ปัจจัยความรุนแรงและความสามารถในการก่อโรคของเชื้อฟลาโวแบคทีเรียม คอลัมแนร์ที่แยกได้จากปลานิลแดงในประเทศไทย

นายเล ดิห์ ฮาย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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VIRULENCE PROPERTIES AND PATHOGENICITY OF *FLAVOBACTERIUM COLUMNARE* ISOLATES RECOVERED FROM DISEASED RED TILAPIA (*OREOCHROMIS* SP.) IN THAILAND

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ฟลาโวแบคทีเรียม คอลัมแนร์เป็นสาเหตุของโรคคอลัมนาริสที่ก่อให้เกิดโรคในปลาน้ำจืดเพาะเลี้ยงหลายชนิดและก่อให้เกิดความสูญเสียทางเศรษฐกิจขึ้นทั่วโลก ปัจจุบันมีการศึกษาที่ศึกษาวิเคราะห์พยาธิกำเนิดของโรคคอลัมนาริส แต่อย่างไรก็ตามบทสรุปในเรื่องของปัจจัยในการก่อความรุนแรงของเชื้อนี้และพยาธิกำเนิดของโรคคอลัมนาริสยังคงไม่เป็นที่ทราบแน่ชัด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความรุนแรงและคุณสมบัติของปัจจัยก่อความรุนแรงต่างๆ ของเชื้อ เอฟ คอมลัมแนร์ ที่แยกได้จากปลานิลแดง ไพรเมอร์จำนวน 6 คู่ได้รับการออกแบบสำหรับตรวจสอบยีนก่อความรุนแรงเป้าหมายทั้ง 6 ยีนด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส (PCR) ผลการวิเคราะห์ด้วย PCR จากตัวอย่างเชื้อเอฟ คอลัมแนร์ 51 ตัวอย่างแสดงถึงกลุ่มของยีนก่อความรุนแรง 2 กลุ่มที่แตกต่างกัน เมื่อทำการทดสอบความรุนแรงของเชื้อโดยการแช่พบว่าความแตกต่างของกลุ่มของยีนก่อความรุนแรงนั้นไม่มีความเกี่ยวข้องกับความรุนแรงในการก่อโรคในปลานิลแดง ค่าลีทอลโดส ฟิฟตี้ (LD_{50}) ของเชื้อเอฟ คอลัมแนร์กลุ่มที่มีความรุนแรงสูง กลาง และต่ำ มีค่าเท่ากับ 6.10 lg_{10}CFU, 6.65 lg_{10}CFU และมากกว่า 8 lg_{10} CFU ml^{-1} มิลลิลิตรของน้ำที่ใช้เลี้ยงปลาตามลำดับ ซึ่งพบว่าปัจจัยการก่อความรุนแรงของเชื้อ เช่น การสร้างไบโอฟิล์ม (biofilm production) ความสามารถในการเกาะติดกับเนื้อเยื่อ (adherent ability) และการสร้างแคปซูล (capsule production) ของเชื้อเอฟ คอลัมแนร์มีความเกี่ยวข้องอย่างมีนัยสำคัญกับความรุนแรงในการก่อโรค แต่ปฏิกิริยาโปรติโอไลติค (proteolytic activity) ไม่มีความเกี่ยวข้องกับความรุนแรงในการก่อโรคของเชื้อ จากผลการทดลองสามารถสรุปได้ว่าการเกาะติดกับเนื้อเยื่อ การสร้างไบโอฟิล์ม และความหนาของโพลีแซคคาไรด์แคปซูลของเชื้อ เป็นคุณสมบัติที่มีบทบาทสำคัญในการก่อความรุนแรงของเชื้อเอฟ คอลัมแนร์ในปลานิลแดง

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LE DINH HAI: VIRULENCE PROPERTIES AND PATHOGENICITY OF *FLAVOBACTERIUM COLUMNARE* ISOLATES RECOVERED FROM DISEASED RED TILAPIA (*OREOCHROMIS* SP.) IN THAILAND. ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, CO-ADVISOR: ASSOC. PROF. DR. NOPADON PIRARAT, 65 pp.

The causative agent of columnarsis disease, *Flavobacterium columnare* is a bacterial pathogen affecting many wild and farmed freshwater fishes, and subsequently resulting in significant economic losses globally. To date, various studies have been elucidated the pathogenesis of columnaris disease. However, comprehension of virulence factors and pathogenesis of *F. columnare* are still unclear. The objective of this study was to investigate pathogenicity of *F. columnare* isolates recovered from diseased red tilapia and some of their virulence-associated properties. Six specific primer pairs targeting each particular putative virulence gene were designed. The PCR results showed two distinct putative virulence gene profiles among 51 *F. columnare* isolates. However, it seems to be unrelated with pathogenicity in red tilapia. The LD_{50} of high, moderate and low virulence isolates were 6.10 $\log_{10}$CFU, 6.65 $\log_{10}$CFU and more than 8 $\log_{10}$ CFU ml^{-1} of rearing water, respectively. The biofilm formation and adherent ability were significantly correlated with the virulence of *F. columnare*. The protease activity of *F. columnare* on skim milk agar was strong, however, lack of association with the levels of pathogenicity. Besides, high and moderate virulent isolates showed the high thickness of polysaccharide capsule when compare with low virulence isolate. In conclusion, adherences and thickness of polysaccharide capsule might play an important role in *F. columnare* virulence and infection in red tilapia.

