhere to and persist in tilapia.

1.3 Objectives of the study

1.3.1 To identify and characterize bacterial pathogens concurrently infected tilapia and striped catfish which showed symptoms of columnaris disease and their pathogenic potential.

1.3.2 To investigate genetic characterization of *F. columnare* isolated from red tilapia, Nile tilapia and stripe catfish.

1.3.3 To compare the virulence and ability of the rhizoid and non-rhizoid morphotypes of *F. columnare* to adhere to and persist in tilapia model.

1.4 Scopes of the study

This study focuses on concurrent bacterial infections and bacterial characterization from columnaris diseased fish. Naturally concurrent bacterial infections of both *Flavobacterium columnare* and non-*Flavobacterium columnare* bacteria have been characterized from diseased tilapia (*Oreochromis* spp.) and striped catfish (*P. hypophthalmus*), which exhibited symptoms of columnaris disease. Subsequently, single or combined challenges have been carried out to mimic natural outbreak and fulfill Koch's postulates. Genetic diversity of *F. columnare* isolated from three different fish species have been evaluated in this study. Virulence, adherence and persistence of two colony morphotypes of *F. columnare* have been determined using red tilapia fry model.

1.5 Advantages of the study

1.5.1 This study had risen up the concept of concurrent infections of dual or multiple infectious pathogens in tilapia and striped catfish during disease outbreaks reflecting the reality of dead-loss in intensively cultured fish farms.

1.5.2 Dual infections of *Flavobacterium columnare* and *Edwarsiella ictaluri* were first addressed in cultured striped catfish in Thailand. The study also had identified an emerging pathogenic bacterium *Aeromonas veronii* causing

hemorrhagic septicemia in Thai tilapia farms. The findings suggested that improved strategic management for pathogen surveillance and disease control is highly required.

1.5.3 Genetic diversity of *F. columnare* revealed that *F. columnare* originated from tilapia was phylogenetically different with those originated from striped catfish and showed relatively low 16S rRNA sequence similarity to the reference strain. This suggests that taxonomy of *F. columnare* isolated from tilapia should be amended.

1.5.4 This study not only emphasized on the main causative agent of columnaris disease but also brought new insights into diversity of opportunistic non-*F. columnare* bacteria associated columnaris disease.

1.5.5 Correlation between *F. columnare* morphotypes and virulence was demonstrated *in vivo* which may shed light on application of avirulent non-rhizoid morphotype as a live vaccine for columnaris disease control.

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CHAPTER II

Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms

Publication

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Abstract

The reality of dead-loss in cultured fish farms due to multiple pathogen infections probably outweighs single infection. Here, we describe the isolation and characterization of the potential pathogens concurrently infected in natural disease outbreaks of Nile tilapia (*Oreochromis niloticus*) and their pathogenicity in the red tilapia fingerling model. Co-infection of bacteria and *Iridovirus* were found in two affected farms. Based on conventional phenotypic tests and sequence analysis of 16S rDNA fragment, most predominant bacteria were identified as *Flavobacterium columnare* and *Aeromonas veronii,* and remainders were *Streptococcus agalactiae, Plesiomonas shigeloides* and *Vibrio cholerae.* Experimental infection with selected single bacterial isolates demonstrated that both alpha- and beta-hemolytic *A. veronii* isolates were highly pathogenic to tilapia fingerling, whereas *F. columnare* and *S. agalactiae* were less virulent and *P. shigeloides* was avirulent. The fish exposed to *A. veronii* or *F. columnare* mimicked major internal and external clinical signs of naturally infected fish respectively. This suggests that *A. veronii* and *F. columnare* are two main pathogens co-responsible for the dead-loss of cultured tilapia farms in the present study, whereas remaining pathogens might serve as opportunistic pathogens in the disease outbreaks.

Keywords: *Aeromonas veronii,* concurrent infections*, Flavobacterium columnare*, Tilapia

Introduction

Nile tilapia (*O. niloticus*) has been commercially produced in most Asian countries (Fitzsimmons et al. 2011). It was introduced to Thailand in 1965 and has become the second most important aquaculture species after shrimp (Bhujel, 2011). Intensive production is undoubtedly threatened by the potential devastation of infectious diseases. The major diseases threatening intensively cultured tilapia farms have been identified as various bacterial infections (Abdel-Fattah and El-Sayed, 2006, Kayansamruaj et al., 2014b). However, most laboratory studies in tilapia diseases are typically aimed at characterization of a single bacterial pathogen but lack an understanding of concurrent infections. The reality of disease outbreaks in cultured farms may in all probability be caused by multiple pathogen infections. Previously, some important pathogens devastating tilapia within intensively produced systems were reported, such as *Streptococcus agalactiae* (Olivares-Fuster et al., 2008; Kayansamruaj et al., 2014a; Kayansamruaj et al., 2014b), *Flavobacterium columnare* (Figueiredo et al. 2005; Dong et al., in press-a,b), *Francisella* sp. (Soto et al., 2009; Jeffery et al., 2010; Leal et al., 2014), *Edwardsiella* spp. (Iregui et al., 2012; Soto et al., 2012), *Aeromonas* spp. (Li and Cai, 2011; Pridgeon and Klesius, 2011; Soto-Rodriguez et al., 2013) and *Iridovirus* (Ariel and Owens, 1997; McGrogan et al., 1998). However, concurrent infections among these pathogens remains poorly understood.

Recently, disease outbreaks occurred annually in floating cage cultured Nile tilapia farms along the Mekong River in Nong Khai province in the northeastern part of Thailand that resulted in high mortality rates and lead to severe economic losses. Unfortunately, the causative agents remain unclear. Diseased fish usually exhibited severe clinical signs resembling bacterial infections but not specific to any single infection. This study therefore aims to isolate and identify potential pathogens concurrently recovered from diseased fish and investigate their pathogenicity in the tilapia fingerling model.

Materials and methods

Fish samples

Representatives of naturally diseased Nile tilapia (*O. niloticus*) (n=10) were randomly collected from two floating cage cultured farms in Nong Khai province, northeastern Thailand during disease outbreaks in August 2014. All fish were examined for clinical signs before aseptically necropsied for bacterial isolation. Small pieces of the liver, kidney and spleen from each diseased fish were preserved in 95% alcohol for PCR assay.

For the experimental challenge, since we were not able to access Nile tilapia fingerlings at their source, red tilapia (*Oreochromis* sp.) were selected as an animal model for pathogenicity tests in the present study. Red tilapia fingerlings (mean weight, 17.8 ± 4.1 g) were kindly donated by Mr. Warren A. Turner (NamSai farm, Prachinburi Province, Thailand). Fish were acclimated for 10 days in laboratory conditions before being used for the challenge studies. Prior to the challenge, all fish appeared normal and a subset of the fish (n=10) were randomly examined for ectoparasites and bacterial isolation and found to be qualified for experimental infection purposes.

Bacterial isolation

Three kinds of media were applied for bacterial isolations in this study. Anacker and Ordal agar (AOA) supplemented with 1 μg mL^{-1} tobramycin (Sigma) (Decostere et al., 1997) was used as a selective medium for *Flavobacterium* sp., whereas cysteine heart agar (CHA) supplemented with 10% sheep blood and Polymycin B 100 units mL^{-1} (Soto et al., 2009) was used for isolation of *Francisella* spp. Tryptic soy agar (TSA) supplemented with 5% bovine blood was aimed for other culturable bacteria. The internal organs (liver, kidney or spleen) of diseased fish were aseptically taken by inserting a sterile loop into the target tissue and streaked directly on three different media. For the purpose of *F. columnare* isolation, bacteria were also isolated from the gills or lesions on the body surface of diseased fish. The plates were incubated at 28 ^{O}C for 24 h (TSA), 48 h (AOA) and 5 days (CHA). Suspected colonies were sub-

cultured on the same cultured media, subjected to bacterial isolation, mixed with a broth medium containing 15 % glycerol and preserved at -80 ^{O}C until needed.

Biochemical characteristics

Conventional biochemical tests were performed with 14 selected culturable isolates (NK01 to NK14) according to Phillips (1993). All isolates were subjected to primary tests including hemolysis ability, Gram staining, oxidation/fermentation (O-F), motility, oxidase, catalase and growth capability on MacConkey agar. Carbohydrates metabolism was examined using nutrient broth supplemented by 1% carbohydrates (glucose, lactose, arabinose, maltose, trehalose and mannitol, fructose, xylose, sorbitol), 0.2 % thymol blue. H_2S production was determined using a triple sugar iron (TSI) medium. Additional tests were performed including citrate, urease, indole and arginine, lysine, ornithine decarboxylase, esculin hydrolysis. Small amounts of bacterial cells were inoculated in a tested medium at 28 ^{O}C for 24 to 48 h prior to the result interpretation according to Phillips (1993). Since *F. columnare* isolates were unable to grow in normal biochemical test media, identification of putative *F. columnare* isolates was performed based on a combination of species-specific PCR and sequencing of 16S rDNA (see below). Additionally, some basic phenotypic assays were performed according to Bernardet (1989) including Gram staining, oxidase, catalase and presence of flexirubin pigment.

DNA extraction from fish tissues and bacterial isolates

Mixed internal organs (liver, spleen, and kidney) of the diseased fish preserved in 95% alcohol mentioned above were subjected to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen). To examine the quality of extracted DNA, PCR primers targeting a 350-bp elongation factor gene of Nile tilapia was used for internal control (Table 1) (Yang et al. 2013). The conditions for PCR amplification were as follows: 5 min at 95°C, 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C 30 s, and extension at 72°C for 7 min.

Genomic DNA of pure bacterial isolates was extracted by the boiling method as previously described by Arias et al. (2004). In brief, overnight cultures of bacterial

isolates in broth medium were collected by quick centrifugation at 6,600 g. Cell pellets were suspended in 100 µL nuclease-free water and boiled for 10 min before being immediately cooled down in cold ice. After centrifugation, the supernatant contained DNA template was used for PCR assay.

Detection of Flavobacterium columnare

PCR detection assays of *F. columnare* were performed using DNA templates extracted from both diseased fish (n = 10) and isolated bacterial colonies (n = 10) grown on AOA plates. Species specific primers FCISRFL and FCISRR1 (Table 1) targeting ITS sequence of *F. columnare* as described by Welker et al. (2005) were applied in the present study. PCR mixures contained 12.5 µL Master Mix (GoTaq®Green, Promega USA), 0.2 µM of each primer and 5 µl of DNA template in a final volume of 25 µL. Amplification was carried out in the thermocycler (TC-96/G/H(b), Bioer China) as follows: denaturation at 94 °C for 5 min; 30 cycles of amplification at 94 °C for 30 sec, annealing at 45 °C for 45 sec; extension at 72 °C for 7 min. Genomic DNA of the isolate CUVET1201 (Dong et al., in press-a) was used as a template for positive control and nuclease-free water for negative control. Amplified products of 400-500 bp were visualized under UV light after being electrophoresed with 1 % agarose gel in TBE and stained with Red Safe (ABC Scientific).

Detection of Francisella spp.

Investigation of *Francisella* spp. infection in diseased fish (n = 10) was carried out using genus-specific primers targeting 16S rRNA gene of *Francisella* spp. (Table 1) (Forsman et al. 1994). PCR mixtures were performed in the same manner as described for *F. columnare* detection. The thermocycling conditions wsere 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 sec, annealing at 60 °C for 60 sec and extension at 72 °C for 60 sec; final extension at 72°C for 5 min. DNA template extracted from isolate of *Francisella noatunensis* subsp. *orientalis* VMCU-FNO131 (accession no. KJ841935) (Nguyen et al., in press) was used for positive control while DNA extracted from healthy fish was used as a negative control. Amplified products were then electrophoresed and visualized under UV light.

Detection of Iridovirus

All primer sequences used for the viral detection were listed in Table 1. Semi-nested PCR primers were designed to target the major capsid protein gene of megalocytivirus (GenBank JQ253374.1), which is a genus within the family *Iridoviridae*. MegaI and MegaII primers for the first step PCR were used to amplify 214-bp according to the method described by Gias et al. (2011). To increase the method's sensitivity, MegaIII primer was newly designed in this study and was used together with MegaI primer in the secondary PCR reaction to amplify a 108-bp product. Each PCR reaction was conducted in a 25 µL reaction volume containing 2.5 µL PCR buffer (10X) 0.75 µL $MgCl_2$ (50 mM), 0.5 µL dNTP (10 mM), 0.5 µL of each forward and reverse primer (10 mM), 19.125 µL water,0.125 µL (1U) Taq DNA polymerase, and 1 µL DNA template. The first PCR amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C 30 s, with a final extension at 72°C for 7 min. For the secondary PCR step, 1 µL of the first-step reaction was used as a template, and the amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 15 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. PCR products were purified using Gel and PCR Clean-up columns (NucleoSpin), according to the manufacturer's protocol. The amplified DNA fragments were ligated into pGEM-T Easy cloning vector (Promega) and transformed in JM109 competent cells as described in Sambrook and Russell (2001). Sequencing of PCR products was carried out by Macrogen, Korea using a BIG DYE 3.1 terminator mix on an ABI 377 Sequencer. BLAST program (NCBI) was used to align the sequences of the amplified products with matching records of iridovirus isolates.

16S rDNA amplification and DNA sequencing

Three sets of universal primer targeting prokaryotic 16S rDNA used in this study (Table 1) included UN20/20F and R1438 (Darwish and Ismaiel, 2005) for isolates of *Flavobacterium* sp.; Universal F1 and Universal R13 (Dorsch and Stackebrandt, 1992) for *Aeromonas* spp.; and Uni-Bact-F and Uni-Bact-R (Weisburg et al., 1991) for

remaining isolates. The PCR mixtures contained 0.25 µM of each primer pair, 0.2 mM of dNTPs, 0.25 µM of $MgCl_2$, 1 unit of Taq polymerase (Invitrogen), 1X reaction buffer, 5 µL bacterial DNA templates or a small amount of bacterial colony and nuclease-free water in a final volume of 25 µL. The PCR conditions were applied as previously described (Weisburg et al., 1991; Dorsch and Stackebrandt, 1992; Darwish and Ismaiel 2005). Noticeably, due to of failure in PCR amplification, a gradient PCR were set up to find out a suitable annealing temperature for *Aeromonas* spp. isolates. Amplified products of ~1.5 kb were purified using a Favogen Gel/PCR purification kit. Purified DNA amplicons were submitted for DNA sequencing at the 1st BASE Pte Ltd. (Malaysia). Assembly process was performed with forward and reverse sequences using ContigExpress software (Invitrogen Corporation, 2006). Homology search was carried out using nucleotide BLAST to the GenBank database in the National Center for Biotechnology Information (NCBI). Multiple sequence alignments of the 16S rRNA gene sequences of all bacterial isolates in the present study and their closed taxa retrieved from GenBank were performed using ClustalW method. Phylogenetic tree was constructed using a Neighbor-Joining method of MEGA version 5 (Tamura et al., 2011) with bootstraps value of 1000 replicates.

Table 1 Primers used in this study.

Organism	Gene	Primer names/Sequences (5'to 3')	References
Other bacteria	16S rDNA	Uni-Bact-F/AGAGTTTGATCMTGGCTCAG Uni-Bact-R/ACGGHTACCTTGTTACGACTT	Weisburg et al., 1991
Aeromonas spp.	16S rDNA	Universal F1/GAGTTTGATCCTGGCTCAG Universal R13/AGAAAGGAGGTGATCCAGCC	Dorsch and Stackebrandt, 1992
Flavobacterium sp.	16S rDNA	UN20/20F AGAGTTTGATC(AC)TGGCTCAG R1438 GCCCTAGTTACCAGTTTTAC	Darwish and Ismaiel, 2005
Flavobacterium columnare	ITS	FCISRFL/ TGCGGCTGGATCACCTCCTTTCTAGAGACA FCISRRL1/ TAATYRCTAAAGATGTTCTTTCTACTTGTTTG	Welker et al., 2005
Francisella spp.	16S	F11/TACCAGTTGGAAACGACTGT	Forsman et al.,

Iridovirus	rDNA	F5/CCTTTTTGAGTTTCGCTCC	1994
	major capsid	Mega I/ GTGTGGCTGCGTGTTAAG MegaII/ TGCCAATCATCTTGTTGTAGC	Gias et al., 2011
		MegaIII/ TGACACCGACACCTCCTTCC	Present study
Tilapia	elongation factor	EF1A-F/GCACGCTCTGCTGGCCTTT EF1A-R/ GGTCTCCAGCATGTTGTCTCCGT-3	Yang et al., 2013

Experimental challenges

Five representatives of four bacterial species identified and designated as *F. columnare* NK-Fc01*, A. veronii* NK01 (ß hemolysis)*, A. veronii* NK06 (α hemolysis), *Plesiomonas shigeloides* NK11, and *Streptococcus agalactiae* NK13 were used for challenging the fish. An overnight culture of a bacterial suspension (log-phase growth) of bacterial isolates was prepared as follows: A single colony of each bacterial isolate was inoculated in 5 mL of either MAT broth (for *F. columnare*) or TSB (for remaining isolates) at 28 ^{O}C for overnight, bacterial suspension was adjusted to OD_{600} equal 0.55 to 0.60 and verified by plate count method before challenging. A total of 60 healthy fish (mean weight, 17.8 ± 4.1 g) were divided into 6 groups, 10 fish each. Groups 1 to 4 were intraperitoneal-injected with 1.36×10^{7} CFU of *A. veronii* NK01 fish^{-1}, 0.82×10^{7} CFU of *A. veronii* NK06 fish^{-1}, 1×10^{7} CFU of *P. shigeloides* NK11 fish^{-1}, and 2.30×10^{8} CFU of *S. agalactiae* NK13 fish^{-1}, respectively. Since *F. columnare* was predominantly found in the skin and gills lesion, group 5 was exposed to 1×10^{7} CFU of *F. columnare* NK-Fc01 fish^{-1} by an intramuscular injection route. The control group was injected with a 0.1 mL TSB medium. Details of the experimental challenge are summarized in Supplemental Table 1. After challenge, fish were maintained in the tanks containing 20 L UV-treated water, fed twice per day with commercial feed (CP, Thailand). Water temperature during the experiment was 26 ± 1 ^{O}C. Clinical signs and mortality of experimental fish was recorded in 15 days. Fresh dead fish were subjected for bacterial isolation and reconfirmed by using species specific PCR (for *F. columnare*) or biochemical tests (for remaining species) as mentioned above.

Results

Description of outbreaks and clinical signs of diseased fish

According to local producers, unknown disease outbreaks occur annually in cage cultured Nile tilapia farms along Mekong River in Nong Khai province, Thailand. The disease has resulted in a cumulative mortality of up to 50% per crop. The diseased fish presented external signs resembling columnaris disease, noticeably gills necrosis, eroded fins and epidermis ulcers (Fig. 1). However, variability in the internal signs that do not refer to columnaris disease were also observed after necropsy, such as the presence of hemorrhagic liver, pale liver with necrotic area, enteritis, swollen gall bladder and abdominal swelling with visceral liquid (Fig. 1).

Figure 1 Naturally diseased Nile tilapia exhibited discoloration and hemorrhage on body surface (a), necrotic gills, swollen gall bladder, hemorrhagic liver (b), and enteritis (c). Red tilapia challenging to *F. columnare* NK-Fc01 exhibited typical skin lesion (d) and severe epidermis ulcer (e). Red tilapia exposed to *A. veronii* NK01 or NK06 showing hemorrhage at basal fins (f) and liver (g).

Bacterial isolation and biochemical characterization

Pure or mixed culture of two to three bacterial colony morphotypes were recovered from the internal organs (liver and kidney) of diseased fish using TSA supplemented with 5% bovine blood. In total, fourteen bacterial isolates representative of all colony types were obtained and subjected to further bacterial identification. The isolates were designated with the following organism code, NK01 to NK14. One bacterial isolate subsequently designated NK-Fc010 was obtained from a fish kidney smeared on AOA agar whereas the collected internal organs of diseased fish did not yield any suspected *Franciscella* colonies on CHA plates. Based on the homology of biochemical characteristics, NK01 to NK14 isolates were categorized into five biochemical profiles (Table 2). The isolates in profiles 1 to 4 were Gram negative, rod-shaped bacteria, positive for oxidase, catalase, motility tests and able to ferment various carbohydrates. The profile 1 (NK01, NK03, NK04) and profile 2 (NK02, NK05, NK06, NK07) were identical in most biochemical characteristics but there was a difference in hemolytic ability (Table 2). These isolates were later identified as *Aeromonas veronii* (see section 3.3). The ß hemolytic *A. veronii* (profile 1) were recovered from farm 1 whereas both α and ß hemolytic *A. veronii* (profile 2) were recovered from farm 2. Profile 5 consists of three Gram positive bacterial isolates (NK09, NK13, and NK14) and was presumptively identified as *Streptococcus* sp.

In parallel with the examination of internal organs of diseased fish, bacterial isolation was also performed using the necrotic gills and skin lesion smeared on AOA plates. The results showed that 8 and 1 bacterial isolates were recovered from gills and skin lesion respectively, from the infected fish from two affected farms. These isolates were assigned organism code NK-Fc01 to NK-Fc09. Morphologically, these isolates together with NK-Fc10 described above formed typical yellow pigmented,

rhizoid colonies on AOA medium. In addition, bacterial cells were Gram negative, and a slender, long rod shape that produced flexirubin pigment and exhibited a positive reaction with both cytochrome oxidase and catalase tests.

Table 2 Biochemical characteristics of bacterial isolates NK01 to NK14 in this study.

Characteristics	Profile 1 (NK01,03,04)	Profile 2 (NK02,05,06,07)	Profile 3 (NK08,12)	Profile 4 (NK10,11)	Profile 5 (NK09,13,14)
Gram	Negative	Negative	Negative	Negative	Positive
Bacterial cell	rod	Rod	rod	rod	cocci
Grown on MacConkey agar	+	+	-	+	-
Oxidase	+	+	+	+	-
Catalase	+	+	+	+	-
Oxidation/Fermentation (O/F)	F	F	F	F	-
Hemolysis	ß	α	γ	γ	ß*
Ornithine decarboxylase	-	-	ND	ND	-
Arginine decarboxylase	+	+	ND	ND	+
Lysin decarboxylase	+	+	ND	ND	-
Esculin hydrolysis	v	v	ND	ND	-
Motility	+	+	+	+	-
Indole	+	+	v	+	-
Citrate	v	-	-	-	-
Urease	-	-	-	-	-
H_2S production	-	-	-	-	-
Gas	+	+	-	-	-
Glucose	+	+	+	+	+
Lactose	-	-	+	+	+
Arabinose	+	+	+	+	V
Galactose	+	+	+	+	-
Maltose	+	+	+	+	-
Fructose	+	+	ND	ND	-
Xylose	-	-	ND	ND	-
Sorbitol	-	-	ND	ND	-

(+), positive; (-), negative; (v), variable; F, fermentation; ND, not determined; (*) at 37 ^{O}C

Bacterial identification

To identify taxonomic classification of the bacteria, NK01 to NK14 and NK-Fc01 to NK-Fc10 isolates were subjected to PCR to obtain the evolutionally conserved 16S rRNA nucleotide sequence. All fragments were fully sequenced from both strands of the DNA and sequence assembly yielded approximately 1.4 kb in length. However, the assembly process failed for PCR products from NK3, NK12, and NK14. BLAST search results using 1.4 kb sequences as queries revealed that the isolates in the biochemical profile 1 & 2 (NK01, NK02, NK04, NK05, NK06, and NK07) were identified as *Aeromonas veronii* based on 99.9% nucleotide identity to the two *A. veronii* bv. *veronii* strain ATCC 35624 (NR118947) and *A. veronii* bv. *sobria* strain HS120920 (KF543779) (Table 3). The isolates in biochemical profile 3 (NK08) were putatively identified as *Vibrio cholerae* based on 99.6% partial 16S rDNA identity with *V. cholerae* strain PIM9 (GQ359963). The isolates in profile 4 (NK10 and NK11) and 5 (NK09 and NK13) were identified as *Plesiomonas shigeloides* and *Streptococcus agalactiae* based on 99.8% nucleotide similarity with *P. shigelloides* strain NCIMB 9242 (NR044827) and 99.9% nucleotide similarity with *S. agalactiae* strain ATCC13813 (NR115728), respectively (Table 3). 16S rRNA sequences of NK-Fc01 to NK-Fc10 isolates exhibited 100% identity to *F. columnare* strains CUVET1201 and CUVET1214 isolated from red tilapia described in our previous study (Dong et al. 2014) (Table 3) and the sequences were completely identical among NK-Fc01 to NK-Fc10 strains. BLAST search was also performed with the 0.7 kb-unassembled fragments derived from NK03, NK12, and NK14. Results showed that sequences had strong similarity to *A. veronii* strain B565 (NR102789) (99% identity), *V. cholerae* strain PIM9 (GQ359963) (98.5% identity), and *S. agalactiae* strain ATCC13813 (NR115728) (99% identity), respectively. Taken together, phenotypic characteristics and molecular data analysis confirmed the result of bacterial identification.

The 16S rDNA gene sequences of ~1.4 kb were deposited in the GenBank database under accession number KP899499 to KP899519. The phylogenetic relationships derived from comparisons of the 16S rDNA sequences are provided in Supplemental Fig. 1. Sequences from 21 bacterial isolates were clustered with their

closest species that were *Flavobacterium columnare, Aeromonas veronii, Streptococcus agalactiae, Plesiomonas shigeloides* and *Vibrio cholerae*, consisting of the above BLAST homology searches.

Table 3 Detail of bacterial isolates isolated from diseased tilapia in this study.

Isolates	Biochemical profile	16S rRNA Genbank accession number	Most closely related species	Identity (%)
NK-Fc01, NK-Fc02, NK-Fc03, NK-Fc04, NK-Fc05, NK-Fc06, NK-Fc07, NK-Fc08, NK-Fc09, NK-Fc10	ND	KP899505-KP899514	*Flavobacterium columnare* CUVET1201(KF274033)	100
NK01, NK03, NK04	1	KP899499-KP899504	*Aeromonas veronii* bv. *veronii* strain ATCC 35624 (NR118947)	99.9
NK02, NK05, NK06, NK07	2		*Aeromonas veronii* bv. *veronii* strain ATCC 35624 (NR118947)	99.9
NK08, NK12	3	KP899517	*Vibrio cholerae* strain PIM9 (GQ359963)	99.6
NK10, NK11	4	KP899515-KP899516	*Plesiomonas shigelloides* strain NCIMB 9242 (NR044827)	99.8
NK09, NK13, NK14	5	KP899518-KP899519	*Streptococcus agalactiae* strain ATCC 13813 (NR115728)	99.9

ND, not determined

PCR diagnosis of diseased fish samples

Ten diseased fish from two affected farms were investigated for the pathogen infection of two bacteria (*Francisella* spp. and *F. columnare*) and one virus (*Iridovirus*) by PCR assays. Successful amplification of a partial sequence of fish elongation factor (EF) gene performed as a control demonstrated sufficient quality of the extracted fish DNA (Fig. 2a). Detection assays indicated negative results for *Francisella* spp. (Fig. 2b) and *F. columnare* (Fig. 2c) in all ten fish samples from two affected farms. In the case of iridoviral detection, while all fish were found to be negative at the first PCR step, two of five fish from the first farm and one of five fish from the second farm were *Iridovirus*-positive at the semi-nested PCR step (Fig. 2d, e). The amplicons of 108 bp were subjected for sequencing analysis, and their sequences had 100% identity to the sequence corresponding to the major capsid protein gene of megalocytivirus (Supplemental Fig. 2).

Moreover, DNA extracted from NK-Fc01 to NK-Fc10 bacterial isolates were subjected to specific PCR detection for *F. columnare* using an established protocol (Welker et al., 2005). The results showed that all isolates were positive with *F. columnare* specific PCR (Fig. 2f).

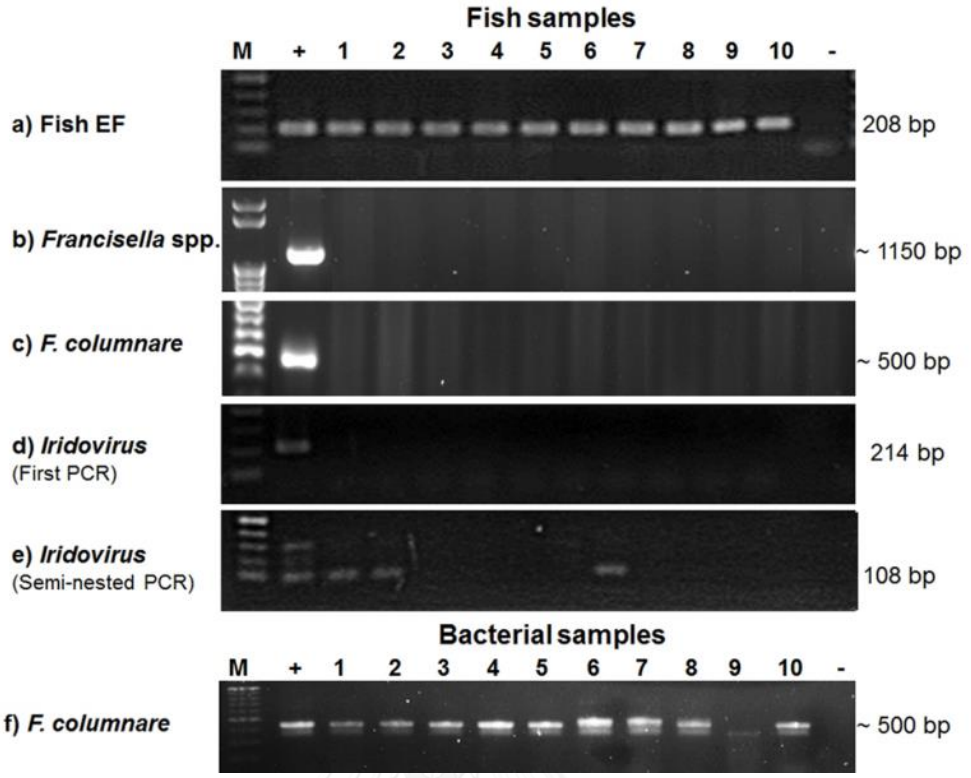

Figure 2 PCR assays of diseased fish and isolated bacteria. DNA extracted from diseased fish samples from farm 1 (numbers 1-5) and farm 2 (numbers 6-10) were subjected to amplification of elongation factor gene (a) and detection of indicated bacterial and viral pathogens (b-e). DNA extracted from NK-Fc01 to NK-Fc10 (numbers 1-10) bacterial isolates were assayed using *F. columnare* specific PCR (f). (+), respective positive control; (-), negative control.

Concurrent infection of bacteria and virus in diseased fish

The data of concurrent infection of bacteria and virus in individual diseased fish collected from two affected tilapia farms was presented in table 4. Among four pathogens infected in farm 1 and five pathogens found in farm 2, *F. columnare* and *A. veronii* were concurrently infected in 100% of the diseased fish from both tilapia farms. The prevalence of *Iridovirus* in each farm was 40% and 20% respectively. *V. cholerae* was detected in 40% of diseased fish in farm 1, whereas *S. agalactiae* and *P. shigeloides* were found only in farm 2 with a prevalence of 60% and 10% respectively. Most diseased fish were concurrently infected by three pathogens (Table 4).

Table 4 Concurrent infection of bacteria and virus found in two affected tilapia farms.

	F. columnare	*A. veronii*	*S. agalactiae*	*Francisella* spp.	*P. shigeloides*	*V. cholerae*	*Iridovirus*	Number of pathogens per fish
Farm 1 (%)	100	100	0	0	0	40	40	
Fish 1	+	+	-	-	-	-	+	3
Fish 2	+	+	-	-	-	-	+	3
Fish 3	+	+	-	-	-	+	-	3
Fish 4	+	+	-	-	-	-	-	2
Fish 5	+	+	-	-	-	+	-	3
Farm 2 (%)	100	100	60	0	20	0	20	
Fish 1	+	+	+	-	-	-	+	4
Fish 2	+	+	+	-	-	-	-	3
Fish 3	+	+	-	-	+	-	-	3
Fish 4	+	+	+	-	-	-	-	3
Fish 5	+	+	-	-	-	-	-	2

Results of experimental challenge

In the two i.p.-challenged groups where the fish received bacterial isolates *A. veronii* NK01 or *A. veronii* NK06, the experimental fish exhibited the highest cumulative percentage mortality (100 and 90% respectively, Fig. 3). All fish died within 24 h and exhibited haemorrhages in the internal organs, noticeably the liver, kidney and intestine (Fig. 1f, g). The group exposed to *F. columnare* NK-Fc01 (Fig. 1d, e) and *S. agalactiae* NK13 exhibited 50% and 20% cumulative percentage mortality respectively (Fig. 3). Clinically sick fish in these groups exhibited typical signs of columnaris disease (epidermis ulcer) or streptococcosis disease (erratic swimming). No mortality was observed in the group receiving bacterial isolate *P. shigeloides* NK11 and control group during 15 days post challenge (Fig. 3). Numerous pure bacterial colonies were recovered from the liver and kidney of all representative fresh dead fish for each challenged group (5 fish per group). These isolates exhibited homologous biochemical characteristics with the original isolates (data not shown). At the end of the experiments, 5 surviving fish in the groups which received *S. agalactiae* and *P. shigeloides* were euthanized for bacterial isolation. *S. agalactiae* was recovered from the brain and kidney of challenged fish but no *P. shigeloides* was found.

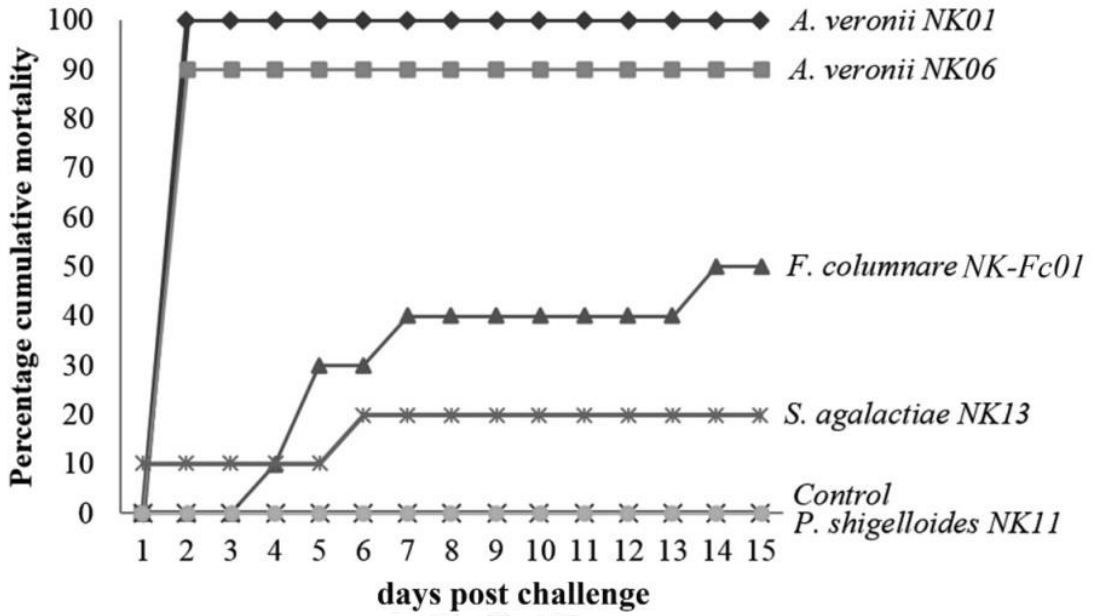

Figure 3 Percentage cumulative mortality of fish challenged with indicated bacteria isolated in this study. Control indicated the group injected with TSB medium.

Discussion

Single important bacterial pathogens affecting intensive cultured tilapia farms have been well-studied in term of pathogenesis. In theory, the floating cages culture system, where the fish are naturally exposed to various potential pathogens, is predictably infected by multiple infections during disease outbreaks. Our results demonstrated that there were five bacterial species and one virus concurrently found in disease outbreaks in this study. It is notable that two bacteria *F. columnare* and *A. veronii* heavily infected all diseased fish, whereas light infection and less prevalence of *P. shigeloides*, *S. agalactiae* and *V. cholerae* and *Iridovirus* was detected. *F. columnare*, *S. agalactiae* and *Iridovirus* are significant pathogens threatening cultured tilapia as well as other freshwater fish (Ariel and Owens, 1997; McGrogan et al., 1998; Figueiredo et al., 2005; Olivares-Fuster et al., 2008; Dong et al., in press; Kayansamruaj et al., 2014a; Kayansamruaj et al., 2014b). In contrast, *A. veronii, P. shigeloides* and *V. cholerae* are not well-described fish pathogens and have been identified as foodborne pathogens that could present a serious issue to consumer health (Joseph et al., 1991; Nadirah et al., 2012; Chen et al., 2013; Chen et

al., 2014; Nguyen et al., 2014). Therefore, further studies should investigate whether these bacterial isolates can threaten human health or not. Interestingly, the data produced in this study revealed the *A. veronii* isolates were highly pathogenic to tilapia fingerling and produced typical hemorrhagic liver, enteritis in challenge fish, mimicked internal clinical signs of natural diseased fish. It should also be noted that this also suggests *A. veronii* is an emerging pathogen affecting cultured tilapia farms in Thailand. Taxonomically, the *A. veronii* species is easily distinguishable from other aeromonas by ornithine decarboxylase-positive characteristic (Hickman-Brenner et al., 1987). Two species *A. veronii* and *A. sobria* are the most phenotypically closed but the DNA inter-connectedness revealed a coincidence and they were proposed as two biogroups of *A. veronii* (*A. veronii* bv. *sobria* and *veronii* respectively). Therefore, *A. veronii* bv. *sobria* is a synonym of *A. sorbia* (Joseph et al., 1991). All *A. veronii* isolates in this study are most closely related to both *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii.* Recent studies reported that *A.veronii* bv. *veronii* is the pathogenic agent causing ulcerative syndrome in Chinese longsnout catfish (*Leiocassis longirostris*) while *A. sobria* caused tail-rot disease in Nile tilapia (Li and Cai, 2011; Cai et al., 2012).

Challenge fish receiving *F. columnare* by intramuscular injection produced typical signs of columnaris disease around the site of injection after 24 h post challenge and mimicked external signs of naturally diseased fish. A severe lesion (epidermis necrosis) was observed afterward in 50% of the infected fish and resulted in death while the remaining fish were able to recover steadily. This clearly indicated that a subset of infected fish was able to resist *F. columnare* infection and this bacterium may partially contribute to the dead-loss of cultured tilapia. The data produced in this study also revealed that *F. columnare* was predominantly isolated from the external lesion (gills, skin) of diseased fish. The species specific-PCR exhibited a negative result in all tested samples, indicating an undetectable level of bacterial cells in the internal tissues of affected fish. Our previous study also revealed the consistent result in diseased red tilapia *Oreochromis* sp. (Dong et al., in press-a). In contrast, systemic infection was found in channel catfish (*Ictalurus punctatus*) and striped catfish (*P. hypophthalmus*) (Shoemaker et al., 2008; Tien et

al., 2012; Dong et al., in press-c). This suggested that *F. columnare* exhibited a different pathogenicity according to its host. The target tissue for disease diagnosis, therefore, should be further investigated within each fish species. On the other hand, challenging the fish to *S. agalactiae* NK13 resulted in a low percentage cumulative mortality (20%). However, the bacteria were able to persist in the brain and kidney of the surviving fish. Moreover, an *in vitro* test revealed that the isolates of *S. agalactiae* in this study exhibited γ, α and β hemolytic ability at 28 ^{O}C, 33 ^{O}C and 37 ^{O}C respectively (data not presented). Hemolysis has been considered as an important virulent factor of *S. agalactiae* (Olivares-Fuster et al., 2008; Kayansamruaj et al., 2014b). Previous studies revealed that a high temperature (35 ^{O}C) significantly promotes expression of various virulent factors of *S. agalactiae* and resulted in a higher percentage cumulative mortality to compare to low temperature (28 ^{O}C) (Rodkhum et al., 2011; Kayansamruaj et al., 2014a). The low water temperature (26 ± 1 ^{O}C) during the experimental challenge might explain the low mortality and persistence of *S. agalactiae* in surviving fish in this study. In contrast, tilapia seem to be more sensitive to *F. columnare* at low temperature (26-28 ^{O}C) during Thailand's rain season (Dong et al., in press-a). However, relatively low mortality from *F. columnare* challenged fish in the present study might suggest other stressors (i.e. stressful environment, a combination with other pathogens, etc.) may be involved in the occurrence of diseases in the field. Our recent work on striped catfish (*Pangasianodon hypophthalmus*) also indicated that dual infections of *F. columnare* and *Edwardsiella ictaluri* caused a significantly higher mortality when compared to individual single infections (Dong et al., in press-c)".

Additionally, the lower infection rates of *P. shigeloides, V. cholerae* and *Iridovirus* found in this study suggests that these pathogens may coincidently occur in the diseased fish. Nadirah et al. (2012) reported that *P. shigeloides* was commonly found in cultured tilapia but its impact on fish health remains unclear. In this study, numerous pure colonies of *P. shigeloides* were isolated from white necrotic lesions in the liver and brain of one diseased fish. The experimental challenge revealed that *P. shigeloides* was non-pathogenic to tilapia fingerlings under experimental conditions

and no lesion was observed in internal organs of the surviving fish. This suggests that *P. shigeloides* might serve as an opportunistic pathogen in tilapia. Pertaining *V. cholera*, a natural inhabitant of aquatic environments which causes severe diarrhea in humans, from previous studies indicated that fish normally serve as reservoirs and vectors of this zoonotic bacterium (Senderovich et al., 2010; Rehulka et al., 2015). While the iridovirus alone was responsible for mortalities in tilapia, both *O. niloticus* and *O. mossambicus* (McGrogan et al., 1998; Ariel and Owens, 1997), the effects of concurrent infections of iridovirus with other pathogens in tilapia have not been fully investigated.

Herein, the present study first revealed concurrent infections of multiple pathogens in natural disease outbreaks in the intensively cultured tilapia system. Besides previously well-described pathogens (*F. columnare, S. agalactiae,* and *Iridovirus*), other bacteria generally known as foodborne pathogens (*A. veronii, P. shigeloides,* and *V. cholerae*) were also concurrently found in this study. Surprisingly, *A. veronii* was highly pathogenic to tilapia and is considered as an emerging pathogen in Thai tilapia farms. The challenge experiments using *A. veronii* and *F. columnare* successfully mimicked major clinical signs of naturally infected fish. This suggests that *A. veronii* and *F. columnare* are two main pathogens concurrently responsible for the disease outbreaks in cultured tilapia farms in the present study, whereas remaining pathogens might serve as opportunistic pathogens in the disease outbreaks. It is possible that primary infection of the fish with *F. columnare* may cause erosions on the gill or skin which results in subsequent infections. However, other pathogen(s) may also be responsible as an initial instigator cannot be excluded. For instance, primarily parasitic infections may create damaged tissues for secondary invasion of multiple bacteria and viruses. Further studies should uncover the primary instigator of the outbreaks as well as aim at the development of a combined vaccine to prevent multiple bacterial pathogens in cultured tilapia.

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CHAPTER III

Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand

Publication

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Abstract

Flavobacterium columnare and *Edwardsiella ictaluri* are two major bacterial pathogens threatening catfish aquaculture globally. Most earlier studies have focused on the characterization of single bacterial infection. In reality, multiple bacterial pathogens are present in aquaculture systems and are probably responsible for disease outbreaks and considerably outweigh single infection. The objectives of this study, therefore, were to investigate whether single or concurrent bacterial pathogens were involved in naturally diseased striped catfish (*Pangasianodon hypophthalmus*) and subsequently investigate pathogenicity of single- and dual-infection through experimental challenges. The investigation revealed coinfections of *F. columnare* and *E. ictaluri* found in naturally diseased Thai striped catfish exhibiting columnaris and edwardsiellosis diseases. Bacterial identification was confirmed by phenotypic tests, species-specific PCR and 16S rDNA sequence analysis. Molecular data analysis also identified that the infected fish species were *P. hypophthalmus*. Experimental challenges of striped catfish juveniles with single and dual bacterial species using both immersion (i.m) and injection (i.p) approaches were performed. Injection of two difference doses of combined bacteria caused markedly high mortality of 86.7-100%, indicating high virulence of the bacterial isolates. Immersion (i.m.) coinfection of *E. ictaluri* (2.6×10^6 CFUs mL^{-1}) and *F. columnare* (1.0×10^4 CFUs mL^{-1}) caused significantly high cumulative mortality (96.7 ± 5.8 %) compared to i.m. of single infection of *E. ictaluri* (80.0 ± 20 %) or *F. columnare* (3.3 ± 5.7 %) with the same dose of bacteria. Both coinfection challenge routes i.p. and i.m. successfully mimicked typical signs and histopathological manifestations of natural coinfection. This study had fulfilled Koch's postulates through single- or dual-challenged tests to mimic the natural disease case in striped catfish.