Department:	Veterinary Pathology	Student's Signature .....
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List of Abbreviations

F. columnare	*Flavobacterium columnare*
%	Percentage
µl	Microliter
µm	Micrometer
ANOVA	Analysis of variance
AO	Anacker and Ordal
BLAST	The Basic Local Alignment Search Tool
bp	Base pair =3.4 Å
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
ECPs	Extracellular products
FCGM	*Flavobacterium columnare* Growth Medium
HV	High virulence
ISR	Intragenic spacer region
LD_{50}	Median lethal dose
LV	Low virulence
ml	Milliliter
MV	Moderate virulence
ºC	Degree Celsius
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate-bufferred saline
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
RFLP	Fragment length polymorphism
rpm	Rounds per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Chapter I. Introduction

1.1. Importance and rationale

In recent years, culture of tilapia has been rapidly increasing worldwide, including in Thailand, the production of tilapia have been contributing to increasing economic value. Unfortunately, the cultural tilapia has been addressing several severe diseases such as parasitic, fungal, viral and bacterial diseases (Plumb and Hanson, 2011). During a considerable period of many last years, many related bacterial diseases and numerous kinds of bacterial pathogens have been characterized as major aquatic pathogens, for instance *Streptococcus* sp., *Flavobacterium* sp., *Edwardsiella* sp., and *Aeromonas* sp. One of the most prime aquatic pathogen in culture freshwater species is *Flavobacterium columnare (F. columnare)* bacterium, the etiologic agent of columnaris disease that is inducible for significant economic losses in aquaculture worldwide (Woo et al., 2011; Declercq et al., 2013)

F. columnare, a non-flagella, Gram negative bacillus bacterium, has been regarded for several nomenclatures, such as *Bacillus columnaris* (Davis, 1922), *Chondrococcus columnaris* (Ordal and Rucker, 1944) and *Cytophaga columnaris* (Garnjobst, 1945) and *Flexibacter columnaris* (Dernardet and Grimont, 1989). This organism is the causative agent of columnaris disease, one of the most important diseases in freshwater fish, which includes with wild and cultured host species (Woo et al., 2011; Declercq et al., 2013). The pathological changes associated with this disease are usually restricted to the external lesions such as the skin damages, gill necrosis and fin rot. Mortality could be fluctuating from 10% to 90% due to *F. columnare* strain (Davis, 1922; Tripathi et al., 2005; Dong et al., 2014). Although columnaris disease was discovered in 1922, during many last decades, various researches have been implemented; characterization of phenotype and genotype of *F. columnare* was comprehend (Lafrentz et al., 2013; Dong et al., 2014). However, many questions related to pathogenesis and virulence factors of this bacterium are still being unanswered.

In recent years, some researches demonstrated that different *F. columnare* isolates exhibited different levels of pathogenicity in (Shoemaker et al., 2008; Shoemaker and LaFrentz, 2014). A number of putative virulence properties such as colony morphotype, adhesion ability, gliding motility, polysaccharide capsule and some extracellular products have been proposed as the important virulence properties in the development of diseases in fish (Suomalainen et al., 2006; Klesius et al., 2010; Declercq et al., 2013).

Among many virulence properties, bacterial adherence to the host surface is the critical incident defining the success of the infection in the primary phase of pathogenesis (Decostere and Haesebrouck, 1999; Bader et al., 2005). It was reported that high virulent *F. columnare* strains were able to adhere superiorly to gill tissue compared with the low virulent strains (Decostere et al., 1999b). Some factors contributing to the adherent capacity of bacteria have been identified such as gliding motility, outer membrane vesicle and capsule formation (Decostere and Haesebrouck, 1999; Kunttu et al., 2011; Laanto et al., 2014).

Similar to other bacteria in phylum Bacteroidetes, *F. columnare* does not possess pili or flagella. Anyhow, this pathogenic organism can move rapidly on the surface based on gliding motility factors. Some gliding motility-related proteins have been identified in *F. johnsoniae,* such as GldA, GldF, GldG, GldH, GldI, GldJ, GldK, GldL, GldM, and GldN (Braun et al., 2005) and it is hypothesized that the mechanism of *F. columnare* motility would be similar. In addition, previous studies found that the flavobacterial gliding motility machinery was related to the newly discovered protein secretion system known as the type IX (McBride and Zhu, 2013). In *F. columnare* pathogens, three genes, including *gldB, gldC* and *gldH,* have been identified, and it was shown that the expression of *gldH* gene was significantly up-regulated after the exposure of bacteria to catfish skin mucus (Klesius et al., 2010).

After attaching to the host, *F. columnare* secrete some extracellular enzymatic products, which contributed to the host-cell interaction. Out of many kinds of enzyme, the chondroitin AC lyase produced by *F. columnare* has been documented in some previous researches (Kitamikado and Lee, 1975; Xie et al., 2005; Suomalainen et al., 2006; Kunttu et al., 2011). Enzyme chondroitin AC lyase degrade

chondroitin sulfates A and C and hyaluronic acid, was demonstrated an important virulence factor of *F. columnare* that implicated to necrotic lesion in high temperature (Dalsgaard, 1993; Suomalainen et al., 2006; Kunttu et al., 2011). In addition, protease products of *F. columnare* have been considered as a virulence factor (Staroscik and Nelson, 2008; Kunttu et al., 2011). Betolini and Rohovec (1992) have found that different *F. columnare* isolates produce protease, which digested protein, is regarded as prime a virulence factor related to necrotic tissues. Besides, the membrane – associated zins metalloprotease contributing to bacterial pathogenesis (Häse and Finkelstein, 1993) have been found in the high virulence of *F. columnare* strain (Xie et al., 2004).