Key words: *Edwardsiella ictaluri*, *Flavobacterium columnare*, striped catfish, *Pangasianodon hypophthalmus*

Introduction

Striped catfish (*P. hypophthalmus*) is native to the Mekong, Chaophraya, and Maeklong basins of Southeast Asia, including Cambodia, Laos, Viet Nam and Thailand (Poulsen et al., 2004; Robert and Vidthayanon, 1991). It has been introduced to other Asian countries such as Bangladesh, China, Indonesia, Malaysia, Myanmar, and India for aquaculture purposes (FAO, 2010-2011). The recent boom in striped catfish culture and significant export levels in Vietnam has brought global attention to the Asian catfish culture industry (Nguyen and Dang, 2009). The high stock density of intensively cultured farms, however, faces devastation through infectious pathogens such as channel catfish virus (Siti-Zahrah et al., in press), parasitic monogenea *Thaparocleidus caecus* and *T. siamensis* (Šimková et al., 2013; Tripathi et al., 2014) or important bacteria *E. ictaluri, E. tarda, F. columnare,* and *Aeromonas hydrophila* (Crumlish et al., 2010; Panangala et al., 2007; Shetty et al., 2014). Among bacterial pathogens, *E. ictaluri* and *F. columnare* are recognized as the most highly pathogenic bacteria that cause enteric septicemia of catfish (ESC) and columnaris disease in freshwater fish respectively (Declercq et al., 2013; Hawke et al., 1981). *E. ictaluri* was reported in channel catfish (*Ictalurus punctatus*) in the United States (Hawke et al. 1981), walking catfish (*Clarias batrachus*) and hybrid catfish (*C. macrocephalus* x *C. gariepinus*) in Thailand (Boonyaratpalin and Kasornchan, 1986; Kasornchandra et al., 1987; Suanyuk et al., 2014), striped catfish in Vietnam and Indonesia (Crumlish et al., 2002; Ferguson et al., 2001; Yuasa et al., 2003), wild ayu (*Plecoglossus altivelis*) in Japan (Nagai et al., 2008), yellow catfish (*Pelteobagrus fulvidraco*) in China (Ye et al., 2009), as well as cultured Nile tilapia (*Oreochromis niloticus*) (Soto et al., 2012). *F. columnare* is one of the oldest known bacterial pathogens, having affected the global population of aquaculture freshwater fish species since the beginning of the last century (Bernardet, 1989; Bernardet and Bowman, 2006; Declercq et al., 2013). A wide range of fish hosts have been reported, 37 fish species were addressed by Anderson and Conroy (1969), and many economic aquaculture fish recently such as Nile tilapia (Figueiredo et al., 2005), red tilapia (*Oreochromis* sp.) (Dong et al., 2014, 2015 in press), Indian carp (*Catla catla*) (Verma and Rathore, 2013), striped catfish

(Tien et al., 2012). It is noticeable that *F. columnare* was considered as the second most important bacterial pathogen threatening the U.S catfish culture industry after *E. ictaluri* (Shoemaker et al., 2007). Earlier studies have focused on characterization of single pathogen infection. Most likely, the reality of the disease manifestation in cultured fish farms frequently occurred as the result of dual or multiple infections. Coinfection between bacteria and parasites has been primarily described in channel catfish and tilapia (Xu et al., 2007, 2012a, 2012b). Understanding the coinfection of important bacterial pathogens, however, remains undetermined, especially in striped catfish. This study aims 1) to characterize a natural coinfection of *E. ictaluri* and *F. columnare* in cultured striped catfish and 2) to experimentally investigate pathogenicity of single and dual infection.

Materials and methods

Infected fish samples and experimental fish

Infected juvenile striped catfish *P. hypophthalmus* (*n*=20) were obtained from two different hatcheries in Ratchaburi, a central province of Thailand through a commercial fish store in Bangkok in June, 2014. Affected fish exhibited typical clinical signs of both columnaris and edwardsiellosis diseases (see details in section 3.1). For experimental challenge tests, healthy striped catfish juveniles (mean weight, 26.7±8.7 g; mean length, 5.4±0.7 inches) were kindly provided by Charoen Pokphand Company (Ayutthaya province, Thailand) and were allowed to acclimate in the aquarium for 11 days prior to infection. Prior to challenge tests, a subset of five fish were randomly subjected for bacterial isolation and found to be free of *F. columnare* and *E. ictaluri*. Remaining fish were observed by the naked eye and none of them had any clinical signs or any abnormalities.

Bacterial isolation and phenotypic assays

Infected fish (n=20) were euthanized in ice-cold water before aseptically necropsied for bacterial isolation. Samples were collected by inserting a sterile metal bacteriology loop into the gills, skin lesion, kidney, and liver of each fish. Collected specimens were then streaked directly onto two different agar plates. Tryptic soy

agar (TSA, Difco) plates supplemented with 5% bovine blood (Department of Veterinary Microbiology, Chulalongkorn University) were generally used for isolation of *Edwardsiella* sp. while Anacker and Ordal's medium (AO) supplemented with 1 µg mL^{-1} tobramycin (Sigma) was employed for culturing of *Flavobacterium* sp. Plates were incubated at 30 °C for 48 h (Hawke et al., 1981; Figueiredo et al., 2005; Dong et al., 2014 in press) and individual bacterial colonies were sub-cultured on respective agar plates to obtain pure isolates. Some conventional phenotypic assays including Gram staining, oxidase, catalase, oxidation/fermentation (O/F), flexirubin pigment were performed as previously described by Bernardet (1989) and Crumlish et al. (2002). It was known later from the phenotypic assays and molecular data (see below) that the identified bacterial strains were *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively. Subsequently, six isolates of *E. ictaluri*, designated T1-1 to T1-3 and T2-1 and T2-3, and six individual colonies of *F. columnare,* named CF1 to CF6, were used in this study.

PCR amplification of bacterial DNA sequences

Universal primers targeting prokaryotic 16S rDNA (Weisburg et al., 1991) and species specific primers for *E. ictaluri* (Sakai et al., 2009) and *F. columnare* (Welker et al., 2005) used in this study are listed in the Table 1. A PCR reaction volume of 20 µL contained a small amount of bacterial colony, 0.25 µM of each primer pair, 0.2 mM of dNTPs, 0.25 µM of $MgCl_2$, 1 unit of Taq polymerase (Invitrogen), and 1X reaction buffer. The PCR conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 40 sec, annealing at 50 °C for 40 sec and extension at 72 °C for 1 min/kb. PCR products were analyzed using 1.0% agarose gel electrophoresis.

PCR amplification of fish DNA sequences

Crude DNA extracts from fish samples were prepared according to a previous report (Kowasupat et al., 2014). Briefly, approximately 5 mg of fish muscle tissue was incubated with 180 µL of 50 mM NaOH at 95 °C for 10 min. The reaction was then neutralized by the addition of 20 µL of 1 M Tris-HCl (pH 8.0). DNA-containing supernatant was used for subsequent PCR reactions. Primers used for PCR

amplification of fish DNA sequences listed in Table 1 included universal primers targeting eukaryotic 18S rDNA (Medlin et al., 1988), specific primers for fish COI (cytochrome c oxidase I), ITS (internal transcribed spacer), and RAG1 (recombinase activating gene 1). PCR reactions and thermocycling conditions were carried out using previously described protocols (Kowasupat et al., 2014).

Table 1 Primers used in this study.

Organism/Virus	Gene	Primer names/Sequences (5'to 3')	Ref.
Fish	18S rDNA	Universal-Euka-F/ AACCTGGTTGATCCTGCCAG	Medlin et al., 1988
		Universal-Euka-R/ TTGATCCTTCTGCAGGTTCACCTAC	
	COI	VF2_t1/TCAACCAACCACAAAGACATTGGCAC	Ward et al., 2005
		FishF2_t1/ TCGACCTAATCATAAAGATATCGGCAC	
		FishR2_t1/ ACTTCAGGGTGACCGAAGAATCAGAA	
		FR1d_t1/ ACCTCAGGGTGTCCGAARAAYCARAA	Ivanova et al., 2007
	ITS	ITS-F2/ACTTGACTATCTAGAGGAAG	Kowasupat et al., 2014
		ITS-R4/TCCACCGCTAAGAGTTGTC	
	RAG1	RAG1-2510F/TGGCCATCCGGGTMAACAC	Li and Orti, 2007
		RAG1-4090R/ CTGAGTCCTTGTGAGCTTCCATRAAYTT	Lopez et al., 2004
		RAG1-2533F/ CTGAGCTGCAGTCAGTACCATAAGATGT	
		RAG1-4078R/ TGAGCCTCCATGAACTTCTGAAGRTAYTT	
Bacteria	16S rDNA	Uni-Bact-F/ AGAGTTTGATCMTGGCTCAG	Weisburg et al., 1991
		Uni-Bact-R/ACGGHTACCTTGTTACGACTT	
Flavobacterium columnare	ITS	FCISRFL/ TGCGGCTGGATCACCTCCTTTCTAGAGACA	Welker et al., 2005
		FCISRRL1/ TAATYRCTAAAGATGTTCTTTCTACTTGTTTG	
Edwardsiella ictaluri	Fimbrial gene	Ed-ictaluri-F/CAGATGAGCGGATTTCACAG	Sakai et al., 2009
		Ed-ictaluri-R/CGCGCAATTAACATAGAGCC	
Infectious myonecrosis virus (IMNV)	Capsid	F13N/TGTTTATGCTTGGGATGGAA	Senapin et al., 2007
		R13N/TCGAAAGTTGTTGGCTGATG	

DNA cloning and sequence analysis

Amplified DNA amplicons were gel purified using a Favogen Gel/PCR Purification Kit and cloned into pPrime cloning vector (5PRIME). Recombinant clones were verified by colony PCR (data not shown) prior to plasmid DNA purification using a FavoPrep Plasmid Extraction Mini Kit. DNA sequencing was performed by 1st BASE Pte Ltd. (Malaysia). A DNA sequence homology search was carried out using BLAST on the GenBank database. Multiple sequence alignments were performed by Clustal W (Thompson et al., 1994) and a Neighbor-Joining (NJ) tree and pairwise distance analysis were conducted using MEGA version 5 (Tamura et al., 2011) with 1000 replicates bootstrap values.

Challenge test by immersion

To minimize the effects from opportunistic pathogens, naïve striped catfish were treated with 1% NaCl for 20 min, kept in 0.1% NaCl for 1 day before being raised in pre-aerated freshwater. The treatment was repeated once after three days. Two bacterial isolates *E. ictaluri* T1-1 and *F. columnare* CF1 were used for challenged experiments. *F. columnare* CF1 and *E. ictaluri* T1-1 were cultured in AO broth and TSB respectively at 30 ^{O}C with shaking (250 rpm) until reaching to an optical density of ~1.0 at 600 nm to get an expected density of ~10^8 CFU mL^{-1}. Conventional plate count method then was performed to determine the CFUs mL^{-1}. For immersion challenge test, designed doses (see below) were prepared by diluting the cells in 50 L water. Fish were divided into 5 groups: Group (1) was infected by *E. ictaluri* 2.6 x 10^6 CFUs mL^{-1}; (2) infected by *F. columnare* 1.0 x 10^4 CFUs mL^{-1}; (3) infected by *F. columnare* 2.5 x 10^7 CFUs mL^{-1}; (4) infected by *E. ictaluri* 2.6 x 10^6 CFUs mL^{-1} plus *F. columnare* 1.0 x 10^4 CFUs mL^{-1}; and (5) non-infected control. In each group, 30 fish were immersed in 50 L water with bacteria for 2 h. Fish not exposed to the bacterium were kept in water for the same time. Following bacterial exposure, fish were delivered into 3 replicate tanks with 10 individuals each. Fish mortality was recorded every 12 h for 20 days. Details of challenge tests are summarized in (Supplemental Fig. 1). Fresh dead fish and moribund fish were necropsied and a

bacteriological examination was made on kidney and liver tissues on TSA with 5% bovine blood and kidney and gills on AOA. Caudal muscle, gills, liver, spleen, and kidney were fixed in 10% buffered formalin for histopathology assessment. During trials, water temperature was maintained in the range 28 - 29 °C and pH was 7 ± 0.5. Fifty percent of the water was replaced every 5 days.

Bacterial co-infections by injection method

Fish were divided into 3 groups, 15 fish each. Group (1) was injected intraperitoneally with *E. ictaluri* 8×10^7 CFUs plus *F. columnare* 6×10^7 CFUs $fish^{-1}$; (2) injected with *E. ictaluri* 8×10^5 CFUs plus *F. columnare* 6×10^5 CFUs $fish^{-1}$; and (3) non-infected control. After injection, fish were distributed to 3 replicate tanks (Supplemental Fig. 1). Experimental conditions were performed similarly with an immersion test. The cumulative mortality of experimental fish was recorded in 20 days. Dead and moribund fish were sampled for bacterial isolation and histological analysis in the same manner as the immersion challenge test mentioned above.

Histopathological assessment and in situ hybridization

Fish organs from representatives of naturally infected fish (n=2) and experimentally infected fish (2 fish per treatment group) were preserved in 10% neutral buffered formalin and paraffin sectioning was proceeded according to standard protocols. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination. Sections from experimentally infected fish were further used for *in situ* hybridization assay. Digoxygenin (DIG)-labelled probes for *in situ* hybridization were prepared using a commercial PCR DIG-labeling mix (Roche Molecular Biochemicals). Recombinant plasmids containing DNA fragments derived from *E. ictaluri* (470 bp) and *F. columnare* (~500 bp) species specific primers were used as templates in the labeling reactions. A control probe was produced from a 282-bp fragment from the infectious myonecrosis virus (IMNV) genome using primers corresponding to nucleotides 5789 to 6070 as previously described (Senapin et al., 2007). The tissue sections were separately incubated with each DIG-labelled probe for 1 h at room temperature, washed, and incubated with anti-DIG antibody

conjugated to alkaline phosphatase for 1 h at 37 °C. Positive *in situ* hybridization signals were then detected using a NBT/BCIP substrate (Roche Molecular Biochemicals). Sections were counterstained with 0.5% Bismarck brown, mounted with permount (EMS, England), and then examined by light microscopy (Olympus BX51 digital microscope).

Statistical analysis

Differences in means were tested for statistical significance using one-way ANOVA.

Results

Clinical signs of naturally and experimentally infected fish

Naturally diseased striped catfish (n=20) exhibited mixed-clinical signs of both columnaris and edwardsiellosis diseases. The presence of “saddle back” lesion, white-yellowish areas on the body surface, necrotic gills and eroded fins were typical signs of columnaris disease (Supplemental Fig. 2A). Internally, multiple 1-3 mm diameter white necrotic and pyogranulomatous foci resembling edwardsiellosis were notably found in the kidney, liver, and spleen of affected fish (Supplemental Fig. 2B). Atypical signs of some clinically sick fish were also observed, including emaciation, swollen abdomen or petechial haemorrhages on the body surface (not shown). Experimentally sick fish in the immersion combined-challenged group (details below) exhibited the consistency of clinical signs with naturally coinfected fish (Supplemental Fig. 2C, D), whereas sick fish in single-treated group with *F. columnare* or *E. ictaluri* exhibited signs of only columnaris or edwardsiellosis disease, respectively.

Bacterial isolation, identification, and phylogenetic analysis

Two kinds of bacterial colony were predominantly recovered from the same all the naturally diseased fish using TSA supplemented by 5% bovine blood and AOA supplemented with Tobramycin 1 μg mL^{-1} tobramycin (Sigma). The whitish, pinpoint colonies (1-2 mm in diameter) on TSA blood recovered from the kidney and liver were Gram negative, slender, variable length. The isolates were fermentative that

performed cytochrome oxidase negative, catalase positive and presumptively diagnosed as *Edwardsiella* sp. The yellow-pigmented, rhizoid colonies on AOA medium were able to be isolated from gill, skin, and kidney of all diseased fish. The isolates were Gram negative, slender long rod-shaped bacteria, produced flexirubin pigment, and exhibited positive reaction with both cytochrome oxidase and catalase tests, presumptively identified as *Flavobacterium* sp. Subsequently, six putative *Edwardsiella* sp. isolates (T1-1 to T1-3 and T2-1 to T2-3) and six putative *Flavobacterium* sp. isolates (CF1 to CF6) were subjected to PCR amplification using universal 16S rDNA primers and respective *E. ictaluri* and *F. columnare* species-specific primers based on published protocols (Weisburg et al., 1991; Sakai et al., 2009; Welker et al., 2005). The results showed that approximately 1.5 kb amplicon using universal 16S rDNA primers were obtained for all bacterial isolates (Fig. 1, left panel). Representative 16S rDNA fragments from T1-1 to T1-3 and CF1 to CF4 isolates were cloned and sequenced. The sequences from T1-1 to T1-3 were 2-4 nucleotides different among them and all strongly matched to *Edwardsiella ictaluri* ATCC 33202 (accession number NR024769) (~99.9% identity). There was a 1-5 nucleotide variation among the amplified CF1 to CF4 sequences and all of them had a highest homology to sequences of *F. columnare* in the GenBank database such as ~99.5% identity to *F. columnare* RDC-1 (accession number AY635167) (Vermaand and Rathore, 2013) and ~98.7% identity to *F. columnare* ATCC 49512 (accession number AY635167). Phylogenetic analysis revealed that *E. ictaluri* T1-1 to T1-3 isolates formed in the same cluster with previously published sequences of *E. ictaluri* originated from Vietnamese catfish (JF274254), American channel catfish (CP001600), Chinese yellow catfish (FJ766525), and Japanese Ayu (AB453281) (Fig. 2A). Sequences from *F. columnare* CF1 to CF4 isolates from Thai striped catfish are most closely aligned with Vietnamese striped catfish (KF274042, KF274043) and American channel catfish (KC912656, AY842900) (Fig. 2B). The 16S rDNA sequences of bacterial isolates reported in this paper have been deposited in GenBank under accession numbers KR080244 to KR080250.

Using *E. ictaluri*-specific PCR protocol (Sakai et al., 2009), *E. ictaluri* (T1-1 to T1-3 and T2-1 to T2-3 isolates) generated one specific band of 470 bp in size (Fig. 1, top right), while *F. columnare*-specific PCR (Welker et al., 2005) yielded 2 amplicons of approximately ~500 bp and 520 bp from *F. columnare* CF1 to CF6 isolates (Fig. 1, bottom right). Sequence analysis revealed that 470 bp amplified products from 6 *E. ictaluri* isolates had completely identical sequences among them and exhibited 99.8% identity with previously published sequences of *E. ictaluri fimbrial (fimA)* gene in the GenBank database. Representatives of ~500 bp (isolates CF1, CF2, and CF5) and ~520 bp (isolates CF1 and CF6) amplified products also exhibited the highest nucleotide homology with 16S-23S rDNA intergenic spacer (ITS) region of published *F. columnare* isolates in the GenBank database. All the sequences derived from species-specific primers have been assigned GenBank accession numbers KR080251 to KR080261.

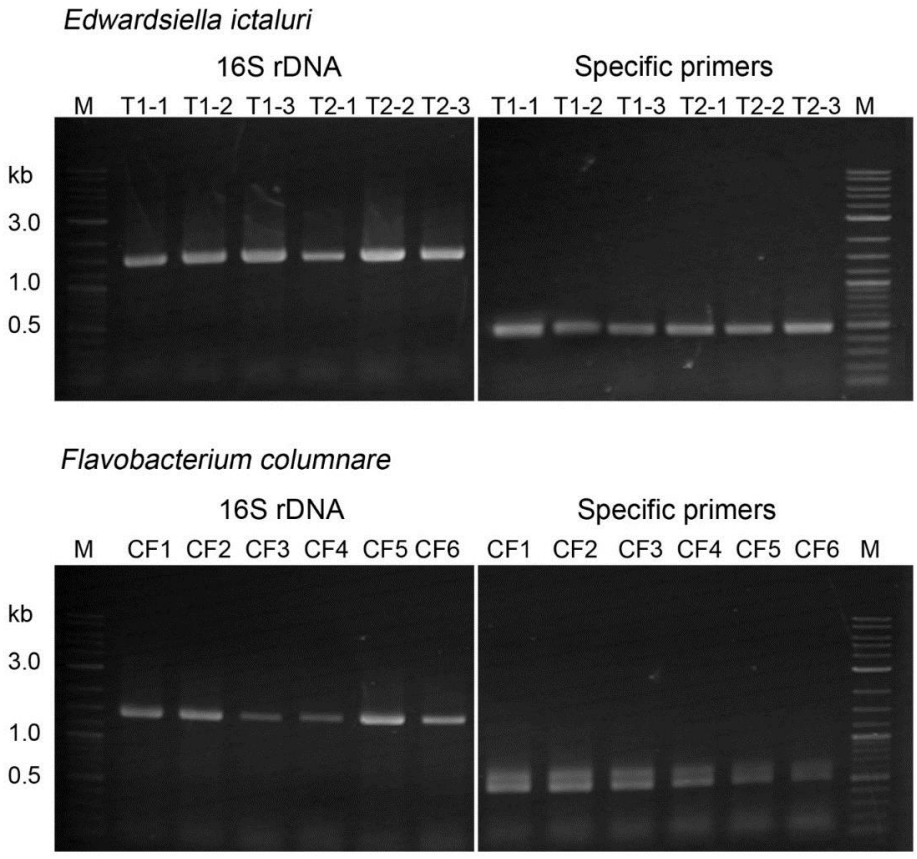

Figure 1 Agarose gels of PCR products from *Edwardsiella ictaluri* and *Flavobacterium columnare* using universal primers targeting prokaryotic 16S rDNA and respective species specific primers. Six individual colonies from *E. ictaluri* and *F. columnare* subjected to the assay. M, DNA marker (2-Log DNA Ladder, New England Biolabs).

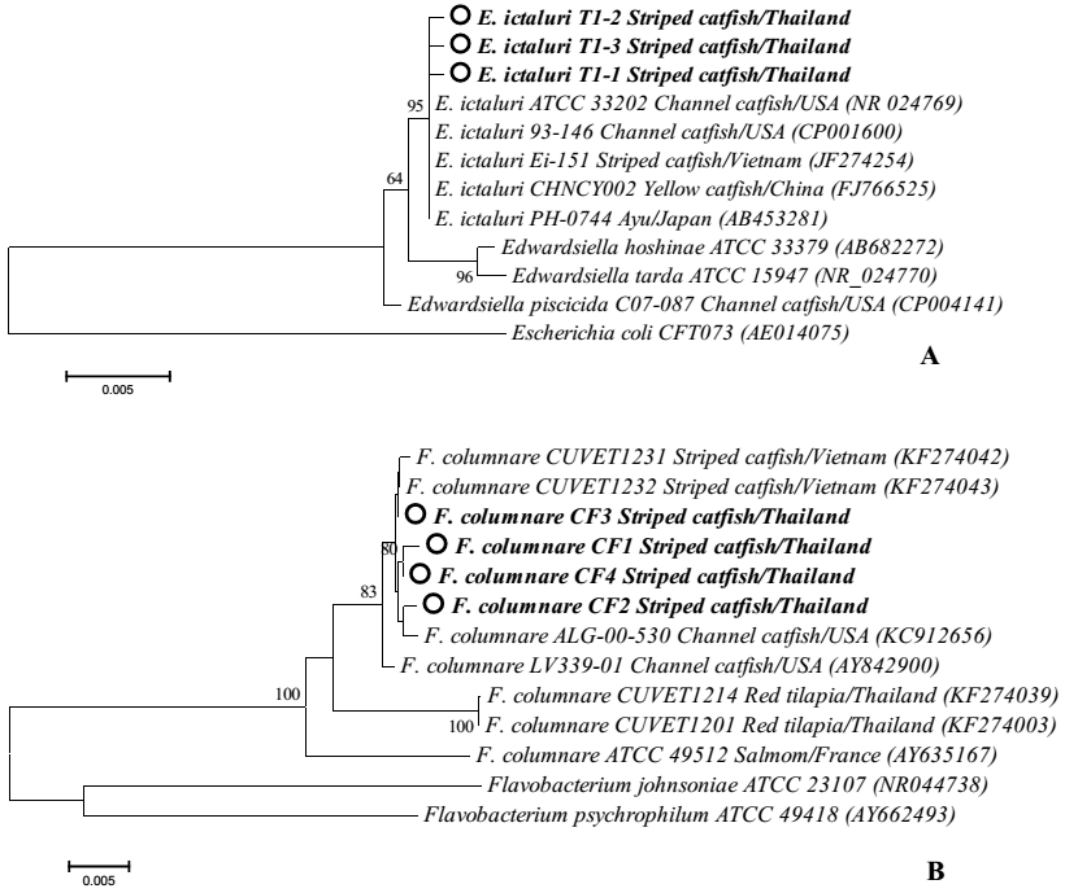

Figure 2 Phylogenetic trees based on 16S rDNA of *E. ictaluri* (A) and *F. columnare* (B) isolates and their closed taxa. Percentage bootstrap values (1000 replicates) are shown at each branch point.

DNA sequences of the striped catfish

The sequences of 18S rDNA, COI, ITS and RAG1 from a representative of naturally infected fish were amplified (Supplemental Fig. 3) and sequenced for the purpose of species confirmation in the present study. BLAST homology search revealed that the ~1.8 kb 18S rDNA amplicon matched 99.4% identical to 18S rDNA nucleotide sequence of striped catfish in the database (accession number AJ876376). In addition, the amplified COI, ITS and RAG1 fragments had 100, 92, and 99.5%

nucleotide identity, respectively, to striped catfish in the GenBank database (accession nos. GN021313, AJ876376, JN979996 respectively). The results confirmed that naturally infected fish obtained in this study were striped catfish (*P. hypophthalmus*). Additionally, a representative experimental challenge fish was subjected to 18S rDNA sequence amplification and analysed in the same manner. The result revealed 99.4% sequence identity between natural and experimental fish assayed in this study (not shown). The sequences were assigned accession numbers KR080262 to KR080265 in the GenBank database.

Challenge tests

In the three single immersion-challenged groups, the group which received a high dose (2.5×10^7 CFUs mL^{-1}) of *F. columnare* was able to induce 100.0 ± 0.0% mortality within one day, whereas, a lower dose (1.0×10^4 CFUs ml^{-1}) caused only 3.3 ± 5.7% mortality in 20 days (Figs. 3A). In the group which received only *E. ictaluri* 2.6×10^6 CFUs ml^{-1}, fish started dying on day 4, and reached the highest cumulative mortality (80.0 ± 20.0%) on day 9 post challenge (Figs. 3A). Cumulative mortality was more pronounced, 96.7 ± 5.8%, in the coinfection treatment with the same bacterial dose (*E. ictaluri* 2.6×10^6 CFUs ml^{-1} and *F. columnare* 1.0×10^4 CFUs ml^{-1}) (Figs. 3A). In the combined intraperitoneal (i.p.) injection-treated groups, the percentage cumulative mortality of the group 1 (received *E. ictaluri* 8×10^7 CFUs & *F. columnare* 6×10^7 CFUs $fish^{-1}$) and the group 2 (received *E. ictaluri* 8×10^5 CFUs & *F. columnare* 6×10^5 CFUs $fish^{-1}$) were 100.0 ± 0.0% and 86.7 ± 11.5% respectively (Figs. 3B). All clinically sick fish administrated with *E. ictaluri* either single or dually with *F. columnare* exhibited edwardsiellosis disease (multifocal pinpoint white spots in the internal organs) (Supplemental Fig. 2D). Clinical signs of both edwardsiellosis and columnaris (“saddle back” lesion, eroded fins) diseases were observed from groups that received both *E. ictaluri* and *F. columnare* by either immersion (i.m.) or injection (i.p.) route (Supplemental Fig. 2C, D). Only clinical signs of columnaris disease were observed in the group which received single *F. columnare*. No mortality or abnormalities were observed in the control group during 20 experimental days. *F. columnare* and *E. ictaluri* were successfully re-isolated from all representative

diseased fish in both i.p and i.m. combined-treated groups (five fish per group). Single types of either *E. ictaluri* or *F. columnare* were recovered from diseased fish in respective single-challenged groups. All these bacterial isolates were re-confirmed by species-specific PCR as mentioned above (data not shown).

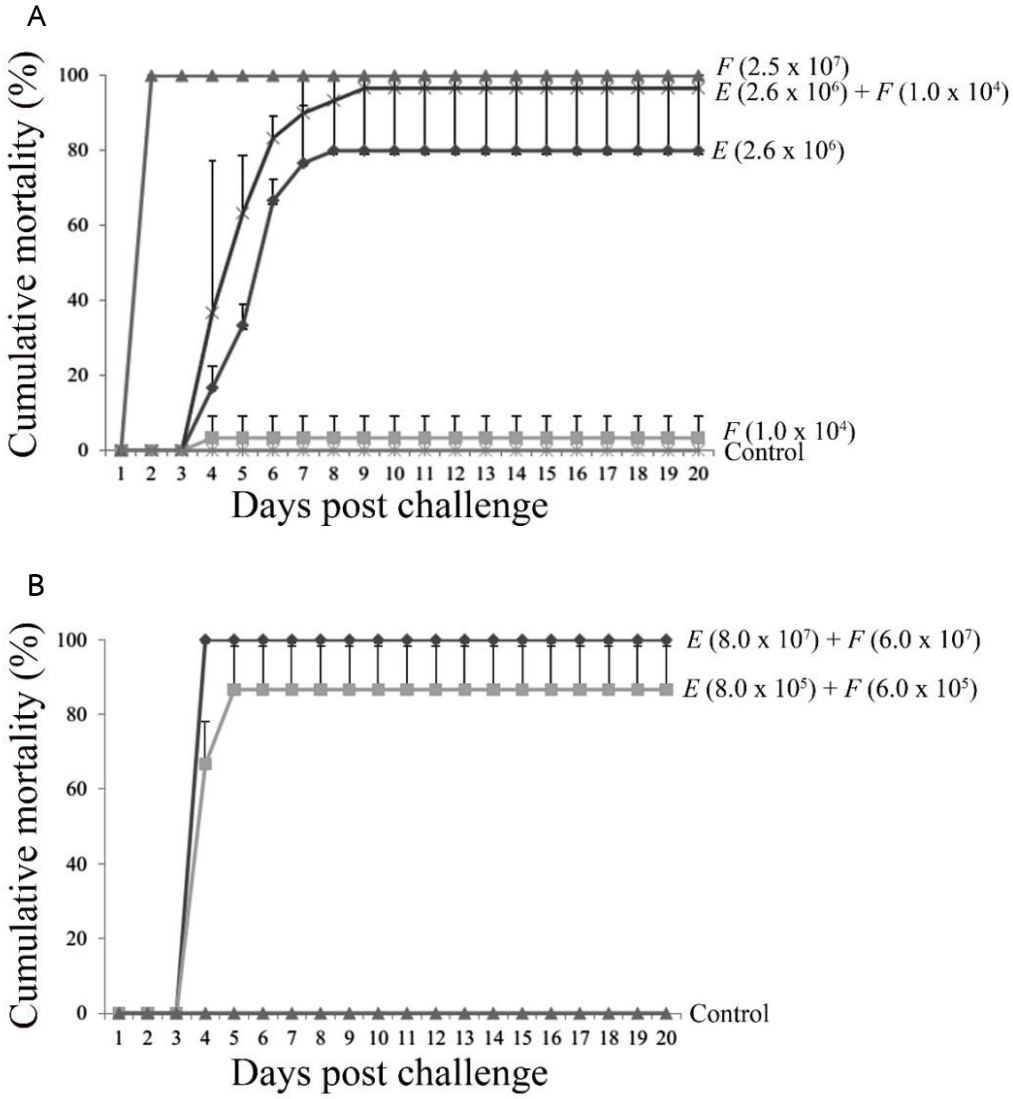

Figure 3 Cumulative percentage mortalities of *P. hypophthalmus* exposed to single *E. ictaluri* and *F. columnare* and combination of both by immersion route (A) and intraperitoneal injection method (B).

Histopathological examination

The severe multi-focal areas of necrosis and pyogranulomas, notably in the kidney, liver, and spleen (Fig. 4A-D) were found in both naturally and experimentally coinfected fish as well as apparently sick fish in the single *E. ictaluri* treated group. There were no significant differences in histopathological manifestation in the internal organs (kidney, liver, and spleen) of the single *E. ictaluri* treated group and combined treatment group. The kidney and spleen of affected fish exhibited more severe damage than the liver (Fig. 4). Varying degrees of degeneration and necrosis were visualized by H&E. The normal architecture of tissue was almost completely obliterated in the center of necrotic areas (Fig. 4A-D). These histopathological manifestations were typically referred to as edwardsiellosis caused by *E. ictaluri.* The typical histopathological changes of columnaris disease were found in the gills of both naturally and experimentally combined-challenged groups as well as single high dose of *F. columnare*, but not in single low dose of *F. columnare*. The lesion was characterized by partially or completely necrotic gill lamellar with presence of numerous inflammatory cells with morphology resembling macrophages, eosinophilic cells, and red blood cells (Fig. 4F). It is noticeable that only hemorrhage without multifocal areas of necrosis in posterior kidney was observed in the fish infected with single *F. columnare* (Fig. 4E) but both hemorrhage and multifocal areas of necrosis were presented in coinfected fish (Fig. 4B-D).

The result of *in situ* hybridization visualized the specific locations of *E. ictaluri and F. columnare* in the infected tissue. Using *E. ictaluri*-specific probe, strong positive bindings were found in the tubule cells of posterior kidney (Fig. 5A, arrow) and weak positive signals in the gill lamellar (Fig. 5B) and anterior kidney (picture not shown) of the fish in both single *E. ictaluri* and combined challenge groups. When using *F. columnare*-specific probe, the strongest positive signals were detected in the gills (Fig. 5D), and weaker signals in the spleen (Fig. 5C), liver, and kidney (picture not shown) of both single *F. columnare* and combined challenge groups. No positive signals were detected using IMNV-specific probe or control group without probe.

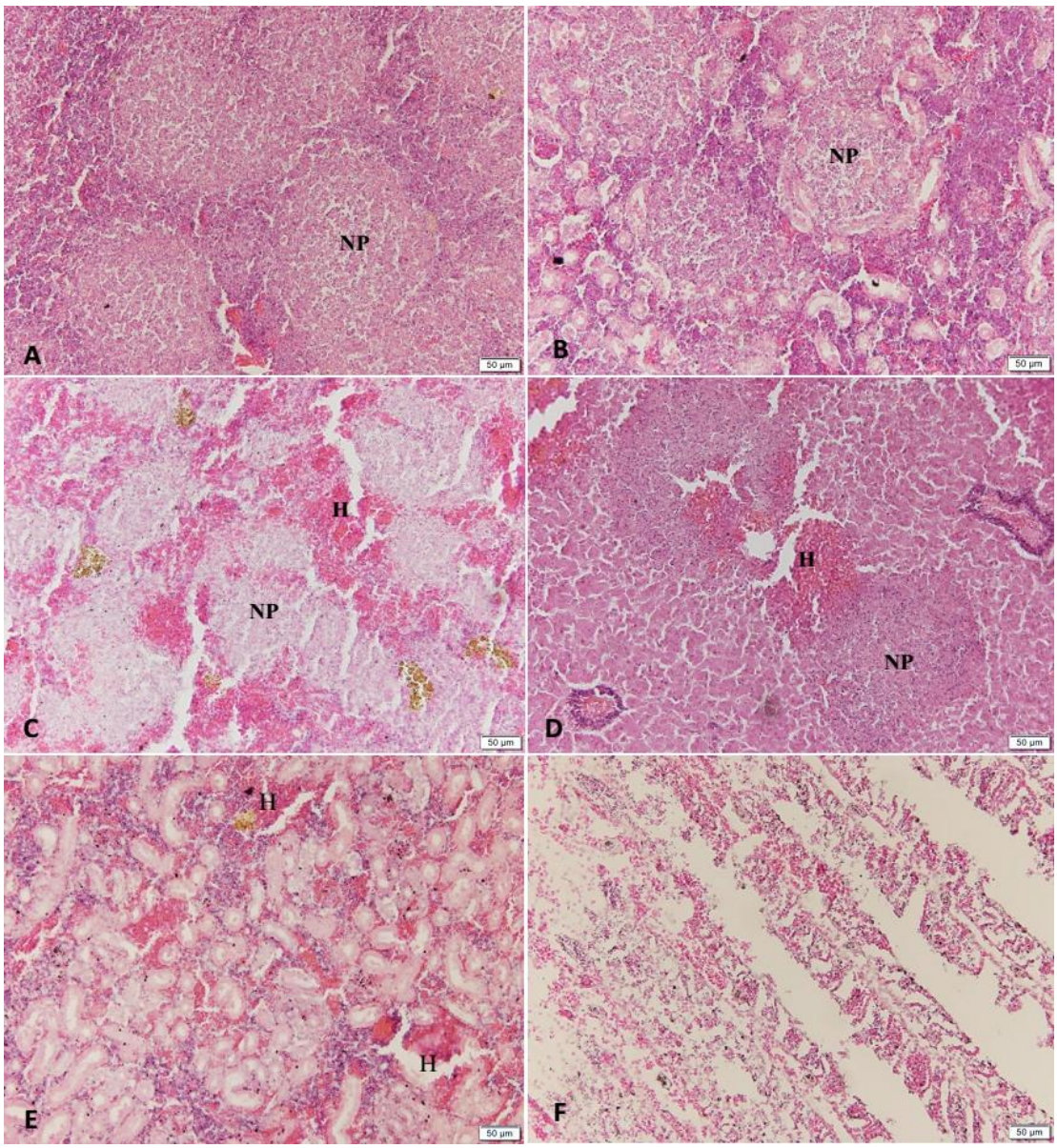

Figure 4 Anterior kidney (A), posterior kidney (B), spleen (C) and liver (D) of striped catfish infected with *E. ictaluri* and *F. columnare*. Posterior kidney (E) and gill lamellar (F) of fish infected with *F. columnare*. NP = multifocal areas of necrosis and pyogranulomas, H = hemorrhages.

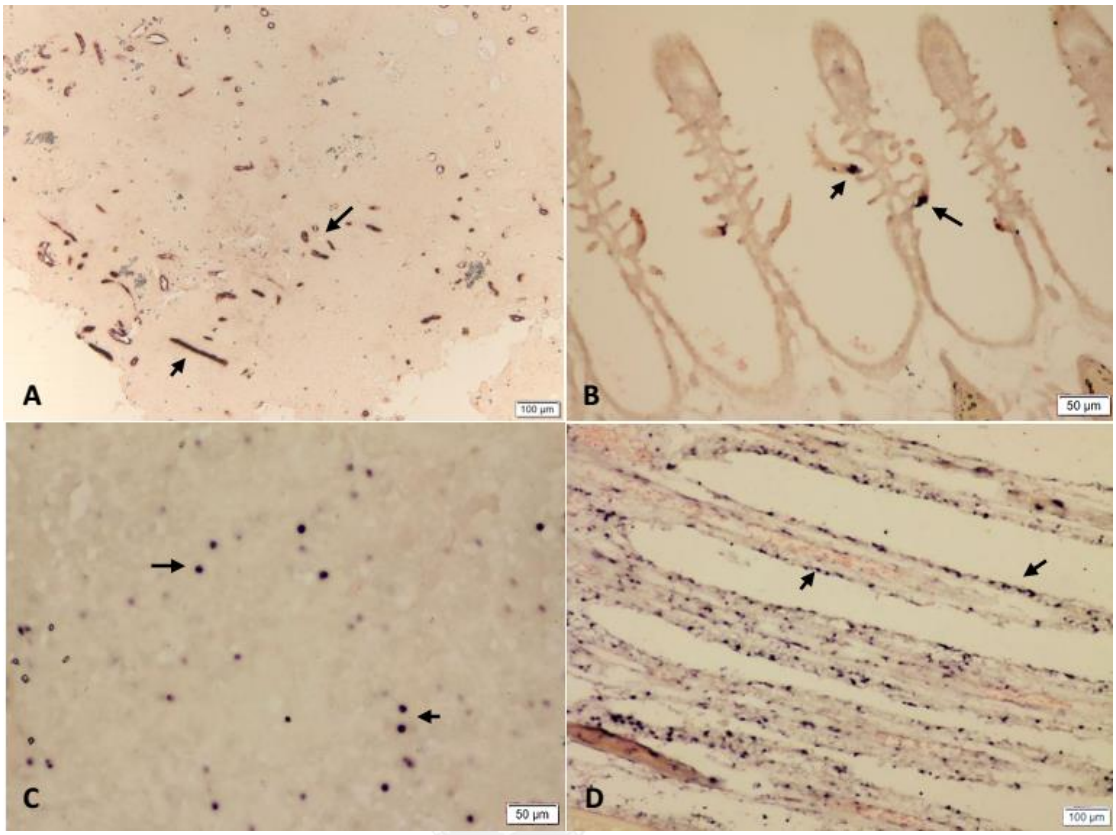

Figure 5 Posterior kidney (A) and gill (B) were positive with *E. ictaluri* (arrow), spleen (C) and gill (D) were positive with *F. columnare* by *in situ* hybridization.

Discussion

Since striped catfish (*P. hypophthalmus*) have been intensively cultured in Asian countries, numerous disease outbreaks resulting in significant financial losses have been reported. Subsequently, various bacterial pathogens causing disease outbreaks have been identified and well-described (Crumlish et al., 2002, 2010; Ferguson et al., 2001; Tien et al., 2012). Most laboratory studies, however, focused on pathogenesis of single bacterial infections whereas concurrent infections remain poorly understood. Here, we first report a naturally concurrent infection of *E. ictaluri* and *F. columnare* in striped catfish and fulfilled Koch's postulates through single- or dual-challenged tests to mimic the natural disease case. Both i.m. and i.p. combined-

treated groups resulted in high cumulative mortality and the infected fish clearly exhibited typical signs of edwardsiellosis and columnaris disease. It should be noted that the fish which received combined-bacterial pathogens by i.m. route exhibited more clearly diseased progress than i.p. route. Additionally, rapid mortality of the group which received a high dose of single *F. columnare* may be correlated to biofilm formation of the bacteria on the gills surface that could inhibit oxygen uptake and result in high mortality. The consistent mortality rate and biofilm formation was previously determined in experimental challenge of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) with highly virulent strains of *F. columnare* (Declercq et al., 2015). In contrast, low dose of *F. columnare* (1.0×10^4 CFUs mL^{-1}) was not able to cause columnaris disease but *in situ* hybridization exhibited positive results in multiple organs (gills, liver, spleen) of the experimental fish. This suggests that under lethal dose, *F. columnare* was able to persist in the fish and may need other stressors to induce disease such as the combination of low dose of *F. columnare* with *E. ictaluri* evidenced in the present study that successfully induced clinical signs of both edwardsiellosis and columnaris disease. Previously, Crumlish et al. (2010) also reported that experimental coinfection of *E. ictaluri* and *Aeromonas hydrophila* mimicked natural outbreaks of the disease in intensively cultured striped catfish farms in Vietnam. Taken together with the data produced in the present study, there is strong evidence to support that the reality of disease manifestation in aquaculture systems could be contributed by concurrent infections of bacterial pathogens.

With an aspect of histopathological manifestation caused by single infection (either *E. ictaluri* or *F. columnare*), our results in this study are consistent with previous reports for Vietnamese striped catfish (Ferguson et al., 2001; Tien et al., 2012). Moreover, this study first describes histopathological changes of both naturally and experimentally coinfected fish and revealed the presence of bacteria in specific tissue through *in situ* DNA hybridization during infections.

Sharing the same diseased pattern with channel catfish (*Ictalurus punctatus*) in the United States, *E. ictaluri* and *F. columnare* have been reported as the most

highly pathogenic bacteria threatening the cultured striped catfish industry in Vietnam (Crumlish et al., 2002; Ferguson et al., 2001; Tien et al., 2012). Recent reports, however, have indicated that the genetic characterization and pathogenicity of *E. ictaluri* were different between Vietnamese and US isolates (Bartie et al., 2012; Rogge et al., 2013), where *E. ictaluri* catfish isolates from Vietnam were non-pathogenic to the US channel catfish (Rogge et al., 2013). The differences in clinical signs and histopathological manifestations caused by *E. ictaluri* in two different hosts may explain the inconsistency in originally described names as enteric septicemia of catfish (ESC) in US channel catfish and "bacillary necrosis in *Pangasius*" in Vietnamese striped catfish (Crumlish et al., 2002; Ferguson et al., 2001; Hawke et al., 1981). With respect to genetic diversity, the phylogenetic analysis based on 16S rDNA sequences exhibited the identity among *E. ictaluri* isolates from Thailand, Vietnam, the United States, China, and Japan (Fig. 2A). In contrast, the 16S rDNA of *F. columnare* from striped catfish in Thailand exhibited similar genetic characteristics with the Vietnamese and US catfish isolates but were different to Thai tilapia (*Oreochromis* spp.)-originated isolates (Fig. 2B) (Dong et al., 2014 in press). In Thailand, striped catfish has been bred for fry/fingerling exporting purposes. Due to the boom in striped catfish production in Vietnam, Thai aquaculture producers have recently paid more attention to striped catfish (Mr. Warren Tuner, personal communication). Although *E. ictaluri* and *F. columnare* have never been reported in cultured striped catfish in Thailand, sharing similar geographical locations and culture technologies with Vietnam, the future catfish farming industry in Thailand is unavoidably predicted to face similar devastation as a result of the problematic pathogens mentioned above. Early management strategies to control the spreading of *E. ictaluri* and *F. columnare,* in a sustainable intensively cultured system in Thailand, therefore, are highly recommended. As a model lesson, disease manipulation strategy against infectious pathogens using monovalent or bivalent vaccine has been developed as a priority and widely applied to fish farms in the United States. (Klesius and Shoemaker, 1999; Shoemaker et al., 2007, 2011). Our further study will focus on the

development of a combined vaccine against coinfection of *E. ictaluri* and *F. columnare* in striped catfish.

Additionally, since the diversity of pangasiid fish has been reported as over ten species that are naturally distributed in the Mekong River basin including, *Pangasianodon gigas*, *P. hypophthalmus, Pangasius bocourti, P. conchophilus, P. krempfi, P. mekongensis, P. larnaudii, P. pleurotaenia, P. elongates, P. macronema* and *P. sanitwangsei* (Poulsen et al., 2004), many of them are similar in morphology that may lead to confusion over species identification. Moreover, this study investigated coinfection of two important bacterial pathogens and provided supplemental data of molecular markers (18S rDNA, ITS, COI, and RAG1) for fish identification.

In conclusion, to our best knowledge, this study is the first to characterize naturally and experimentally concurrent infection of two important bacterial pathogens *F. columnare* and *E. ictaluri* in striped catfish.