More recently, Laanto et al. (2014) found that OmpA family outer membrane protein P60 was produced in a large amount by *F. columnare* rhizoid colony morphotypes that often show high levels of pathogenicity, whereas it was not or rarely seen on the cell of the soft colony morphotype. Therefore, OmpA family outer membrane protein was regarded an as virulence property of *F. columnare*.

F. columnare is considered an important pathogen in tilapia, the ability of *F. columnare* to cause disease depends on the degree of the expression of virulence factors (Plumb and Hanson, 2011). However, comprehension of pathogenesis of columnaris disease and functional virulence factors of *F. columnare* is limited and unclear. The aim of this study is to determine the relationship between the pathogenicity and *in vitro* virulence-related properties of *F. columnare.*

1.2. Research questions

- Are there any relationships among the virulence gene profiles of *F. columnare* and their levels of pathogenicity in red tilapia?

- What are the differences in virulence properties among high, moderate and low levels of pathogenicity of *F. columnare* isolates?

1.3. Hypothesis

- The differences in putative virulence gene profiles are associated with different levels of pathogenicity among *F. columnare* isolates.

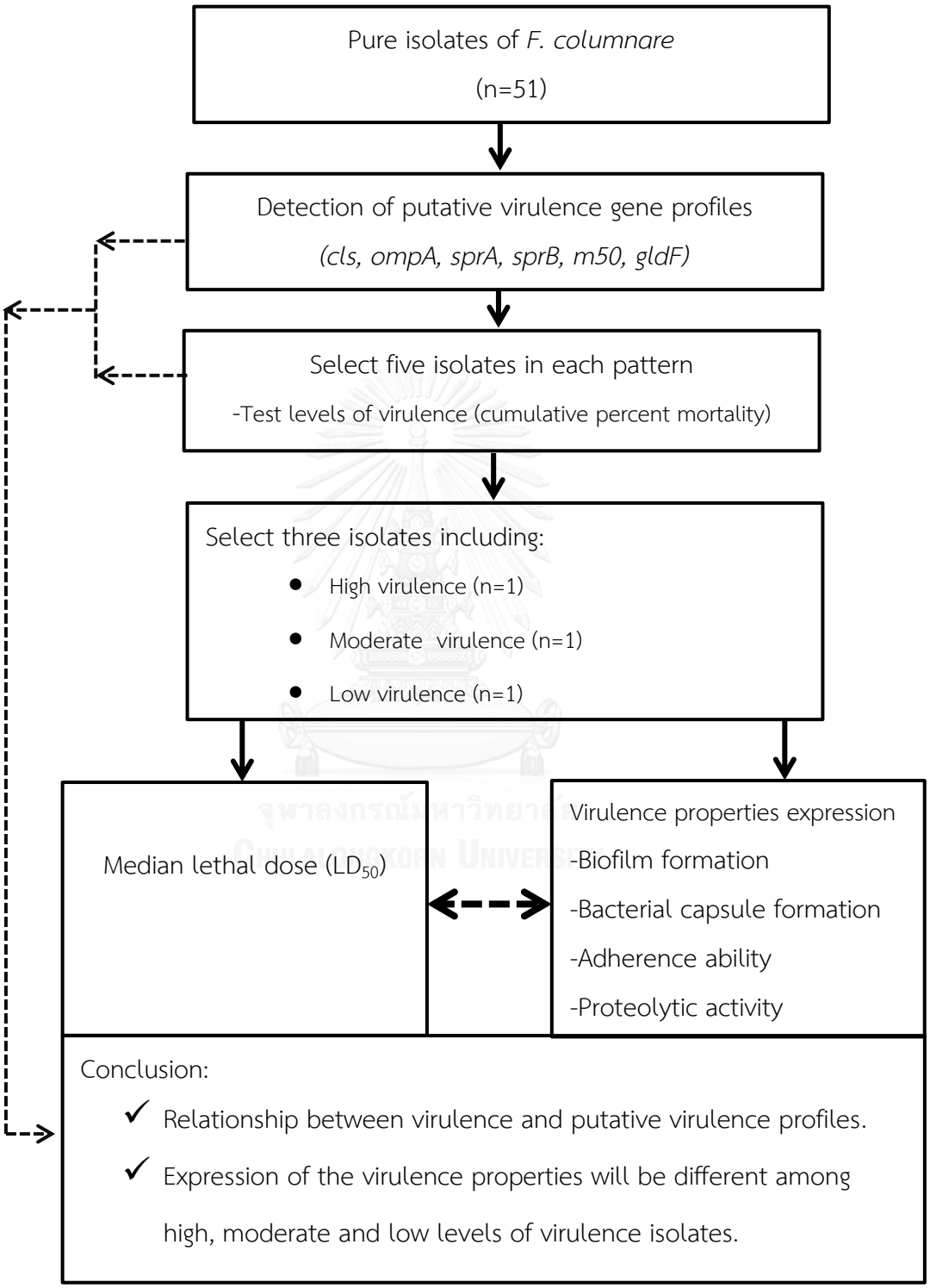
- The expression of several virulence properties can be different among high, moderate and low levels of pathogenicity of *F. columnare* isolates.