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CHAPTER IV

Diversity of non-*Flavobacterium columnare* bacteria associated with columnaris-like diseased fish

Publication

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Abstract

Numerous yellow and pink pigmented bacterial isolates were recovered from diseased fish exhibiting columnaris-like disease in Thai tilapia farms and the predominant species was previously identified as *Flavobacterium columnare* while taxonomic classification of the remaining isolates and their pathogenic potential remain undetermined. An additional yellow bacterial strain was also obtained from a koi carp sample showing columnaris-like symptoms. In continuing our previous work, we described the identification of ten representatives of unknown culturable non-*Flavobacterium columnare* bacteria based on a combination of phenotypic characteristics and nucleotides homology of 16S rRNA gene and subsequently investigated their pathogenicity in Nile tilapia (*Oreochromis niloticus*) fingerlings. The majority of the yellow pigmented bacteria were identified as *Chryseobacterium* spp. while the remainders were identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus*, and *Flavobacterium indicum*. The pink pigmented bacteria were identified as *Flectobacillus roseus*. Five representative species of the identified bacteria isolated from diseased tilapia were individually subjected to a pathogenicity test in healthy Nile tilapia fingerlings. The experimental challenge results revealed that the tested bacteria exhibited low or no virulence to the fish (0 - 20% cumulative mortality). This suggests that the identified bacteria merely served as opportunistic pathogens that may require stressors for disease manifestation.

Keywords: 16S rRNA, non-*Flavobacterium columnare* bacteria, columnaris-like

Introduction

Columnaris disease caused by *Flavobacterium columnare* has been reported in over 37 fresh water fish species worldwide (Anderson and Conroy, 1969; Bernardet and Bowman, 2006; Noga, 2010). Diseased fish usually exhibit typical external lesions such as eroded fins and epidermis ulcers or necrotic gills which results in mass mortality and severe financial losses for aquaculture producers (Davis 1922; Figueiredo et al., 2005; Bernardet and Bowman, 2006; Declercq et al., 2013; Dong et al., 2015a). With respect to disease diagnosis in the majority of fish disease laboratories, suspected cases of columnaris disease were generally subjected for *F. columnare* isolation using recommended selective media such as Anacker and Ordal's agar (Anacker and Ordal, 1955) or modified Shield agar (Decostere et al., 1997). Our previous study (Dong et al., 2015a) was performed in the same manner and found that numerous yellow and pink pigmented bacterial isolates were concurrently recovered from diseased fish exhibiting columnaris-like symptoms in Thai tilapia farms. The predominant species was identified as *F. columnare* and exhibited high virulence through experimental challenge using the fry red tilapia model (Dong et al. 2015a, b). Taxonomic classification of the remaining isolates and their ability to cause disease in fish remains undetermined. The reality in intensively cultured fish farms is that the manifestation of disease involves multiple potential pathogens (Dong et al., 2015 c, d). Therefore, beside well-described pathogens, the presence of the other pathogens and their potential contribution to disease manifestation should be uncovered. The objectives of this study, therefore, are to identify the remaining unknown culturable non-*Flavobacterium columnare* bacteria, which involve a columnaris-like disease in fish, and investigate their pathogenicity in Nile tilapia fingerlings.

Materials and methods

Bacterial isolates and growth conditions

Eight yellow and two pink pigmented bacterial isolates previously recovered from necrotic gills, skin ulcers, or kidney of red tilapia (*Oreochromis* sp.), Nile tilapia (*Oreochromis niloticus*), and koi carp (*Cyprinus carpio*) exhibiting columnaris-like disease (Dong et al., in press-a) were subjected for species identification in this study. All isolates used in this study were designated with the organism code, CUVET as summarized in Table 1. Isolation of the bacteria was described in an earlier work (Dong et al., in press-a). Briefly, a sterile loop that inserted into the diseased fish tissues was primarily streaked on an Anacker and Ordal's agar (AOA) (Anacker and Ordal, 1955) supplemented with Neomycin (Sigma) 0.5 mg mL^{-1} and Polymycin B (Sigma-Aldrich) 200 IU mL^{-1} (Anacker and Ordal, 1955) or modified Shield agar added with Tobramycin (Sigma) 1 µg mL^{-1} (Decostere et al., 1997). Suspected colonies were then sub-cultured for further isolation on AOA without antibiotic. Bacterial preservation was performed using AO broth containing 20% glycerol and preserved in -80 °C until needed. Prior to phenotypic and experimental challenge assays, fresh isolates were recovered by spreading 30 µL of bacterial stock on AOA supplemented 0.5 g $MgSO_4.7H_2O$ and 0.5 g $CaCl_2.2H_2O$ L^{-1}, incubated at 28 °C for 24-36 h.

Table 1: Description of yellow and pink pigmented bacterial isolates obtained from fish exhibiting columnaris-like disease.

Isolates/Color	Host	Organ	16S rRNA GenBank accession number	Most closely related species	Identity (%)
1.**CUVET1205** Yellow	NT	Skin	KJ190166	*Candidatus Chryseobacterium massiliae* (AF531766)	99.2
2.**CUVET1206*** Pale yellow	NT	Gill	KJ190167	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* (AB078045)	99.9
3.CUVET1211* Pale yellow	NT	Gill	KJ190172	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* (AB078045)	99.9
4.**CUVET1217** Yellow	RT	Skin	KJ190174	*Chryseobacterium taichungense* (JX042458)	98.1
5.CUVET1218 Yellow	RT	Kidney	KJ190175	*Chryseobacterium taichungense* (JX042458)	98.1
6.**CUVET1219** Yellow	RT	Kidney	KJ190176	*Chryseobacterium indologenes* (EU221399)	99.1
7.CUVET1220 Yellow	RT	Kidney	KJ190177	*Candidatus Chryseobacterium massiliae* (AF531766)	99.3
8.CUVET1225 Yellow	KC	Skin ulcer	KJ190180	*Flavobacterium indicum* (NR074422)	99.7
9.**CUVET1207** Pink	NT	Skin ulcer	KJ190168	*Flectobacillus roseus* GFA-11 (EU420062)	99.8
10.CUVET1227 Pink	NT	Gill	KJ190182	*Flectobacillus roseus* GFA-11 (EU420062)	99.8

NT, Nile tilapia; RT, red tilapia; KC, koi carp

Grey shade marks fish that was also infected with *F. columnare* (Dong, et al 2015a)

Underline bold-faced codes represent isolates subjected for experimental challenge

* indicates isolates subjected for ISR sequence analysis

Phenotypic tests

Conventional biochemical characteristics of the ten bacterial isolates were performed as described by Bernardet (1989) and Dong et al. (2015). Isolates were examined for colony morphology, bacterial cell shape, Gram stain, ability to grow on MacConkey and tryptic soy agar (TSA) media, catalase, oxidase, and the presence of flexirubin pigment. Carbohydrate metabolism was determined using modified Anacker and Ordal's medium (AO) (Anacker and Ordal, 1955) that contained 0.5 % tryptone, 0.5 % yeast extract, 0.2 % phenol red as indicator, and 1 % carbohydrate (arabinose, glucose, sucrose, maltose, mannitol, lactose and trehalose). AO agar supplemented with gelatin (1 %), skim milk (5 %) or starch (5 %) was used to test for degradation of gelatin, casein, and starch, respectively. AO agar supplemented with 0.1% esculin and 10% bile salts was prepared for esculine hydrolysis test. Sodium chloride tolerance was performed by growing isolates in AO broth containing 0, 1, 1.5, and 2 % NaCl for 5 days at 28 °C (Table 2).

16S rRNA amplification, DNA sequencing, and phylogenetic analysis

Genomic DNA from each isolate was extracted as described by Arias *et al.* (2004). Briefly, a single colony of each pure isolate was suspended in 100 µL nuclease-free water, boiled for 10 min, cooled rapidly on ice, and then briefly centrifuged. Supernatant containing genomic DNA was used as template for PCR reactions. A fragment of the 16S rRNA gene from the bacterial isolates was amplified using universal primers UN20 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R1438 (5'-GCC CTA GTT ACC AGT TTT AC-3') according to Darwish and Ismaiel (2005). The PCR reaction mixtures were prepared in 50 µL volume containing 25 µL Master Mix (GoTaq®Green, Promega, USA), 0.2 µM of each primer, 5 µL genomic DNA templates and 18 µL nuclease-free water. The PCR amplification was performed in a thermocycler (TC-96/G/H(b), Bioer, China) as follows: denaturation at 94 °C for 5 min, followed by 30 cycles of amplification at 94 °C for 30 sec, 45 °C for 30 sec, 72 °C for 2 min and final extension at 72 °C for 4 min (Darwish and Ismaiel 2005). Amplified products were purified using NucleoSpin® Extract II Kit (Macherey-Nagel, Germany)

and subjected for sequencing (1st BASE Pte Ltd) using UN20 and R1438 primers as mentioned above. Assembly of forward and reverse sequences of each isolate was conducted using ContigExpress software (Invitrogen Corporation, 2006). Nucleotide sequences were deposited in the GenBank database (Table 1). The similarity of the 16S rRNA gene sequences was compared with published sequences available in the GenBank database using Nucleotide BLAST program from National Center for Biotechnology Information (NCBI). Isolates were identified to species level based on at least 99 % identity of the 16S rRNA gene with sequences of type strains or published studies, whereas isolates that exhibited lower than 99 % identity were only identified to genus. A phylogenetic tree was constructed based on 1302 nucleotides of 16S rRNA gene sequences (position 87-1388 *E. coli* numbering) of the 10 isolates in this study and closely related taxa retrieved from GenBank. The 16S rRNA gene sequence of *Aeromonas hydrophila* ATCC7966 (NR074841) was used as outgroup. The phylogenetic tree was generated by the neighbor-joining method using p-distance model of MEGA 5.2 package (Tamura et al., 2011) after discarding gaps and unidentified bases (complete deletion option). The tree topology was evaluated by bootstrap analysis of 1000 replicates.

16S-23S rRNA intergenic spacer region (ISR) amplification and sequencing

Two isolates, designated as CUVET1206 and CUVET1211, were suspected for a novel potential pathogen first discovered in the present study. To provide more genetic characteristics and evidence for bacterial identification, the ISR of these isolates was further investigated. The universal primer 16S14F (5'-CTT GTA CAC ACC GCC CGT C-3') and 23S1R (5'-GGG TTT CCC CAT TCG GAA ATC-3') targeted to 16S rRNA and 23S rRNA genes respectively were used to amplify full length of ISR (Zavaleta et al., 1996). The preparation of PCR reaction mixtures was performed in the same manner mentioned above. The thermocycler conditions were as follows: denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 3 min (Zavaleta et al., 1996). Purification of PCR products and DNA sequence analysis were performed in the same manner described above.

Experimental challenge

The potential of the yellow and pink pigmented bacteria to cause disease in tilapia was investigated by experimental challenge using an intramuscular injection method. Five representative bacterial isolates were cultured on modified AO agar (AOA supplemented 0.5 g L^{-1} $MgSO_4.7H_2O$ and 0.5 g L^{-1} $CaCl_2.2H_2O$) at 28 ^{o}C for 36 h. Colonies of each bacterial isolate were harvested and suspended in sterile normal saline (0.85 % NaCl) to reach to approximately 10^9 CFUs mL^{-1}. The bacterial density was then verified by the plate count method and the exact injection doses of each bacterium are summarized in Table 3. Sixty apparently healthy Nile tilapia fingerlings (mean weight, 14.5 ± 1.5 g) were divided into 6 groups. Each group of 10 fish was intramuscularly injected with 0.1 mL volume containing either *Candidatus Chryseobacterium massiliae* CUVET1205, [*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206, *Chryseobacterium* sp. CUVET1217, *Chryseobacterium indologenes* CUVET1219 or *Flectobacillus roseus* CUVET1207 (Table 3). The control group was injected with 0.1 mL normal saline water. Fish were fed daily with tilapia feed (CP, Thailand). The water temperature during the experiment was 26.6 ± 1.4 ^{o}C.

Results

Bacterial identification

In the present study, 1, 4, and 5 bacterial isolates obtained from koi carp (*Cyprinus carpio*), red tilapia (*Oreochromis* sp.), and Nile tilapia (*Oreochromis niloticus*) exhibiting columnaris-like disease (Table 1) were selected for further investigation. The ten bacterial isolates were Gram negative, formed yellow, pale yellow or pink colonies on AO agar and were unable to grow on MacConkey agar (Table 2). Most of the isolates were positive for oxidase, catalase, flexirubin pigment, and some were able to grow on TSA agar. Detailed biochemical results are presented in Table 2. Based on the phenotypic tests, ten bacterial isolates were classified into six different phenotypic profiles. Colony morphologies of the six representative isolates from each of the biochemical phenotypes on modified AO agar are shown in Fig. 1.

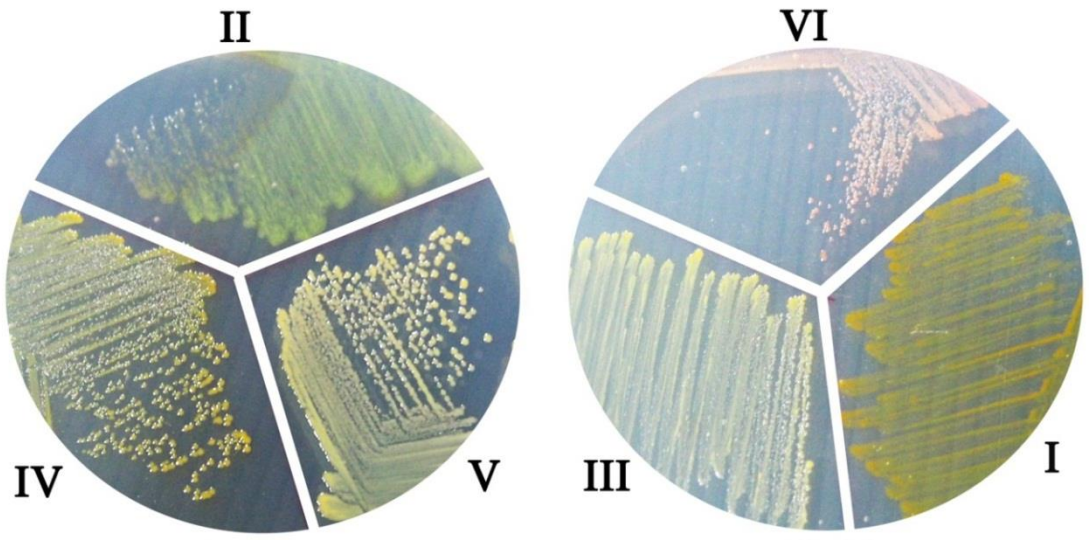

Figure 1: Colony morphology of bacterial species on modified AO agar after 36 h incubation at 28 ^{O}C. Roman numerals represent six individual biochemical characteristics. See Table 2 for a key to isolates classified into biochemical characteristics I to VI.

Further characterization was performed by sequencing approximately 1300 bp of the 16S rRNA gene of all ten bacterial isolates. Consistent with the phenotypic tests, homology search by BLAST analysis indicated that ten bacterial isolates belonged to six different species including *Candidatus Chryseobacterium massiliae, Chryseobacterium* sp., *C. indologenes*, *Flectobacillus roseus*,[*Flexibacter*] *aurantiacus* subsp *excathedrus*, and *Flavobacterium indicum* (Tables 1-2). Specifically, the isolates CUVET1205 and CUVET1220 were putatively identified as *Candidatus Chryseobacterium massiliae* based on > 99 % nucleotide identity to *C. massiliae* (AF531766). The isolates CUVET1219 and CUVET1225 were putatively identified as *Chryseobacterium indologenes* and *Flavobacterium indicum* based on 99.1% and 99.7% nucleotide identity to *C. indologenes* H2S10 (EU221399) and *F. indicum* GPTSA100-9 (NR074422), respectively. Two pale yellow pigmented isolates, designated CUVET1206 and CUVET1211, were putatively identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus* IFO 16024 (AB078045) based on 99.9 % identity. Isolates CUVET1217 and CUVET1218 exhibited a highest 16S rRNA sequence identity

to *Chryseobacterium* sp. and were most closely related to *C. taichungense* (JX042458) with 98.1% nucleotide identity. Isolates CUVET1207 and CUVET1227 were identified as *Flectobacillus roseus* based on 99.8 % identity to *F. roseus* GFA-11 (EU420062). All ten 16S rRNA sequences of the bacterial isolates were deposited in the GenBank database and their accession numbers were presented in Table 1. Additionally, the 16S-23S rRNA intergenic spacer region sequences of the two [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates (CUVET1206 and CUVET1211) were first sequenced in the present study and deposited in GenBank under accession numbers KM977904 and KM977905, respectively. These sequences exhibited the highest similarity with ISR sequences of *Flavobacterium columnare* isolates (85-88%) in the GenBank database.

Note that the isolates identified as *Chryseobacterium* sp., *C. indologenes*, *C. massiliae*, *F. indicum* and *F. roseus* (CUVET1217 - CUVET1220, CUVET1225, CUVET1227) in the present study were recovered from diseased fish that exhibited columnaris-like disease in which *F. columnare* was also isolated (Dong et al., in press-a) (Table 1). The isolates identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus* (CUVET1206), *C. massiliae* (CUVET1205) and *F. roseus* (CUVET1207) were concurrently recovered from Nile tilapia that exhibited columnaris-like disease, but *F. columnare* was not isolated from these fish (Table 1).

Table 2: Biochemical characteristics of yellow and pink pigmented bacterial isolates in this study.

Phenotypic profile	I	II	III	IV	V	VI
Isolate	*F. indicum* (CUVET1225)	*F. aurantiacus* subsp. *excathedrus* (**CUVET1206** & CUVET1211)	*C. indologenes* (**CUVET1219**)	*C. Chryseobacterium massiliae* (**CUVET1205** & CUVET1220)	*Chryseobacterium* sp. (**CUVET1217** & CUVET1218)	*F. roseus* (**CUVET1207** & CUVET1227)
Colony	Yellow	Yellow	Yellow	Yellow	Yellow	Pink
Gram	Negative	Negative	Negative	Negative	Negative	Negative
Oxidase	-	+	+	+	+w	+

Catalase	-	-	+	+	+	+
Flexirubin pigment	+	-	+	+	+	ND
TSA	-	-	+	+	+	-
MacConkey	-	-	-	-	-	-
Glucose	+	-	+	+	+	+
Sucrose	-	-	-	v	-	+
Maltose	+	ND	+	+	+	ND
Mannitol	+	ND	-	-	+	ND
Lactose	-	ND	-	-	+	-
Trehalose	-	ND	+	-	+	ND
Arabinose	-	ND	-	-	+	ND
Degradation of						
Gelatin	ND	+	-	v	+	+
Casein	+	-	+	+	+	-
Starch	-	-	+	v	+w	-
Esculin	-	ND	+	+	+	ND
Sodium chloride tolerance						
NaCl 0 %	+	+	+	+	+	+
NaCl 1 %	-	-	+	+	+	-
NaCl 1.5 %	-	-	+	+	+	-
NaCl 2 %	-	-	+	-	+	-

ND, not determined; v, variable; w, weak

Underline bold-faced codes represent isolates subjected for experimental challenge

16S rRNA phylogenetic analysis

Together with the phenotypic and molecular analysis described above, eight yellow pigmented bacteria in this study were composed of 2 *Candidatus Chryseobacterium massiliae,* 2 *Chryseobacterium* sp., 1 *C. indologenes*, 1

Flavobacterium indicum, and 2 [*Flexibacter*] *aurantiacus* subsp *excathedrus* while 2 isolates of the pink bacteria belonged to *Flectobacillus roseus* (Tables 1-2). Phylogenetic analysis based on the 16S rRNA gene sequences of the ten bacterial isolates and closely related species retrieved from GenBank clearly separated into two distinct phylogenetic groups each with members of previously reported and newly identified yellow and pink bacteria (Fig. 2). Within the yellow bacterial group, there are two clusters consisting of bacteria in the genera a) *Candidatus Chryseobacterium* and *Chryseobacterium* and b) *Flavobacterium* and [*Flexibacter*] (Fig. 2).

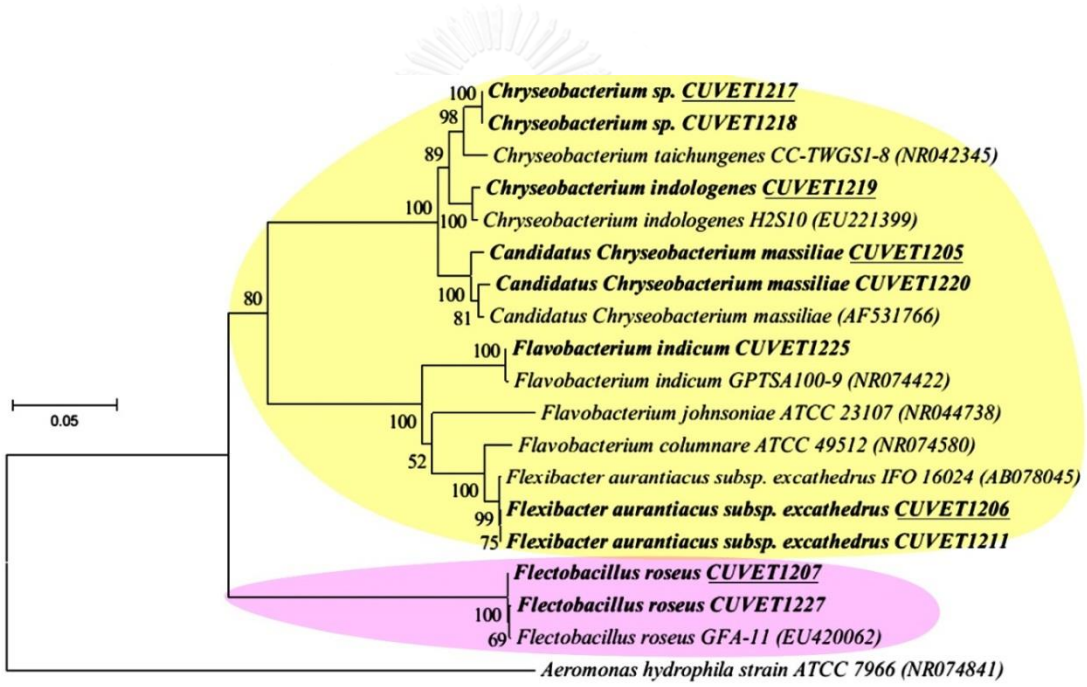

Figure 2: Phylogenetic tree was constructed based on 16S rRNA gene sequences (position 87-1388 *E. coli* numbering) of eight yellow and two pink pigmented bacteria in this study and closely related taxa by the neighbor-joining method using p-distance model. The tree topology was evaluated by bootstrap analysis of 1000 replicates. *Aeromonas hydrophila* ATCC 7966 was used as outgroup. Underline bold-faced codes represent isolates subjected for experimental challenge.

Evaluation of pathogenic potential of yellow and pink pigmented bacteria

In the present study, bacterial isolates from phenotypic profiles II to VI were chosen for evaluation of the pathogenic potential in Nile tilapia fingerlings. This study mainly focused on bacteria recovered from diseased tilapia, the koi carp isolate (phenotypic profile I), therefore, was not selected for the challenged test. After 14 days post challenge by injection method, the highest cumulative percentage mortality of 20 % was observed in the group administered with the bacterial isolate *Chryseobacterium indologenes* CUVET1219 (Table 3). Three other groups, which received either *Candidatus Chryseobacterium massiliae* CUVET1205, [*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206 or *Flectobacillus roseus* CUVET1207, exhibited only 10% cumulative percentage mortality while no mortality was observed in the group injected with *Chryseobacterium* sp. CUVET1217 and saline buffer (Table 3). All dead fish were observed within the first 7 days of the experimental period. The moribund fish which received *C. indologenes* CUVET1219 or *Candidatus Chryseobacterium massiliae* CUVET1205 exhibited discoloration areas on the body surface resembling columnaris disease (Fig. 4) and yellow pigmented bacteria were re-isolated from these fish. Observation of clinical signs and bacterial isolation were not performed with the dead fish from two other groups (injected with CUVET1206 and CUVET1207) because only one fish died (10 % mortality) and this happened during the night.