1.4. Objectives

- To evaluate the association between the putative virulence gene profiles and levels of pathogenicity among *F. columnare* isolates.

- To investigate the expression of several virulence properties among high, moderate and low levels of pathogenicity of *F. columnare* isolates.

1.5. Conceptual framework



Chapter II. Literature review

2.1. History, taxonomy, genotype and phenotype of *F. columnare*

F. columnare, the etiological agent of columnaris disease was first described by Davis in 1922. It belongs to the family of *Flavobacteriaceae*, in the phylum of *Bacteroidetes*. Over the years, the name of this pathogen and its taxonomy have been changed several times such as *Bacillus columnaris* (Davis, 1922), *Chondrococcus columnaris* (Ordal and Rucker, 1944) and *Cytophaga columnaris* (Garnjobst, 1945). More recently, in 1996, by molecular techniques, this bacterium was transferred to the genus *Flavobacterium* and this bacterium was named as *F. columnare* (Bernardet et al., 1996).

In 1999, based on differences in 16S rRNA sequences, fragment length polymorphism (RFLP) and DNA-DNA- hybridization *F. columnare* was genotyped in three different genomovars involving I, II and III (Riyanto and Hisatsugu Wakabayashi, 1999). Olivares-Fuster et al. (2007) have found coexistence of two subgroups in genomovar I and II, which were listed as genomovar I-B and genomovar II-B. LaFrentz et al. (2014) demonstrated a new gemomovar as I/II, by RFLP assay. Therefore, until now coexistence of five genomovars in various *F. columnare* isolates have been found including I, II, II-B, III and I/II (Lafrentz et al., 2013). *F. columnare* isolates recovered from diseased red tilapia in Thailand were presented in genomovar I and II, but predominate in genomovar II (Dong et al., 2014). In 2012, the completion genome of *F. columnare* ATCC49512 was performed and published, this will help to understand more clearly about this bacterium (Tekedar et al., 2012).

F. columnare is described as an aerobic, Gram negative, long, slender, non-flagellated bacillus that measured about 0.5μm in width and 5 μm to 12 μm in length. Colonies of *F. columnare* on cytophaga agar have different forms such as rhizoid, rough and soft morphotypes. The color of *F. columnare* colony is various from pale yellow, greenish, yellow to golden yellow. G + C content is about 32 % (Griffin, 1992). This bacterium can grow at temperatures from 4 to 37°C but optimized temperature was 28°C. The physiological characteristics of *F. columnare* are described as strict aerobic growth; reduces nitrate to nitrite; producing catalase,

creating H_2S, hydrolyzing gelatin, and no growth on medium with more than 1% NaCl. They produced no acid from carbohydrates; oxidase positive; they can hydrolyze gelatin, casein, and tyrosine. The capsule of *F. columnare* was related to their virulence. In general, capsule of high virulence isolates were thicker than low virulence (Dernardet and Grimont, 1989; Decostere and Haesebrouck, 1999; Dong et al., 2014).

2.2. Epidemiology

F. columnare, etiologic columnaris disease is distributed in broad geographic in the worldwide with an extensive host range. Up to date, at least thirty six fish species have been found to be susceptible to *F. columnare* in both wild and cultured freshwater fish, cold and warm water, such as catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), tilapia (*Oreochronmis* sp), bass (*Micropterus salmoides*), bluegill *(Lepomis macrochirus*), and crappie (*Pomoxis nigromaculatus*) and numerous other fish (Decostere et al., 1998; Figueiredo et al., 2005; Klesius et al., 2008).

2.3. Pathogenesis of columnaris disease and bacterial virulence factors

2.3.1. Bacterial pathogenicity

The different isolates of *F. columnare* have exhibited various levels of pathogenicity from acute to chronic course, which depend on the age and immune state of the host and virulence of bacteria (Decostere et al., 1999b; Morley and Lewis, 2010; Declercq et al., 2013). Base on bacterial pathogenicity, *F. columnare* isolates were grouped into three terms “high virulence”, “moderated virulence” and ‘low virulence’. The isolates induced more than 80% fish mortality within 72 hours post-challenge was listed in the high virulence pattern. The isolates showed a mortality of fish from 20% to 80% were considered as moderate virulence, while remainder isolates were assigned as low virulence (Declercq et al., 2015). The association between virulence and genomovar of *F. columnare* have revealed in some host species, it was shown that *F. columnare* genomovar I isolates were less virulent than genomovar II, for instance, in channel catfish, rainbow trout and zebrafish (Shoemaker et al., 2008; Li et al., 2010). However, more recently, by immersion challenge, Shoemaker and LaFrentz (2014) demonstrated that the

relationship between genomovars and virulence of bacteria in tilapia is still indistinct. By using PFGE-derived profiles, Soto et al. (2008) found that two major genetic patterns existed among 30 isolates of *F. columnare* with more than 60 % similarity. Their experiment in channel catfish showed significant different virulence between two genetic groups.

In culture condition, *F. columnare* can form different kinds of colony morphologies, particularly being rhizoid colony and non-rhizoid colony. Virulence of different bacterial morphology colonies was also investigated by some previous researches and it still is a controversial issue. A majority of studies believed that rhizoid colony morphology were more virulent than non-rhizoid colony mophotypes (Kunttu et al., 2011; Laanto et al., 2014). However, Kunttu et al. (2009) have demonstrated that the relationship between virulence and its colony morphology were unclear. Besides, the effect of temperature to the pathogenicity of *F. columnare* have reported, here high temperature influenced the transmission of columnaris disease (Suomalainen et al., 2005). On the other hand, the cultured circumstance also played important for increasing pathogen transmission at the fish farm have been demonstrated (Declercq et al., 2013)

2.3.2. Virulence factors of *F. columnare*

F. columnare are one of the most threats to develop aquaculture freshwater fish. As well as other bacteria, the capacity to cause disease depends on the levels of expression of virulence factors, which help *F. columnare* to colonize, evade host defenses and invade the host, finally result pathological influence. Thus, it is necessary to the study of bacterial virulence factors in order to develop the novel chemotherapeutic to against the bacterial infections. During the last decades together with the development of molecular techniques, many virulence factors of fish pathogens have been discovered. This allowed understanding well-known pathogenic mechanisms. *F. columnare* cause columnaris disease with external infections affecting the gills, causing fin rot and skin lesion. Some virulence factors have proposed, but function of them was unclear.

2.3.2.1. Adherent ability

Many virulence properties and processes participated to cause disease in the host by bacterial pathogens. After contacting the host, bacteria express some virulence factors achieve the host reaction. Among many virulence factors, adherence is a primary step in the host-pathogen interaction process that help bacteria bind to molecules of host tissue cells and overcome the reaction of host beginning of infection stage (Wilson et al., 2002).

F. columnare the causative agents related to external organs, hence, the ability of adhesion plays most important function for the successful colonization of bacteria in the host tissue. Previous studies have estimated the capacity of colonization of *F. columnare* to host tissues and showed that the pathogen can attach to both gill and skin tissues rapidly after exposing the host. Decostere and his colleagues have illustrated that the high pathogenicity strains were more adherent to the host than low virulence strains in black mollies *in vivo* and *in vitro* (Decostere et al., 1998; Decostere et al., 1999a; Decostere et al., 1999b). Virulence of *F. columnare* in fish was reduced by 75% in mutated adhesion strain when compared with the strain that still had adhesion capacity (Bader et al., 2005). Besides, adherent ability of *F. columnare* was significantly decreased when bacteria were incubated with d-glucose, N-acetyl-dglucosamine, d-galactose and d-sucrose or treating them with sodium metaperiodate (Decostere et al., 1999b). Adherence capacity has been determined was significantly related to temperature following high temperature promoted the adhesion of *F. columnare* (Kunttu et al., 2011). More recently Declercq et al. (2015) showed that the attached bacteria to gill tissue of high virulence seem much more efficiency than moderate and low virulence.

In opposite observation, it was believed that the adhesion capacity of *F. columnare* isolates was not related to their virulence clearly (Kunttu et al., 2009) Similar to that opinion, Olivares-Fuster et al. (2011) revealed that the adhesion ability to fish tissues is not enough to induce columnaris disease.

2.3.2.2. Gliding motility structural components

F. columnare cell, as well as other member bacterium of in the phylum Bacteroidetes does not process flagella or pili. However, they can move on the host surface based on some other factors as known gliding motility bacteria, instead motive ability of bacteria over surfaces depends on a factor known as gliding motility, which help the bacteria to spread and adhere to the host species (Sato et al., 2010). Gliding motility was a protein complex composed of ~20 various proteins, which were encoded by *gld* and *spr* genes. Some essential gliding motility proteins have been determined in *F. johnsoniae* such as GldA, GldF, GldG, GldH, GldI, GldJ, GldK, GldL, GldM, and GldN (Braun et al., 2005). Among them GldA, GldF, and GldG are ABC transporter permease protein (Agarwal et al., 1997; Hunnicutt et al., 2002). Besides, GldK, GldL, GldM, and SprA are responsible for secretion of the Adhesins SprB, one of cell surface gliding motility adhesions (Shrivastava et al., 2013).

Some research showed that there was a link between colony morphology, bacterial adhesion capacity and virulence of *F. columnare* (Decostere et al., 1999b; Kunttu et al., 2009; Kunttu et al., 2011). Laanto et al. (2012) also demonstrated that rough colony morphotypes was lost gliding ability and their virulence when compare with rhizoid colony. The relational the levels of gene transcription of some gliding motility gene (*gldB, gldC, gldH*) have been revealed by Klesius et al. (2010), the results of their study showed that it was significant unregulated the level of transcription of *gldH* gene after bacterium post-exposure to the catfish mucus at 5 minutes. However, no upregulation of *F. columnare* gliding motility genes were found when the bacterium was pretreated with D-mannose. Observation of Laanto et al. (2014) in rhizoid colony that showed high virulence revealed that numerous filaments on the cell surface but was rarely seen in rough colony. These filaments may connect to gliding motility of bacteria. Besides, in that study, one gliding protein GldF has been identified (Laanto et al., 2014). Gliding protein GldF was related to flavobacterial gliding motility need for the formation of spreading colonies on agar and for the secretion of SprB on the cell surface (Rhodes et al., 2011).

2.3.2.3. Outer membrane proteins (OMPs)

The important virulence function of outer membrane proteins has reported in many aquatic pathogens such as *Aeromonas hydrophila* (Khushiramani et al., 2012), *Vibrio* (Ben Abdallah et al., 2009), *Francisella* sp (Huntley et al., 2007). They are responsible to contribute the bacterial adhesion and against host defense. Besides, they participated in stimulating the host immune system. Therefore OMPs is also considered as good bacterial vaccine candidate substance (Austin and Austin, 2007).