Table 3 Cumulative mortality in Nile tilapia fingerlings upon 14 day-experimental challenge with different bacterial isolates

Biochemical characteristics group	Bacteria administrated	Number of fish	Route of infection	Challenge dose (CFUs $fish^{-1}$)	Cumulative percentage mortality (%)
II	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206	10	Intramuscular injection	2.7×10^7	10
III	*Chryseobacterium indologenes* CUVET1219	10	Intramuscular injection	8.2×10^8	20
IV	*Candidatus Chryseobacterium massiliae* CUVET1205	10	Intramuscular injection	9.3×10^8	10
V	*Chryseobacterium* sp. CUVET1217	10	Intramuscular injection	4.1×10^8	0
VI	*Flectobacillus roseus* CUVET1207	10	Intramuscular injection	0.7×10^8	10
Control	0.85 % NaCl	10	Intramuscular injection	0.1 mL	0

CFUs, colony forming units

Figure 4 Experimental fish exposed to *Chryseobacterium indologenes* CUVET1219 (upper) and *Candidatus Chryseobacterium massiliae* CUVET1205 (lower) exhibited discoloration areas on the body surface resembling columnaris disease.

Discussion

Since *Flavobacterium columnare* (previously known as *Bacillus columnaris*, *Chondrococcus columnaris*, *Cytophaga columnaris* or *Flexibacter columnaris*) was first discovered as the aetiological agent of columnaris diseases (Davis, 1922), most later disease laboratory studies typically aimed at different aspects of single *F. columnare* infection and other bacteria involved are comparatively ignored in the literature. Our recent studies first revealed concurrent infections of multiple pathogens in natural diseased Nile tilapia (*O. niloticus*) and striped catfish (*Pangasianodon hypophthalmus*), which exhibited clinical signs resembling columnaris disease (Dong et al., 2015c, d), and have proposed the concept that "The reality of natural disease outbreaks in fish farms caused by multiple pathogen infections probably outweighs single infection". The present study therefore aimed at further investigation of other bacterial infections, with an emphasis on unknown culturable yellow and pink pigmented bacteria associated with columnaris diseased fish samples. Ten representative yellow and pink pigmented bacteria isolated from

tilapia and koi carp exhibiting columnaris-like disease belonged to six different species including *Candidatus Chryseobacterium massiliae, Chryseobacterium* sp., *C. indologenes*, *Flavobacterium indicum*, [*Flexibacter*] *aurantiacus* subsp *excathedrus*, and *Flectobacillus roseus*. To the best of our knowledge, all bacteria identified in our study have never been reported in tilapia and koi carp.

Regarding bacterial taxonomy, [*Flexibacter*] *aurantiacus* Lewin 1969 contains two subspecies [*Flexibacter*] *aurantiacus* subsp. *excathedrus* and [*Flexibacter*] *aurantiacus* subsp. *copepodarum* (Lewin, 1969). Based on fatty acid profiles and DNA-DNA hybridization assay, two type strains of [*Flexibacter*] *aurantiacus* have been transferred to *Flavobacterium johnsoniae* [6]. However, phylogenetic analysis based on 16S rRNA (Fig. 2) and 16S-23S rRNA intergenic spacer region (Fig. 3) of the isolates (CUVET1206 and CUVET1211) in this study and closely related taxa clearly indicated that [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates were closest relatives of *Flavobacterium columnare* ATCC 49512. This suggests that taxonomy of [*Flexibacter*] *aurantiacus* subsp. *excathedrus*/ [*Flavobacterium*] *johnsoniae* subsp. *excathedrus* should be further amended.

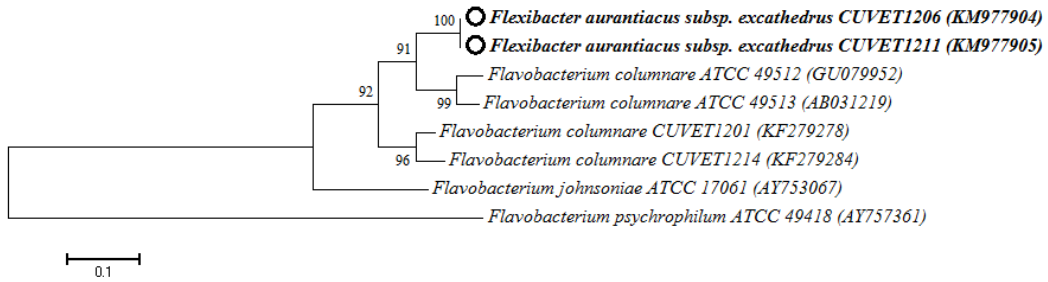

Figure 3 Neighbor-joining tree was constructed from complete 16S-23S rRNA intergenic spacer region sequences of the two [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates (CUVET1206 and CUVET1211) and closely related taxa. The tree topology was evaluated by bootstrap analysis of 1000 replicates.

The majority of the collected unknown yellow pigmented bacteria were identified as *Chryseobacterium* spp. Over the last decade, a large number of new *Chryseobacterium* species have been described from diseased fish (Bernardet et al., 1995; de Beer et al., 2006; Ilardi et al., 2009; Kämpfer et al., 2011; Zamora et al.,

2012a, b, c, d; Pridgeon et al., 2013; Loch and Faisal 2014). For example, *C. indologenes* was reported as a pathogenic bacteria causing disease in American yellow perch, *Perca flavescens*, (Mitchill) (Pridgeon et al., 2013). The pink pigmented bacterium, *F. roseus* was originally isolated from a freshwater environment (Sheu et al., 2009) and was recently reported as the causative agent of a new disease (Flectobacillosis) in roho labeo (*Labeo rohita*) fingerlings (Adikesavalu et al., 2015).

In contrast to published reports (Pridgeon et al., 2013, Adikesavalu et al., 2015), *C. indologenes* and *F. roseus* identified in the present study exhibited low virulence to healthy Nile tilapia fingerlings even when a high dose of different bacterial isolates was used. These contradictory findings might be implicated in different host organisms and their susceptibility to the pathogens. With respect to bacterial pathogenicity, the challenged experiment revealed that single bacterial infection failed to establish their virulence to result in high mortality in the challenged fish. However, the collaboration of multiple bacteria in disease manifestation according to our concurrent infections concept has not yet been evaluated. In addition, it might be possible that previously identified *F. columnare* isolates could play a role as primary instigator of the disease outbreaks followed by secondary infections of the yellow and pink pigmented bacteria. If this proves to be the case, the newly identified bacteria might serve as opportunistic pathogens that need stressors (i.e. primary pathogens, environmental factors etc.) for disease manifestation in tilapia farms.

It is interesting to note that concurrent infections of three bacterial species were first addressed in this study. [*Flexibacter*] *aurantiacus* subsp. *excathedrus* (CUVET1206), *C. massiliae* (CUVET1205), and *F. roseus* (CUVET1207) were recovered from the same fish specimen of Nile tilapia showing clinical signs resembling columnaris disease in which *F. columnare* seemed to be absent. The causative agent(s) of this case was not revealed through single infections within our current experimental challenges. However, it might be worthwhile to establish an experiment for multiple concurrent infections in fish model and to survey these

bacteria as potential pathogens in natural outbreaks in order to gain better understanding of the reality of natural disease manifestation in fish farms.

Herein, this study first reported various non-*Flavobacterium columnare* bacteria involved columnaris-like diseased fish and primarily investigated their pathogenic potential to tilapia fingerlings without stressors. Future works will investigate virulence of the bacteria through different routes of infections under environmental stress conditions or multiple infections.

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CHAPTER V

Virulence assay of rhizoid and non-rhizoid morphotypes of *Flavobacterium columnare* in red tilapia, *Oreochromis* sp., fry

Publication

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Abstract

Numerous isolates of *F. columnare* were previously recovered from red tilapia, *Oreochromis* sp., exhibiting columnaris-like disease in Thai farms, and the phenotypic and genetic characteristics were described. The objective of this study was to determine the virulence of two morphotypes (rhizoid and non-rhizoid colonies) of *F. columnare* and to determine their ability to adhere to and persist in red tilapia fry. The results showed that the typical rhizoid isolate (CUVET1214) was a highly virulent isolate and caused 100 % mortality within 24 h following bath challenge of red tilapia with three different doses. The non-rhizoid isolate (CUVET1201) was avirulent to red tilapia fry. Both morphotypes adhered to and persisted in tilapia similarly at 0.5 and 6 h post challenge as determined by whole fish bacterial loads. At 24 and 48 h post challenge, fry challenged with the rhizoid morphotype exhibited significantly higher bacterial loads than the non-rhizoid morphotype. The results suggested that an inability of the non-rhizoid morphotype to persist in tilapia fry may explain lack of virulence.

Keywords: Colony morphotypes, *Flavobacterium columnare*, virulence, tilapia.

Introduction

Columnaris disease (also known as myxobacterial disease, peduncle disease, saddleback, fin rot or cotton wool disease) was first described by Davis (1922) and is one of oldest known diseases of freshwater fish (Bernardet & Bowman, 2006; Noga 2010). *Flavobacterium columnare* is the aetiological agent of columnaris disease (Bernardet 1989; Amin *et al.* 1988; Declercq *et al.* 2013) and numerous wild and cultured fish are susceptible (Decostere *et al.* 1998). A list of thirty-seven susceptible fish species was suggested by Anderson & Conroy (1969), and this number has increased in recent years with Nile tilapia *Oreochromis niloticus* (L.) (Figueiredo *et al*. 2005; Eissa *et al.* 2010), red tilapia *Oreochromis* sp. (Dong *et al.* 2014), Indian carp *Catla catla* (Hamilton) (Verma & Rathore 2013), striped catfish *Pangasianodon hypophthalmus* (Sauvage) (Tien *et al.* 2012) and rainbow trout *Oncorhynchus mykiss* (Walbaum) (LaFrentz *et al.* 2012) reported as susceptible. Naturally infected fish usually show lesions on the external body surface and gills such as skin damage, fin rot, "saddle back" lesion or gill necrosis (Bernardet & Bowman, 2006; Declercq *et al.* 2013).

Dong *et al*. (2014) reported columnaris-like disease in intensively cultured tilapia farms in Thailand. Numerous yellow pigmented bacteria associated with the diseased fish were recovered and the majority were identified as *F. columnare* and characterized (Dong *et al*. 2014). A few of the isolates recovered exhibited rhizoid colonies upon primary isolation and following subculture in the laboratory, the colony morphology changed to non-rhizoid. This has been reported previously, and the change has been associated with a loss of virulence in rainbow trout (Kunttu *et al.* 2009). The objectives of this study were to verify that *F. columnare* was the aetiological agent involved in the columnaris-like disease in red tilapia by fulfilling Koch's postulates and to determine the virulence and ability of the rhizoid and non-rhizoid morphotypes to adhere to and persist in red tilapia fry.

Materials and methods

Bacterial isolates

Two isolates of *F. columnare*, designated CUVET1214 and CUVET1201, were previously isolated from columnaris-like diseased red tilapia and were assigned to genomovar II (Dong *et al.* 2014). Morphologically, CUVET1214 formed typical rhizoid colonies on Anacker and Ordal (AO) agar, whereas CUVET1201 was originally rhizoid but transformed to non-rhizoid under the preservation condition in the laboratory. Photographs of bacterial colonies on AO agar plates were taken by digital camera under light microscope (Olympus 686726, Japan). These two different colony morphotypes were used for experimental challenges to determine virulence and fulfill Koch's postulates, and to determine the ability to adhere to and persist in red tilapia.

Fish for experimental challenge

Apparently healthy red tilapia fry (*n*=240; mean weight, 0.37 ± 0.1 g) were obtained from a commercial hatchery at Samut Sakhon province and transported to the Veterinary Microbiology laboratory (Chulalongkorn University) for experiments. Fish were acclimated for 5 d in UV-treated water. The temperature and pH of the water was monitored daily during the acclimation period and experimental challenges, was averaged 29 ± 2 ^{O}C and pH of 7.5 ± 0.3, respectively. Fish were fed twice daily with commercial pellet food (CP, Thailand). Before experiments, 15 fry were randomly selected for bacteriological and parasitic examination and found to be negative with *F. columnare* and ectoparasites. The fish used in this study were approved by the Chulalongkorn University Animal Care and Use Committee no. 11/2556.

Experimental challenge of F. columnare

Flavobacterium columnare CUVET1201 (non-rhizoid) and CUVET1214 (rhizoid) were cultured in 100 mL AO broth at 29 ± 1 ^{O}C with shaking for 24 h. The cultures from both isolates were adjusted to an optical density of 0.8 at 540 nm using sterile AO broth and the CFUs mL^{-1} of each were determined by plate count method. For

each isolate, three challenge doses were prepared by diluting the cells in 0.5 L water and the plate count results indicated challenge doses of 2×10^5, 2×10^6, and 2×10^7 CFUs mL^{-1} for *F. columnare* CUVET1214 and 1×10^5, 1×10^6, and 1×10^7 CFUs mL^{-1} for *F. columnare* CUVET1201. Three control tanks contained 0.5 L water without bacteria. Single groups of ten red tilapia fry were immersed in each challenge dose or control for 1 h and then transferred to new tanks containing static 1.5 L specific-pathogen-free water with gentle aeration (Fig. 1A). Experimental fish were fed twice per day and water was changed at seventy percent every two days. Mortality of infected fish was monitored for 15 d. Bacteria were isolated from the gills and skin of moribund or fresh dead fish using selective AO agar supplemented with tobramycin 1 μg mL^{-1} (Sigma) (Decostere, Haesebrouck & Devriese 1997). All putative *F. columnare* were confirmed by species-specific PCR previously described by Welker *et al.* (2005).

Quantification of adherence and persistence of F. columnare to red tilapia fry

F. columnare CUVET1201 (non-rhizoid morphotype) and CUVET1214 (rhizoid morphotype) were further used to determine the ability of each to adhere to and persist in red tilapia fry. Single groups of forty five fry were immersed for 0.5 h in 1 L water containing *F. columnare* CUVET1201 (5.8×10^5 CFUs mL^{-1}), *F. columnare* CUVET1214 (6.8×10^4 CFUs mL^{-1}), and no bacteria as the control group. After the 0.5 h immersion, five fish from each group were immediately collected for bacterial quantification. Remaining fish from each group were divided into two tanks (20 fish per tank) containing 10 L *F. columnare*-free water with gentle aeration. Fish were fed twice per day and environmental parameters were monitored every day. Three fish per each replicate tank and treatment were sampled at 6, 24 and 48 h post-challenge for bacterial quantification (Fig. 1B). Bacterial loads were estimated based on plate count method described by Shoemaker *et al.* (2008) with slight modification. Fish were euthanized with ice-cold water and internal organs were carefully removed to minimize contamination from fish gut. Each fish was then weighed by an electronic balance (Mettler Toledo) in order to back calculate the CFUs per gram fish tissue. The whole fish were then homogenized in 1.5 mL microtube using disposable polypropylene pestle. The tissue was then transferred to

10 mL sterile AO broth supplemented with tobramycin 1 μg mL^{-1}. Fifty microliters of non-diluted, 10-fold and 100-fold diluted samples was spread onto AO agar and incubated at 29 ^{O}C for 36-48 h. The number of *F. columnare* colonies were counted and expressed as the average number of CFUs g^{-1} fish. The differences in bacterial loads in each experimental group were compared using a t-test analysis of Statistical Package for the Social Science (SPSS 17.0).

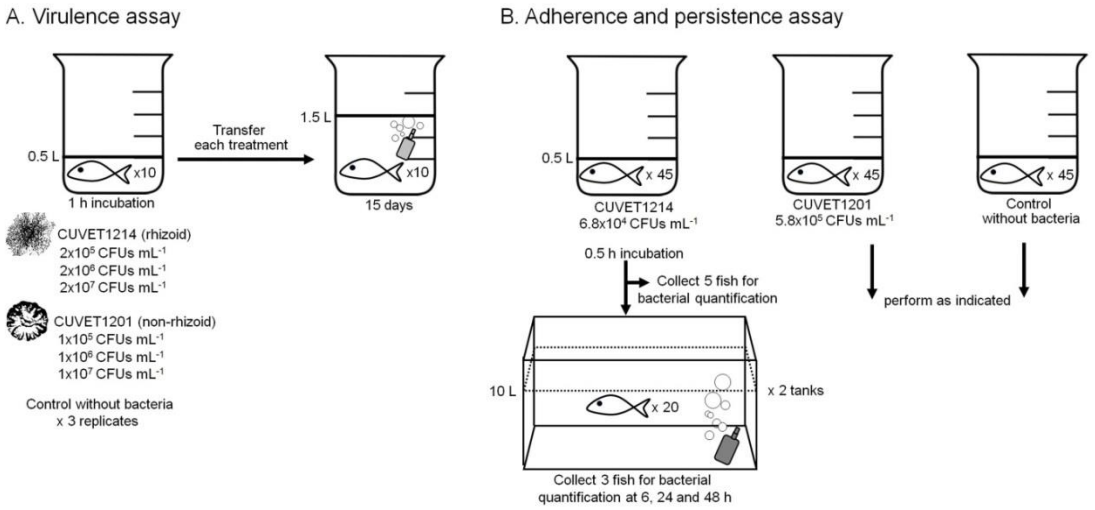

Figure 1: Experimental designs of virulence assay (A) and the adherence and persistence assay (B).

Results

Colony morphology of F. columnare

Upon primary isolation, both *F. columnare* CUVET1201 and CUVET1214 formed yellow pigmented rhizoid colonies on AO agar. These isolates were preserved in AO broth. Briefly, one single colony of the bacterial isolates was cultured in 30 mL sterile AO broth, incubated at room temperature (29 ± 1 ^{O}C) for approximately six months before recovering on AO agar. Under this preservation condition in our laboratory, the CUVET1201 isolate transformed to a non-rhizoid morphotype whereas the CUVET1214 isolate remained as the rhizoid morphotype (Fig. 2). The colony morphotype of the CUVET1201 isolate did not revert to rhizoid following multiple passages on the same medium.

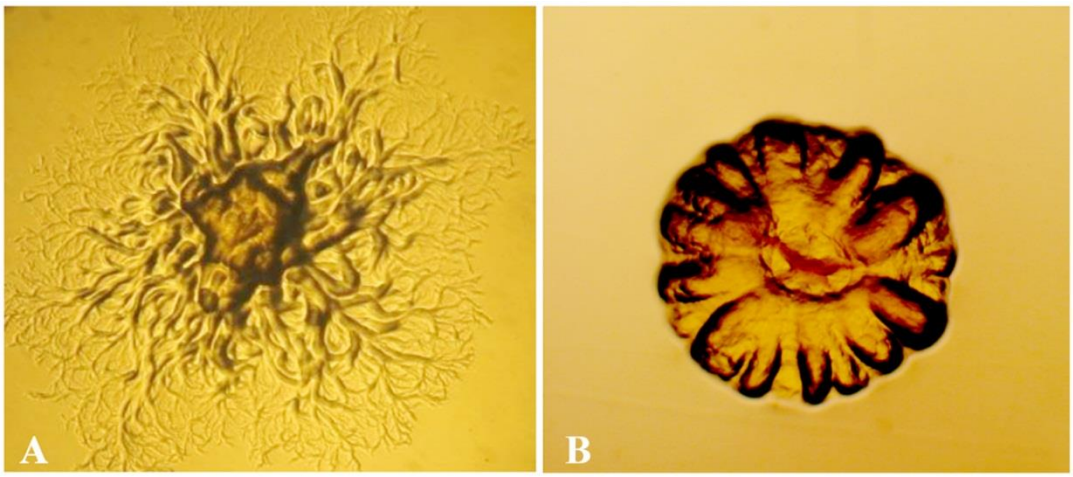

Figure 2: Rhizoid morphotype of *F. columnare* CUVET1214 (A) and non-rhizoid morphotype of *F. columnare* CUVET1201 (B) on AO agar after incubation at 29 ^{O}C for 48 h.

Virulence of F. columnare isolates in red tilapia fry

The virulence of *F. columnare* CUVET1201 (non-rhizoid morphotype) and CUVET1214 (rhizoid morphotype) were determined in red tilapia fry by immersion challenge method. *F. columnare* CUVET1214 induced 100 % mortality within 24 h at each challenge dose (2×10^5, 2×10^6, and 2×10^7 CFUs mL^{-1}). No mortality was observed following challenge of fish with three doses of *F. columnare* CUVET1201 (1×10^5, 1×10^6, and 1×10^7 CFUs mL^{-1}) as well as the control tanks. Moribund fish exhibited typical skin discoloration on the body surface and congested gills (Fig. 4B). The caudal skin lesion was clearly observed under stereo microscope (Fig. 4C). Isolates of putative *F. columnare* were successfully recovered from the gill and skin of all thirty dead or moribund fish. Fifteen randomly selected isolates were confirmed by PCR as *F. columnare* (data not shown).

Figure 4: Experimentally diseased tilapia fry (B, C) exhibiting typical caudal skin lesions that were similar to the clinical signs of naturally diseased fish (A). Pictures were taken by digital camera in normal light condition (A, B) and under stereo microscopy (C).

Adherence and persistence of F. columnare in red tilapia fry

To determine the ability of the two morphotypes of *F. columnare* to adhere to and persist in red tilapia fry, bacterial quantification from fish challenged by immersion was performed in a time-course manner. For this experiment, a shorter duration of challenge (0.5 h) and a lower dose (6.8×10^4 CFUs mL^{-1}) was used for the virulent *F. columnare* CUVET1214 to allow for fish survival. At 0.5 h post challenge (hpc), ~ 10^6 CFUs g^{-1} fish tissue were recovered from tilapia challenged with CUVET1201 and CUVET1214 (Fig. 3). The bacterial loads were lower (~ 10^5 CFUs g^{-1}) in both groups at 6 hpc. In the group challenged with *F. columnare* CUVET1201 (non-rhizoid), bacterial loads reduced to ~ 10^3 CFUs g^{-1} fish and 0 CFUs g^{-1} fish at 24 and 48 hpc, respectively. In contrast, the bacterial loads of *F. columnare* CUVET1214 (rhizoid) remained relatively constant at ~ 10^5 CFUs g^{-1} fish at 24 and 48 hpc. The differences in bacterial loads between the two isolates were significant ($p < 0.05$) at 24 and 48 hpc (Fig. 3).