In *F. columnare* pathogen OMPs alos have identified previously (Liu et al., 2008; Dumpala et al., 2010) however, the virulence function of them is unidentified. Laanto et al. (2014) have found a major number of OmpA-family outer membrane protein P60 in rhizoid colony that showed in high virulence strain but rarely in non-rhizoid colony strain. Although the association of virulence and OmpA-family outer membrane protein P60 was not distinct, however its function in adherent process was demonstrated. Indeed, OmpA has been documented as an antigenic target of the immune system of rainbow trout against *F. psychrophilum* (Dumetz et al., 2008). On the other hand, OmpA that encoded by *omp*A gene has been demonstrated as a potent virulence factor in several kinds of bacteria such as *Escherichia coli* (Nicholson et al., 2009), *Riemerella anatipestifer* (Hu et al., 2011) and *Vibrio* spp. (Emrah Altindis et al., 2014). Relationship between major OMPs and biofilm formation have been found in *E. coli*. It was demonstrated that during biofilm formation, the overexpression of gene encode major OMP was identified. Therefore, OmpA may play an important role in bacterial biofilm formation that help bacteria maintain adhesion and colonization (Orme et al., 2006).

2.2.3.4. Biofilm formation

Biofilms are the combination of several substances such as extracellular polysaccharides, proteins, DNA and lipids. They provide for their member cells several benefits including protection against the harsh environments, resistance to antibiotics and chemicals (Flemming and Wingender, 2010). Besides, biofilm formation was considered as virulence factors of bacteria. Some outbreaks of pathogens related to biofilm formation have been reported in *Listeria* sp., *Yersinia*

sp., *Campylobacter* sp., *Salmonella* sp., *Staphylococcus* sp. and *Escherichia coli* O157:H7 bacteria (Myszka and Czaczyk, 2011).

Biofilm formation of the fish pathogen *F. columnare* associated with its infectivity and the cause of columnaris disease in channel catfish was revealed. Cai et al. (2013) have reported that biofilm was formed after six hours post-inoculation. The role of bacterial biofilm formation was approved in channel catfish fingerlings. It was shown that high virulence was strong to form biofilm in chamber while it was weak in low virulence. Besides, the formation of biofilm was affected by some physicochemical parameters. In particular, temperature (21-25), pH (6.2-7.3), salinity (0.5ppt) and carbohydrates (mannose) were suitable to biofilm formation (Cai et al., 2013). Laanto et al. (2014) also have showed the structure of biofilm was different between virulent rhizoid colony and non-rhizoid colony. Rhizoid colony was great to form biofilm while non-rhizoid colony was weak.

2.2.3.5. Polysaccharide capsules

Polysaccharide capsules is a layer of polypeptides and polysaccharides that cover cell wall was found in many bacterial pathogens. It plays important in mediate interactions between the pathogen and its surroundings including the host. Therefore It i have been considered as important virulence properties for many bacterial pathogens (Reckseidler-Zenteno et al., 2005). A great number of virulence functions of capsule have been demonstrated in bacteria such adherence (mediating the interaction between the cell surface and host) protection of desiccation, especially, protection from specific and nonspecific host immune response and protection of antibiotic therapy (Bazaka et al., 2011).

Capsular formation was able to intensify adherence of pathogens, which may mediate to cell to cell and cell to host surface attachment, leading to form biofilm and persistence of bacteria during infection. (Kim et al., 1986). The ability of adhesion to fish cell line of bacteria with polysaccharide capsules has been reported. It showed that bacteria with capsule was strong to adhere and invade than those made by bacteria without capsule (Austin and Austin, 2007).

Polysaccharide capsule are responsible to resist specific and non-specific immunity defense of the host. In functional resistance of non-specific immunity, capsule formation becoming the barrier helps bacteria to escape from macrophage. The capsule can also combine with O antigen resist to complement-mediated killing. Besides, the capsule is considered as a fence between C3b on phagocyte and the C3b present on the bacterial cell surface. This is due to bacterial resist complement mediated phagocytosis initiated by an opsonic. In the role of resistance against specific immunity of the host defense, polysaccharide capsule is a similar structure of polysaccharides encountered on host tissue; therefore, they are weak to stimulate the antibody response in the host.

In *F. columnare*, capsule of high virulence is thicker than low virulence (Decostere et al., 1999b). Klesius et al. (2010) have found the thickness of capsule of *F. columnare* was reduced when the bacteria was treated by sodium metaperiodate (Klesius et al., 2010). It was hypothesized that sodium metaperiodate may inactivate or remove the lectin chemotactic receptor related to the capsule formation by deleting the C–C link between vicinal hydroxyl groups of sugar, therefore reducing the capsule of *F. columnare*. Besides, chemotactic responses of *F. columanare* to catfish skin mucus and adhere to gill tissue was inhibited when bacteria were treated by D-mannose, D-glucose and N-acetyl-D-galactosamine. Therefore, it was suggested that capsule formation together with carbon binding receptors were involved in adherent response of *F. columnare*.

2.2.3.6. Extracellular products (ECP)

In general, when bacterial pathogenic attachment onto host surfaces, they release enzymes to break down proteins of the host's extracellular matrix. Thus, they had the potential to degrade proteins and peptides that participate in a broad range of biological functions, including the infection process (Barrett, 1994). In *F. columnare*, some extracellular products have proposed as putative virulence factors of bacteria such as chondroitin AC (Suomalainen et al., 2006), proteases (Xie et al., 2004), collagenases (Li et al., 2010).

Chondroitin AC lyase

Chondroitin AC lyase is an enzyme encoded by *cls* gene that destroys chondroitin sulfates A and C and hyaluronic acid - a mucopolysaccahride in animal connective tissues. Therefore, chondroitin AC lyase helps bacteria to maintain infection in the host; it was also believed that chondroitin AC lyase maybe the cause of the saddleback regions present in the columnaris disease fish (Kitamikado and Lee, 1975; Jackson et al., 1991; Xie et al., 2004; Suomalainen et al., 2006; Klesius et al., 2008).

The function of chondroitin AC in *F. columnare* was significant correlation to virulence in high temperature. The activity of chondroitin AC lyase at 25°C was significantly better than 20°C (Suomalainen et al., 2006). Although Chondroitin AC lyase plays an important role in virulent bacterial pathogens, however, to damage to the host, it would be combined with other factors such as gliding motility, capsule formation, protease of the bacterium (Declercq et al., 2013).

Proteases

The role of extracellular protease has been studied and documented as an important virulence of many bacteria including in aquatic pathogenes. For instance, a mutation of gene encode protease in *A hydrophila*, virulence in rainbow trout was reduced approximately 100 times (Allan and Stevenson, 1981; Khalil and Mansour, 1997). In *F. columnare*, extracellular proteases were characterized by substrate SDS-PAGE and displays great similarity of proteases created by diverse isolates from a variety of fish species (Betolini and Rohovec, 1992). Activity of protease was increased in the presence of fish mucus and therefore it was suggested to have a correlation between the host-cell interaction and protease activity in *F. columnare* (Staroscik and Nelson, 2008).

In addition, the relationship between the functional extracellular zinc metalloprotease and protease have been demonstrated in *Burkholderia cepacia*. It was shown that *zmp*A mutant gene isolate that encode zinc metalloprotease was created less protease than the original isolates in *Burkholderia cepacia* bacterium (Corbett et al., 2003). The membrane-associated zinc metalloprotease gene was

determined in *F. columnare* with 449 amino acids in length that was encoded by *map* gene of 1800 bp in length (Xie et al., 2004).

Collagenase

Collagenases, the enzyme break the peptide bond in collagen (a component of the animal extracellular matrix) and destroy extracellular structures have been considered as virulence factor in bacterial pathogens (Ascencio et al., 2000; Han et al., 2008). It showed that collagenase plays an important virulence function in *Aeromonas veroinii*, virulence of them was reduced when collagenese associated genes was deleted (Han et al., 2008). Besider the virulent role of collagense have demostrated in *Vibrio hydrophila* (Ascencio and Wadstrom, 1998). In *F. columnare,* although the role of collagenolytic activity in pathogenesis was unidentified, however its function in bacterial virulence have been mentioned by Li et al. (2010).

2.2.3.7. Lipopolysachrides (LPS)

Lipopolysachrides (LPS) is complex molecules that find on the cell wall outer membrane. Its structure is composed of three substances, including "O antigen" that is an outer polysaccharide region, lipid A that is responsible for toxicity in Gram-negative bacteria. The virulent function of LPS have been demonstrated in some aquatic pathogens such as *Aeromonas*, *Edwardsiella* and *Vibrio* (Plumb and Hanson, 2011). Although virulent role of LPS in *F. columnare* was not identified clearly, however, some previous studies have performed and showed different LPS structures were shown in different levels of pathogenicity of this pathogen. In particular, different LPS structures have found in low and high virulence strains, which recovered from channel catfish. It was revealed that low virulence was absent from the he higher molecular bands in the LPS (Zhang et al., 2006). Kunttu et al. (2011) have compared LPS-protein profiles of various colony morphologies and found that it was an identical single LPS band. However, different colony strains showed different size of LPS even they have the same source.

2.2.3.8. Iron acquisition

Iron element is a necessary factor for almost bacteria in the process of its metabolic cellular pathways and the growth. In the host, iron reserved in two forms

ferrous iron—Fe^{2+} or Fe(II) and iron—Fe^{3+} or Fe(III) and they existed as compound iron binding proteins, for examples transferrin in serum and extracellular fluids, lactoferrin on mucosal surfaces, and ovotransferrin in eggs (Payne, 1994). However, it is not easy for pathogens to uptake for using, because of all most of them existed as compounds and there was a little or no free iron to support bacterial growth. During bacterial infection, they faced an extremely limitation of iron factor. To absorb iron, many bacteria can bind directly to the transferrin, lactoferrin or haem containing proteins by substrate-specific receptors in the bacterial outer membrane or synthesize low molecular mass molecules, which bound to specific receptors in the bacterial outer membrane when they combined with iron within host tissues (Cornelissen and Sparling, 1994; Wandersman and Stojiljkovic, 2000). Therefore, the ability to acquire iron is also considered an important factor in pathogenicity and virulence of bacteria.