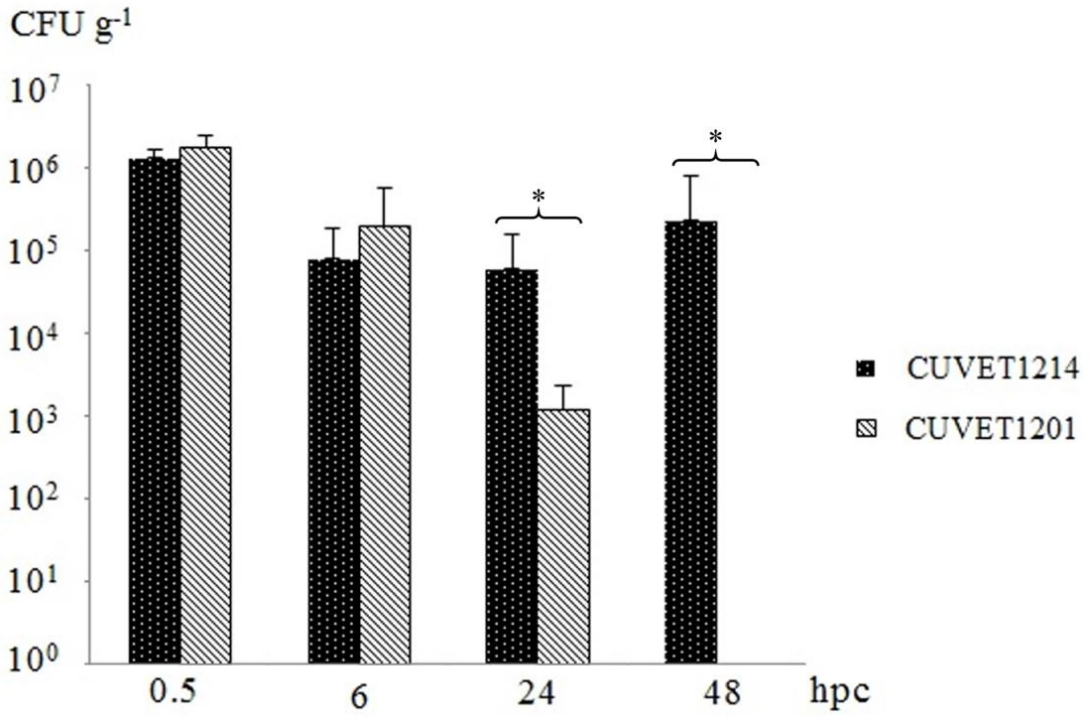

Figure 3: Bacterial loads (CFU g^{-1}) quantified from red tilapia fry following challenge with the rhizoid morphotype of *Flavobacterium columnare* (CUVET1214) and the non-rhizoid morphotype (CUVET1201). Error bars indicate standard deviation and an asterisk (*) indicates a significant ($P < 0.05$) difference between bacterial loads.

Discussion

Dong *et al.* (2014) characterized *F. columnare* isolates recovered from red tilapia (sub-adult, fingerling and fry) in Thailand and results from sequencing the 16S rRNA gene and 16S-23S rRNA intergenic spacer region indicated the isolates were a distinct genetic group. In the present study, red tilapia fry were challenged with two representative morphotypes, rhizoid and non-rhizoid, of *F. columnare* to fulfill Koch's postulates and to determine the virulence of these isolates. Tilapia fry (~ 0.37 g) were used in the challenge experiments because fry were one of the size classes of tilapia impacted by columnaris disease in our previous study, and this size of fish is practical for virulence and adherence/persistence studies of *F. columnare*. Tilapia

challenged with the rhizoid isolate (CUVET1214) exhibited typical clinical signs of columnaris disease (Fig. 4B, C) that were similar to naturally diseased fish (Fig. 4A) and *F. columnare* was isolated from infected fish; thus, the challenge experiment fulfilled Koch's postulates. This demonstrated that *F. columnare* was the causative agent of the columnaris-like disease previously described in red tilapia (Dong *et al.* 2014). Koch's postulates were not fulfilled with the non-rhizoid isolate (CUVET1201); however, it should be noted that upon primary isolation, this isolate exhibited the rhizoid colony morphology and following manipulation in the laboratory the morphology changed to non-rhizoid.

The observation of *F. columnare* forming different colony morphologies in the laboratory has been previously described. Kunttu *et al.* (2009) described four colony types and subsequently refined the classification system to three colony types (Kunttu et al. 2011). These included rhizoid, rough, and soft colony morphotypes (Kunttu et al. 2011). The change from rhizoid to the other colony types has been associated with routine passage under laboratory condition (Kunttu *et al.* 2009), and exposure to bacteriophages (Laanto *et al.* 2012, Zhang *et al.* 2014). The non-rhizoid isolate used in the present study (CUVET1201) exhibited the rough morphotype (Fig. 1), and the change of this isolate from rhizoid to rough was associated with longterm preservation in AO broth (Dong *et al.* 2014). Research has demonstrated the change from rhizoid to the rough and soft morphotypes is associated with a loss of virulence in rainbow trout (Kunttu et al. 2009; Kunttu et al. 2011; Laanto et al. 2014). Similarly, challenge of red tilapia with a rhizoid and non-rhizoid (rough) morphotype of *F. columnare* demonstrated differences in the ability of these isolates to induce disease and mortality. Challenge of tilapia with the rhizoid isolate (CUVET1214) resulted in 100% mortality within 24 h at all doses, while challenge with the non-rhizoid isolate (CUVET1201) did not result in any mortality. Previous studies classified the virulence of *F. columnare* into four grades based on the time required to induce mortality in challenged fish (Kou *et al.* 1981, Pacha & Ordal 1963). Isolates that induced 100% mortality within 24 h were categorized as high virulence, 24-96 h were considered as moderate virulence, more than 96 h were

low virulence and no mortality were classified as avirulent (Kou *et al.* 1981, Pacha & Ordal 1963). Based on this classification, the rhizoid morphotype (CUVET1214) was considered a highly virulent isolate whereas the non-rhizoid morphotype (CUVET1201) was considered avirulent.

Kunttu *et al.* (2009) demonstrated that the virulence of the different morphotypes was not associated with the ability of the isolates to adhere to polystyrene and gill tissue. Similarly the rhizoid and non-rhizoid (rough) isolates in the present study did not appear to differ in adherence. At 0.5 and 6 hpc, bacterial loads from challenged tilapia were similar for both isolates which suggests that there were no difference in the ability to adhere to fish. However, the ability of the isolates to persist was different. At 24 and 48 hpc, bacterial loads from tilapia challenged with the rhizoid isolate were constant at ~ 10^5 CFUs g^{-1} tissue, while the bacterial loads from fish challenged with the non-rhizoid isolate were significantly reduced at 24 h and no *F. columnare* were recovered at 48 hpc. This observation suggests that the lack of virulence in the non-rhizoid (rough) isolate may be due to an inability to invade and persist in the fish following adhesion. This is supported by recent research that demonstrated rough and soft colony morphotypes exhibited distinct differences from the rhizoid morphotype in secreted proteins and outer membrane vesicles, putative virulence factors of *F. columnare* (Laanto *et al.* 2014). Interestingly, the non-rhizoid isolate maintained this morphotype following passage through the fish *in vivo*.

Herein, this study demonstrated that previously characterized *F. columnare* (Dong *et al.* 2014) was the etiologic agent of the columnaris-like disease in red tilapia and also demonstrated differences in virulence and the ability of the rhizoid and non-rhizoid (rough) colony morphotypes to persist in tilapia fry. Future work will develop strategies to prevent or treat columnaris disease in red tilapia.

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CHAPTER VI

Genetic diversity of *Flavobacterium columnare* isolated from Nile tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and striped catfish (*Pangasianodon hypophthalmus*)

Abstract

Flavobacterium columnare is an important threat of cultured freshwater fish species worldwide. Disease outbreaks associated with *F. columnare* were recently reported in Nile tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and striped catfish (*Pangasianodon hypophthalmus*) in Thailand. Genetic characterization of *F. columnare,* however, was well-described in only red tilapia. Here, we aimed to assign genomovar for isolates of *F. columnare* isolated from Nile tilapia and striped catfish and investigate genetic diversity of these isolates based on 16S rRNA gene. All isolates of *F. columnare* isolated from diseased Nile tilapia and striped catfish were assigned to genomovar II. Genetic diversity of *F. columnare* based on 16S rRNA revealed that *F. columnare* originated from Nile tilapia and red tilapia was identical and formed a unique cluster and phylogenetically different with those originated from striped catfish.

Key words: Genetic diversity, Genomovar, *Flavobacterium columnare*, striped catfish, tilapia

Introduction

Nile tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and striped catfish (*Pangasianodon hypophthalmus*) are major aquaculture freshwater fish in Thailand. These species were reported as the susceptible hosts of *Flavobacterium columnare* (Tien et al., 2012; Dong et al., 2015a; Dong et al., 2015b; Dong et al., 2015c). Genetic diversity of *F. columnare* was first described by Triyanto and Wakabayashi (1999) and first classified *F. columnare* isolates into three genomovars (I, II & III) based on the restriction fragment length polymorphism of 16S rRNA gene (16S-RFLP). Several later studies have been atempt different methods to investigate genetic diversity of *F. columnare* such as random amplified fragment length polymorphic DNA (RAPD) (Thomas-Jinu and Goodwin, 2004), amplified fragment length polymorphism (AFLP) fingerprinting (Arias et al., 2004), single-strand conformation polymorphism (SSCP) (Olivares-Fuster et al., 2007b). Up to date, only 16S-RFLP technique has been proposed as a standard method for genotyping of *F. columnare* and widely applied in later studies (Darwish and Ismaiel, 2005; LaFrentz et al., 2014). LaFrentz et al. (2014) validated 16S-RFLP methods and proposed new genomovars I/II and II-B.

Genetic characterization of *F. columnare* originated from red tilapia was recently described. Co-existence of two genomovars (I and II) of *F. columnare* was reported in red tilapia but genomovar II appeared as major population and clustered in a unique 16S rRNA phylogenetic group (Dong et al., 2015a). However genetic characterization of *F. columnare* isolated from Nile tilapia and striped catfish or whether genetic characterization of *F. columnare* exhibited host-specific remains undetermined. The objectives of the present study are to assign gemomovar for isolates of *F. columnare* isolated from Nile tilapia and striped catfish and phylogenically compared 16S rRNA sequences of these isolates.

Materials and Methods

Bacterial isolates

Twenty one isolates of putative *F. columnare* (designated NT, NS and NK in Table 1) isolated from external lesion of naturally diseased Nile tilapia, which showed clinical signs of columnaris disease, were subjected for species identification, 16S-RFLP and 16S rRNA sequencing in this study. Eleven isolates (designated BU, Fc-NK) and 6 isolates (designated SCF), which were previously isolated from diseased Nile tilapia and striped catfish respectively (Table 1) (Dong et al., 2015b; Dong et al., 2015c), were subjected for genomovar assignment based on 16S-RFLP in this study. Additionally, 16 isolates (designated CUVET, Table 1) from diseased red tilapia (n=14) and Vietnamese striped catfish (n=2) characterized in our previous study were included for genetic diversity comparison.

Table 1: Bacterial isolates used in this study.

Isolate	Numbers of isolate	Host	Geographic origin	Genomovar	% Similarity to *F. columnare* ATCC 49512
NT (01-09)	9	NT	Suphanburi, TL	II	~98
NS (01, 02, 04, 09-12)	8	NT	Prachinburi, TL	II	~98
NK (06-09)	4	NT	Nongkhai, TL	II	~98
BU	1	NT	Chachoengsao, TL	II	~98
Fc-NK (01-10)	10	NT	Nongkhai, TL	II	~98
SCF (01-06)	6	SCF	Ratchaburi, TL	II	~99
CUVET (1231-1232)	2	SCF	Cantho, VN	II	~99
CUVET (1201-1204, 1212-1215, 1336, 1338, 1343-1346, 1350)	14	RT	Ratchaburi, Phetchaburi, Kanchanaburi, Chachoengsao, TL	I & II	~98

NT, Nile tilapia; RT, red tilapia; SCF, striped catfish; TL, Thailand.
Underline bold-faced codes represent the isolates were assigned Genomovar in this study.
Grey shade marks the isolates which were subjected for 16S rRNA sequencing in this study.

Identification of F. columnare based on specific PCR

Flavobacterium columnare-specific primers FCISRR and FCISRR1 (Table 2) targeting 16S-23S ISR (Welker et al., 2005) was used for identification of 21 putative *F. columnare*. PCR mixtures contained 12.5 µl Master Mix (GoTaq®Green, Promega USA), 0.2 µM of each PCR primer and 150-200 ng genomic DNA. Amplification was performed as following thermocycler conditions: 94 °C for 5 min; 30 cycles of 94 °C for 30 sec, 45 °C for 45 sec, 72 °C for 1 min and final extension at 72 °C for 7 min. DNA template from *F. columnare* CUVET1201 was used as the positive control and no template was used as the negative control. PCR products were detected under UV light after electrophoresis.

16S rRNA amplification and genomovar assignment based on 16S-RFLP analysis

1.45 kb of 16S rRNA of *F. columnare* isolates was amplified using universal primers UN-20/20F and R1438 (Table 2). The PCR reactions will be performed as described by Darwish & Ismaiel (2005). The isolate *F. columnare* CUVET1201 was used as the positive control and a negative control without DNA template also will be included. Digestion of PCR products with restriction endonucleases *Hae*III (Thermo Scientific, USA) was performed following PCR amplification. Mixtures of 20 µL PCR products, 1 µL *Hae*III, 2 µL buffer and nuclease-free water in final volume of 30 µL were incubated at 37 ^{O}C for 2-16 hours. DNA banding patterns were visualized under UV light of gel documentation following electrophoresis of 20 µL digested mixtures in 3% agarose gel in TBE at 100V for 80 min. The isolates CUVET1215 and CUVET1201, assigned to genomovar I & II respectively in our previous study (Dong et al., 2015a), were used as internal control in the present study.

Table 2 Primers used in this study

Primer name	Primer sequence (5'-3')	Target	Size of PCR product	Reference
UN-20/20F	AGAGTTTGATC(AC)TGGCTCAG	16S rRNA	1450 bp	Darwish and Ismaiel (2005)
R1438	GCCCTAGTTACCAGTTTTAC			
FCISRFL	TGCGGCTGGATCACCTCCTTTCTAGAGACA	ITS	400-500 bp	Welker *et al.* 2005
FCISRR1	TAATYRCTAAAGATGTTCTTTCTACTTGTTTG			

16S rRNA sequencing and phylogenetic analysis

Amplification of ~1.45 kb of 16S rRNA was conducted using universal primers UN-20/20F and R1438 (Table 2) as previously described by Darwish and Ismaiel (2005). PCR products were cleaned up using NucleoSpin® Extract II Kit (Macherey-Nagel, Germany) and submitted for direct sequencing (1st BASE Pte Ltd). The sequences of two directions were assembled using ContigExpress (VNTI version 10.1). Multiple alignments (clustal W, MEGA 6) of 16S rRNA sequences in this study and sequences retrieved from GenBank which represented each of the five described genomovars of *F. columnare*. The phylogenetic tree was generated by the neighbor-joining method with p-distance model and bootstrap of 1000 replicates.

Results

Identification of putative F. columnare isolates

All putative *F. columnare* isolates (n=21) were recovered from external lesion of naturally diseased Nile tilapia, which exhibited symptoms of columnaris disease (Fig. 1A). These isolates were Gram-negative, slender long rod-shaped bacteria that formed yellowish, flat, rhizoid colonies on AOA medium (Fig. 1B) and produced flexirubin pigment. Species-specific PCR yielded two to three PCR products with approximate size of 400-600 bp (Fig. 2) and definitive identification as *F. columnare*.

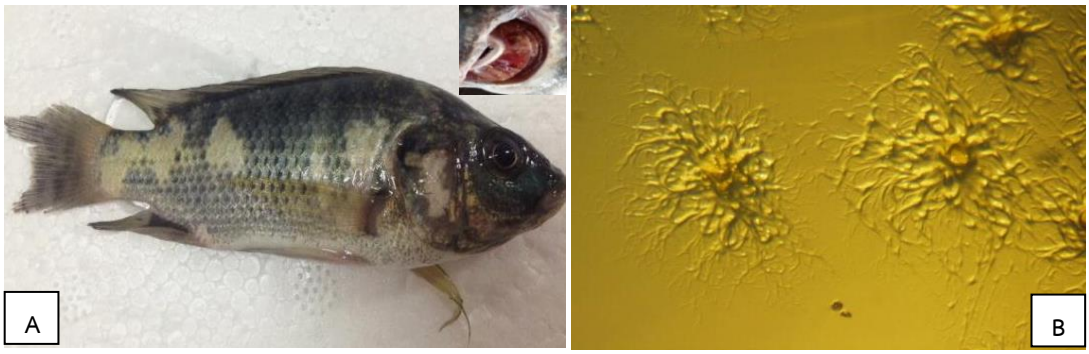

Figure 1: Naturally diseased tilapia caused by *Flavobacterium columnare* (A); Colonies morphology of *F. columnare* on Anacker and Ordal's medium (B).

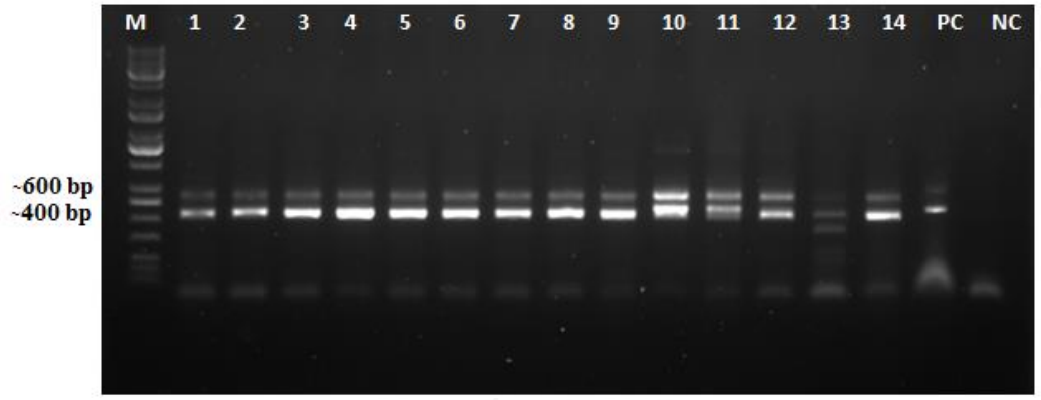

Figure 2: *F. columnare*-specific PCR generated one amplified fragment ranged from 400-600 bp. Lane M: Marker; Lane 1-14: Isolate NS01, NS02, NS04, NS09 to NS12, NT01, NT02, NK06 to NK09; PC: positive control; NC: negative control.

Genomovars assigments

16S rRNA amplification using universal primers yielded an amplicon of approximately 1450 bp in all examined isolates. The 16S-RFLP generated the same DNA banding patterns with the genomovar II internal control isolate, including four DNA bands approximately 600, 290, 279 and 180 bp in size, in all isolates in the present study (Fig. 3). All isolates from Nile tilapia (n=37) and striped catfish (n=6) therefore, were assigned to genomovar II.

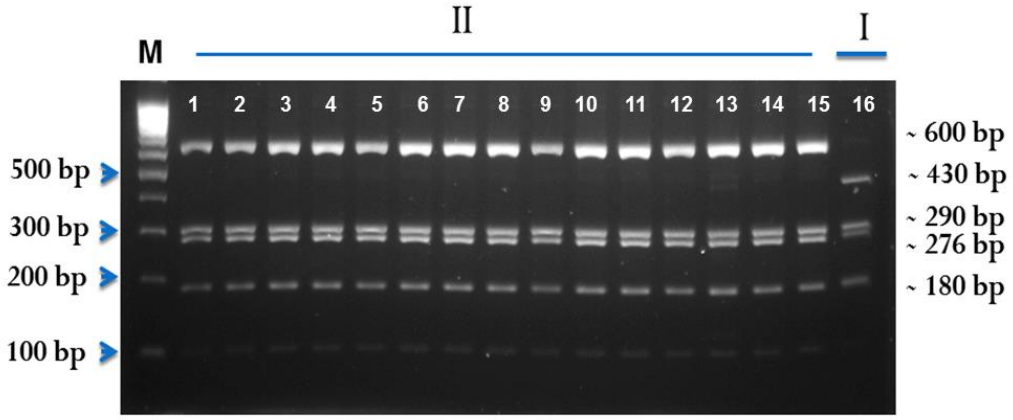

Figure 3: 16S-RFLP patterns of representative *F. columnare* isolates following digestion with *Hae* III. Lane M: Marker; lane 1-14: Isolate NS01 to NS12; SCF01 to SCF06; lane 15 and 16 were internal control CUVET1201 (genomovar II) CUVET1215 (genomovar I) respectively.

Phylogenetic analysis

A proportion of 16S rRNA gene sequences (~1350 bp) were obtained from all 21 new isolates of *F. columnare* isolated from Nile tilapia (designated NT, NS) after assembly. The BLAST results showed 98% similarity with *F. columnare* ATCC 49512 and ~100% similarity to isolates from red tilapia in our previous studies. The 16S rRNA-based phylogenetic tree generated three clusters. The first cluster (Genomovar I & I/II) contained isolates of genomovar I and one isolate of genomovar I/II (*F. columnare* F10-HK-A). The second cluster (Genomovar II & II-B) is the dominant cluster which contained majority of genomovar II isolates and one isolate of genomovar II-B. This cluster was sub-divided into two sub-clusters namely “Tilapia sub-cluster” and “Catfish sub-cluster” (Fig. 4), which was supported by boostrap 67 (Fig. 4). The “Tilapia sub-cluster” is an unique cluster which formed by only isolates recovering from diseased Nile tilapia and red tilapia. Wherease “Catfish sub-cluster” formed by majority of isolates recovering from striped catfish (*P. hyphophthalmus*) and channel catfish (*Ictalurus punctatus*), few genmomovar II isolates from other host and one isolate of genomovar II-B (*F. columnare* PT-14-00-151). The third cluster

(Genomovar III) formed by previously published genomovar III isolates and one isolates of genomovar I (CUVET1215).

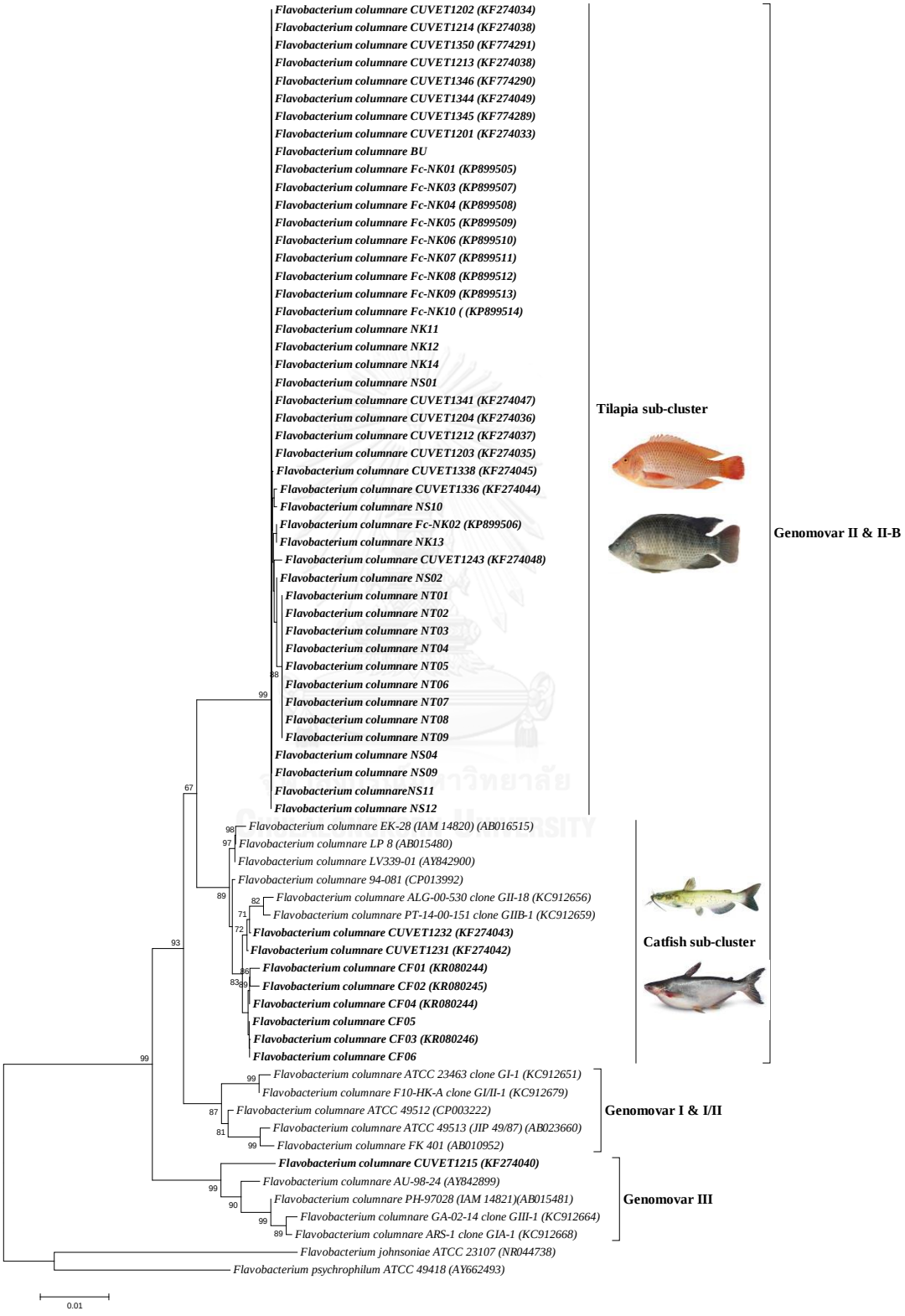

Figure 4 Phylogenetic tree based on 16S-rRNA of *F. columnare* isolates. *F. johnsoniae* ATCC 23107 and *F. psychrophilum* ATCC 49418 were used as outgroup. Bold codes indicated isolates of *F. columnare* from our laboratory collection.

Discussion

Occurrence of columnaris disease caused by *F. columnare* in commercial farms of red tilapia, Nile tilapia and striped catfish was recently reported in Thailand (Dong et al., 2015a; Dong et al., 2015b; Dong et al., 2015c). Genetic diversity of *F. columnare* was previously described. However, the majority of isolates were isolated from diseased red tilapia and only one isolate from Nile tilapia (BU) and two isolates from striped catfish (CUVET1231, CUVET1232) was previously included. In continuing previous work, greater number of isolates originated from Nile tilapia and striped catfish was first assigned genomovar and included for phylogenetic analysis in this study. The result is consistent with previous studies that the majority of *F. columnare* recovering from warm water fish in Asia belongs to genomovar II (Schneck and Caslake, 2006; Olivares-Fuster et al., 2007a). It is noted that the universal primers UN20/20F & R1438 used for 16S rRNA amplification in this study were obtained from (Darwish and Ismaiel, 2005) since amplification failed to yield expected amplicon by using UN20/20F & R1500 which were suggested by (Triyanto and Wakabayashi, 1999; LaFrentz et al., 2014). Similarly, Darwish and Ismaiel (2005) experienced the same problem with few isolates for *F. columnare*. This suggests that the primers used for standard method (16S-RFLP) should be further amended.

Dong et al. (2015a) revealed that *F. columnare* isolated from red tilapia exhibited ~98% 16S rRNA similarity to the reference strain *F. columnare* ATCC 49512 and formed a novel sub-cluster in the main cluster that formed by genomovar II isolates. Greater number of genomovar II isolates recovered from Nile tilapia and striped catfish were used for genetic comparison in this study. Interestingly, all isolates from Nile tilapia and red tilapia were phylogenetially identical except the isolate CUVET1215. Six Thai striped catfish isolates (CF1 to CF6) were phylogenetically identical with two Vietnamese striped catfish isolates (CUVET1231 and CUVET1232), four isolates of American channel catfish (ALG-00-503, PT-14-00-

151, 94-081 and LV-339-01) and two isolates of other fish species (LP 8 and EK 28). Taken together, two sub-clusters “Tilapia sub-cluster” and “Catfish sub-cluster” within Genomovar II & II-B cluster were proposed based on host origin of majority of isolates. This suggests that 16S rRNA might be a good gene marker for differentiation isolates of *F. columnare* originated from tilapia. Since 16S rRNA of *F. columnare* isolates from tilapia exhibited quite low similarity (98%) to the reference strain *F. columnare* ATCC 49512 and phylogenically unique, this suggests that these isolate probably should be separated into a new subspecies. Further analyses such as DNA-DNA hybridization, acid fat profile should be performed to support for taxonomic emendation.

Acknowledgements

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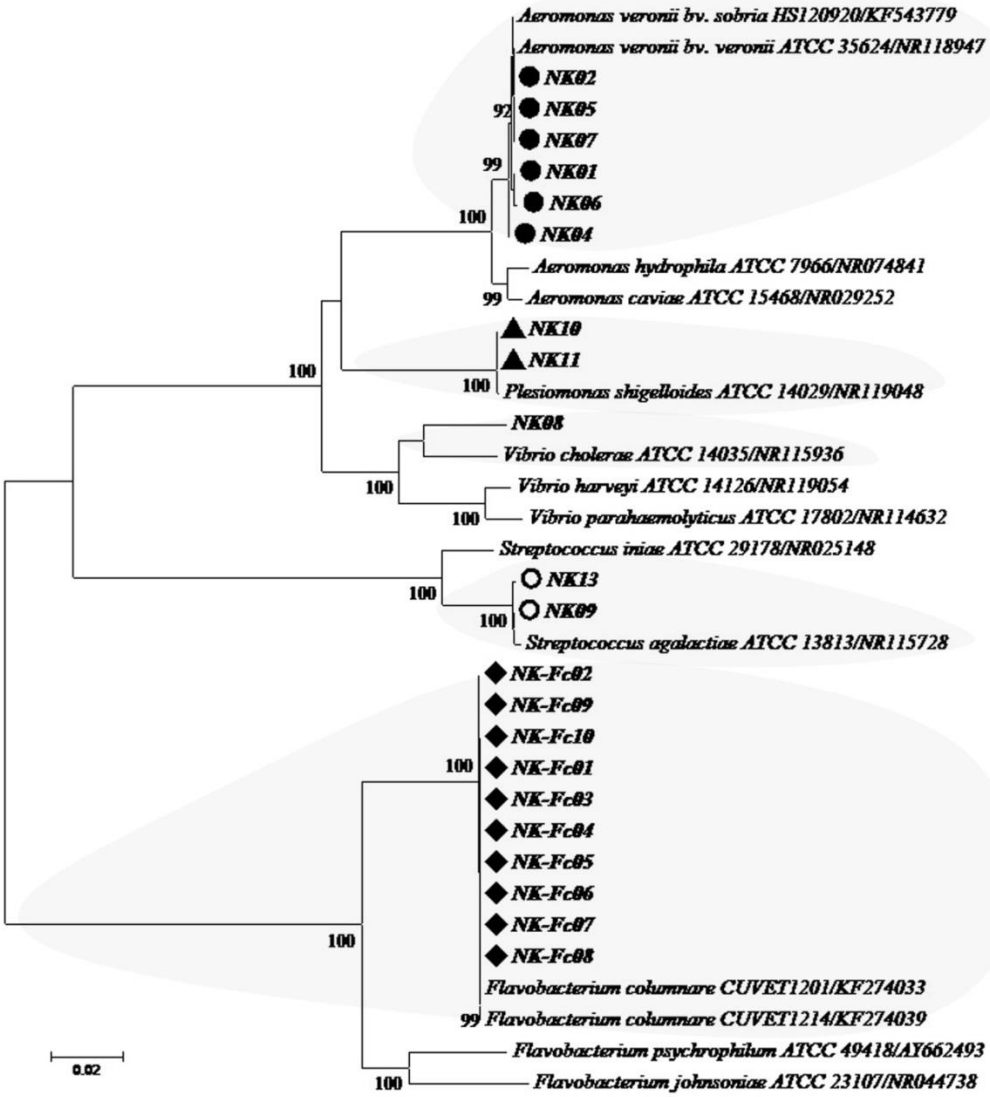
APPENDIX

Supplemental data of Chapter II

Supplemental Table 1 Detail of experimental challenge assays.

Treatment group	Number of fish	Bacteria administered	Infection route	Challenge dose
1	10	*A. veronii* NK01	I.P. injection	1.36×10^7 CFUs $fish^{-1}$
2	10	*A. veronii* NK06	I.P. injection	0.82×10^7 CFUs $fish^{-1}$
3	10	*S. agalactiae* NK13	I.P. injection	2.30×10^8 CFUs $fish^{-1}$
4	10	*P. shigeloides* NK11	I.P. injection	1×10^7 CFUs $fish^{-1}$
5	10	*F. columnare* NK-Fc01	Intramuscular injection	1×10^7 CFUs $fish^{-1}$
6	10	TSB (Control)	I.P. injection	0.1 mL sterile TSB

CFUs, colony forming units; I.P., intraperitoneal; TSB, tryptic soy broth

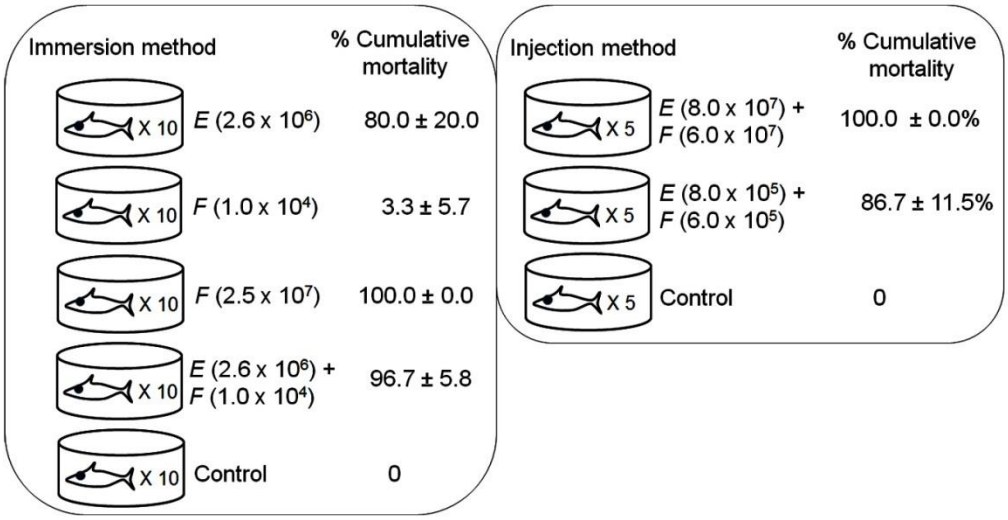


Supplemental Figure 1 Phylogenetic tree based on 16S rRNA of bacterial isolates in this study and their closest taxa. Percentage bootstrap values (1000 replicates) are shown at each branch point.

5'GTGTGGCTGCGTGTTAAGATCCCCTCCATCACATCCAGCAAGGAGAACAGCT
ACATCCGCTGGTGCGACAATCTGATGCACAATCTAGTGGAGGAGGTGTCGGTG
TCA3'

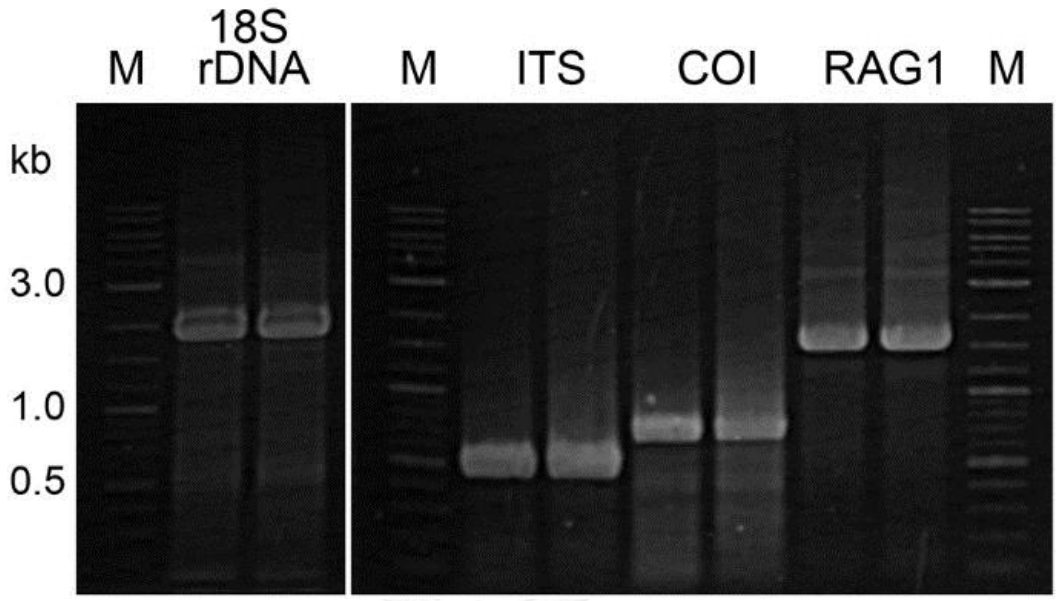
Supplemental Figure 2 Nucleotide sequence of iridovirus obtained from the 108-bp semi-nested PCR amplicon. The underlined text indicates primer binding sites.

Supplemental data of Chapter III



Supplemental Figure 1 Experimental plans for bacterial challenge tests of striped catfish, *P. hypophthalmus* with *E. ictaluti* T1-1 (*E*) and/or *F. columnare* CF1 (*F*) isolates. Number in brackets represent dose of bacterial cells (details in text). Each treatment was performed in triplicates. Cumulative percent mortality during a 20-day experimental assay is present in each treatment.

Supplemental Figure 2 Naturally diseased fish (A, B) and experimentally diseased fish (C, D) exhibited clinical signs of columnaris disease (A, C) and typically multifocal white spots of edwardsiellosis in the head kidney, liver, and spleen (B, D). Arrows mark typical lesions of diseases.



Supplemental Figure 3 Amplification of DNA fragments from a naturally infected fish sample. Primers specific for eukaryotic 18S rDNA, ITS (internal transcribed spacer), COI (cytochrome c oxidase I), and RAG1 (recombinase activating gene 1) were employed in the PCR reactions. Each amplified products were loaded into two wells. M, DNA marker (2-log ladder, New England Biolabs).

Anacker and Ordal's 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	12 g
Distilled water	1000 ml

Preservative formula for *F. columnare*

Sterile glycerol 60%	100 ml
Bovine serum	200 ml
Bacterial culture in Cytophaga broth	700 ml

TBE electrophoresis buffer (10X)

Tris base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 ml
RNase-free H_2O	1000 ml

Prepare with RNase-free H_2O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 month at room temperature

In situ hybridization protocol

A. Preparation of tissue sections

1. Embed the fixed tissue in paraffin.

2. Cut 5 μm section from the paraffin-embedded material and place the section onto coated glass slides (commercial, positively charged microscope slides are recommended.)

3. Place the slide on their long edge on tissue paper in an overnight oven at 45-50°C or 30 min at 60°C.

4. Dewax section 3-5 min with fresh xylene.

5. Rehydrate the tissue as follows:

2-3 min in 100% Ethanol

2-3 min in 95% Ethanol

1-3 min in 80% Ethanol

1-3 min in 70% Ethanol

1-3 min in 50% Ethanol

1-3 min in Distilled water

1-3 min in TNE

6. Prepare fresh working Proteinase K 20 μg/ml in 1x TNE buffer. Treat each section with 500 μl of working Proteinase K solution and incubate for 30 min at 37°C in a humid chamber.

7. After the treatment with Proteinase K, fix the section with ice cold 0.4% formaldehyde solution for 5 min.

8. Immerse the section in 2XSSC for 5 min. Then, blot off the excess solution.

9. Add section in 0.5 EDTA pH 8.0 for 1 hr.

10. Immerse the section 2x5 min in 2XSSC.Then, blot off the excess solution.

11. Incubate section at 37°C for at least 10 min with pre-hybridization buffer (prepare for 10ml add 5ml formamide + 2ml 20XSSC + 3ml DEPC)

B. In situ hybridization step

12. Prepare hybridization buffer in a tube as follows.

250 µl 50% deionized formamide

50 µl (w/v) dextran sulfate 50%

10 µl 50x Denhardt's solution (Sigma)

100 µl 20X SSC

12.5 µl 10 mg/ml salmon sperm DNA (Invitrogen)

Distilled water to make a total volume of 500 µl

13. Add approximately 5-10 µl of DIG-labeled probe into the hybridization buffer and mix by vortexing.

14. Denature the probe by boiling at 95°C for 10 min followed by immediate chilling on ice for at least 2 min.

15. Carefully remove the pre-hybridization buffer from the sections and add 100µl of hybridization buffer containing the DIG-labeled probe.

Note: prepare a negative control by covering one section with "blind" probe, i.e. no the DIG-labeled probe in the hybridization buffer.

16. Cover each section with a separate cover slip and incubate at 42°C overnight in a humid chamber.

C. Post-hybridization

17. Remove cover slips from section by immersing slides for 10 min in 2x SSC at RT.

Caution: Do not place samples that are hybridized with different probe in the same container.

18. Wash the section in a water bath as follows:

1st wash for 15 min at 37°C with 2X SSC

2nd wash for 15 min at 42°C with 1XSSC

3rd wash for 15 min at 42°C with 0.1X SSC

D. Immunological detection

19. Wash the section in 1x buffer I for 5 min at RT.

20. Incubate each tissue sections with buffer II (blocking solution) for 30 min at RT.

21. Decant buffer II from the slides and incubate sections for 1 h at RT in a humid chamber with 300 μl of the antibody conjugate (anti-DIG-alkaline phosphatase) 1:1000 in buffer II

22. Immerse sections 2x10min with buffer I at RT.

23. Incubate sections in buffer III for 5 min at RT.

24. Prepare a color development solution containing 200 μl of NBT/BCIP Stock Solution (Roche) to 10 ml of buffer III. Always prepare this solution fresh, immediately before use.

25. Add to each section approximately 300 μl of color development solution and incubate slides in a humid chamber for 1-2 h at 37°C. Then, incubate at RT overnight in the dark.

Note: NBT/BCIP produces a blue precipitate product. A positive color reaction may be seen after a few minutes in the dark but it is possible to incubate for up to 24 h.

26. When color development is optimal, stop the reaction by washing slides in distilled water for 3x5 min.

27. Counterstain sections for in 0.5% Bismarck brown for 2 min.

28. Wash sections in running tap water for 3 min.

29. Dry the sections or Dehydrate section;

1st 1 min in 95% Ethanol

2nd 1 min in 100% Ethanol

3rd 2 min in Xylene

30. Mount by using an aqueous mounting solution.

31. View under the light microscope.

Reagents

10x Tris-NaCl-EDTA (TNE)

500mM Tris-Cl	60.57 g
100mM NaCl	5.84 g
10mM EDTA	3.72 g
Distilled water	990 ml

Adjust pH to 7.4; autoclave to sterilize; store at RT.

Proteinase K

Prepare a stock solution of Proteinase K 5 mg/ml in H2O

Proteinase K 50 mg

Autoclave ddH2O 10 ml

Keep frozen at -20C.

Working solution of Proteinase K (20µg/ml)

Stock solution of Proteinase K (5 mg/ml) 20 µl

1x TNE 5 ml

Prepare just prior to use.

0.4% Formaldehyde Solution

40% Formaldehyde solution	5 ml
Autoclaved ddH2O	495 ml

Store at 4°C for up to 2 months

Pre-hybridization Buffer (4xSSC + 50% deionized formamide)

100% deionized formamide	5 ml
20xSSC	2 ml
DEPC	3 ml

Hybridization Buffer (50% deionized formamide + 5% w/v Dextran sulfate + 1x Denhardt's solution + 4xSSC + 0.25 mg/ml salmon sperm DNA)**.**

100% deionized formamide	250 µl
50% Dextran sulfate (w/v)	50 µl
50x Denhardt's solution (Sigma)	10 µl
20xSSC	100 µl
10 mg/ml salmon sperm DNA (Invitrogen)	12.5µl
Distilled water to make a total volume of 500 µl	77.5 µl

Hybridization buffer minus salmon sperm DNA can be stored at -20°C for several months.

50x Denhardt's solution (commercially available from Sigma)

One vial contains 50 mg BSA, 50 mg ficoll and 50 mg PVP; reconstitution with 5 ml of autoclaved ddH2O yields a 50x concentrate; store at -20°C.

50% Dextran sulfate

Dextran sulfate	50 g
Autoclaved ddH_2O	100 ml

Dissolve dextran sulfate in water, heat on "low" to facilitate mixing, dispense 10 ml per tube and store frozen at -20°C.

Salmon Sperm DNA (10 mg/ml)

Salmon Sperm DNA	500 mg
Autoclave ddH2O	50 ml

Add DNA slowly to water while stirring and heating on "low"; qs to 50 ml. Shear DNA by passing through an 18 gauge needle several times. Autoclave to sterilize and to further denature the DNA, dispense 1 ml in small tubes and store at -20°C.

20xSSC (Sodium chloride/ Sodium citrate) Buffer

3M NaCl	175.32 g
0.3M Na citrate	88.23 g
Distilled water	900 ml

Adjust pH to 7.0; autoclave to sterilize; store at RT.

To make 2xSSC, dilute 100 ml of 20xSSC in 900 ml autoclaved ddH_2O; store at RT.

To make 1xSSC, dilute 50 ml of 20xSSC in 950 ml autoclaved ddH_2O; store at RT.

To make 0.1xSSC, dilute 5 ml of 20xSSC in 995 ml autoclaved ddH_2O; store at RT.

10x Buffer I

1M Tris-HCl	121.14 g
1.5M NaCl	87.66 g
Distilled water	900 ml

Adjust pH to 7.5; autoclave to sterilize; store at RT.

To make 1x Buffer I, dilute 100 ml of 10x stock solution in 900 ml autoclaved ddH2O; store at RT.

Buffer II (0.5% Blocking solution)

Blocking agent (Roche)	0.5 g
1x Buffer I	100 ml

Buffer III

100 mM Tris-HCl	12.114 g
100 mM NaCl	5.844 g
50 mM MgCl2·6H2O	10.16 g
Distilled water	990 ml

Adjust pH to 9.5; autoclave to sterilize; store at RT.

0.5% Bismarck Brown Y

Bismarck Brown Y	1 g
Distilled water	200 ml

Stir until completely dissolved; filter through Whatman#1 filter paper; store at RT.

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