In *F. columnare* the iron uptake mechanism have analysis and identified *in vitro* condition. An outer membrane protein of 86 kDa, which have identified as a TonB-dependent ferrichrome-iron receptor precursor (FhuA) was upregulated under iron-limited condition. Fur protein of the *F. columnare* was similar in structure of iron uptake regulation of other bacteria (Guan et al., 2013)

2.4. Clinical signs of columnaris disease

The expression of clinical signs of columnaris disease depends on the host, virulence of bacteria and environments. They may be chronic, sub-acute or acute stage, typically affects the gills, the skin and fins. The clinical signs of this disease observed in young fish are usually in acute but in adult fish, the signs may be chronic, sub-acute or acute stages. In some cases, the fish died before the clinical signs appear (Pacha and Ordal, 1967; Foscarini, 1989). The symptoms of columnaris disease can begin externally on the body surface such as gill, skin and fin lesions. Fish in columnaris disease showed lethargy, listlessness, swimming near the surface of the water. Fish maybe died after 48 hours after the appearance of clinical disease (Decostere et al., 2002). In tilapia, signal symptoms were skin discoloration and

increased opercula movement and begin died after 24 hour post-challenge (Shoemaker and LaFrentz, 2014).

Skin lesions begin as areas of discoloration. Discoloration typically begins at the base of the dorsal fin and then they extend to a pale white band around the body called a “saddleback” lesion, lesions may also be seen on the head. The area around the lesion will normally become reddish. Fish skin ulcers may occur in fish, because of the bacteria attached to the surface of skin and penetrate the epidermis and corium, forming deep skin ulcers, brings to necrosis of the underlying muscle (Decostere and Haesebrouck, 1999; Decostere et al., 2002).

Chapter III. Materials and Methods

3.1. Detection of putative virulence genes of *F. columnare* isolates

3.1.1. Bacterial isolates

Fifty - one bacterial isolates of *F. columnare* have been recovered from different organs of infected red tilapia (gill, kidney, skin, tail and liver) from 2012 to 2013 in different geographical regions in Thailand including Ratchaburi, Phetchaburi, Kanchanaburi, Chachoengsao and Ayutthaya provinces. Phenotype and genotype of all isolates have been characterized in a previous study, most of the isolates are genomovar II, only one isolate belong to genomovar I (CUVET1215) (Dong et al., 2014). Bacterial stocks were preserved in 20% glycerol added 10% bovine serum aluminum and stored in -80°C at Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. In this study, *F. columnare* strain CUVET1232 (GenBank accession number KF274043) which have been sequenced 16S rRNA gene and 16S-23S ISR was used as the positive control in all experiments. The detail of 51 *F. columnare* using in this study was presented in table 1.

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

Isolates	Geographical source	Fish organ	Year	Colony mophology
CUVET1201	Ratchaburi, TL	gill	2012	Non-Rhizoid
CUVET1202	Ratchaburi, TL	gill	2012	Rhizoid
CUVET1203	Ratchaburi, TL	gill	2012	Rhizoid
CUVET1204	Ratchaburi, TL	tail fin	2012	Rhizoid
CUVET1212	Phetchaburi, TL	tail fin	2012	Rhizoid
CUVET1213	Phetchaburi, TL	kidney	2012	Rhizoid
CUVET1214	Phetchaburi, TL	gill	2012	Rhizoid
CUVET1215	Phetchaburi, TL	kidney	2012	Rhizoid
CUVET1336	Kanchanaburi, TL	skin	2013	Rhizoid
CUVET1338	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1340	Kanchanaburi, TL	liver	2013	Rhizoid
CUVET1341	Kanchanaburi, TL	gill	2013	Rhizoid

CUVET1343	Kanchanaburi, TL	skin	2013	Rhizoid
CUVET1344	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1337	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1339	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1342	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1345	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1346	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1347	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1348	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1349	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1350	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1351	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1352	Chachoengsao, TL	gill	2013	Rhizoid
CUVET1353	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1354	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1355	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1357	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1358	Chachoengsao, TL	skin	2013	Rhizoid
CUVET1359	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1360	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1361	Kanchanaburi, TL	kidney	2013	Rhizoid
CUVET1362	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1363	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1364	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1365	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1367	Kanchanaburi, TL	kidney	2013	Rhizoid
CUVET1368	Kanchanaburi, TL	skin	2013	Rhizoid
CUVET1369	Kanchanaburi, TL	gill	2013	Rhizoid
CUVET1370	Kanchanaburi, TL	kidney	2013	Rhizoid
CUVET1371	Kanchanaburi, TL	gill	2013	Non-Rhizoid

CUVET1372	Kanchanaburi, TL	skin	2013	Non-Rhizoid
CUVET1373	Kanchanaburi, TL	skin	2003	Non-Rhizoid
CUVET1374	Ayutthaya, TL	gill	2013	Rhizoid
CUVET1375	Ayutthaya, TL	gill	2013	Rhizoid
CUVET1376	Ayutthaya, TL	skin	2013	Rhizoid
CUVET1377	Ayutthaya, TL	gill	2013	Rhizoid
CUVET1378	Ayutthaya, TL	gill	2013	Rhizoid
CUVET1379	Ayutthaya, TL	skin	2013	Rhizoid
BU1	Chachoengsao, TL	skin	2013	Rhizoid

3.1.2. Primer design and sequence alignment

In the present work, specific primers that amplify to putative virulence gene of *F. columnare* was designed based on the complete genome sequence of *F. columnare* strain ATCC 49512 (GenBank accession number CP003222) by using Primer3 software. According to published literature, six nucleotide sequences of strain ATCC 49512 which was identified as putative virulence gene were selected for primer design. Six specificity of primer pairs targeted six putative virulence genes, including *cls, sprB, ompA, sprA, gldF* and *m50* which encode Chondroitin AC lyase, adhesin SprB, OmpA family outer membrane protein P60, SprA protein, gliding motility protein GldF and membrane-associated zinc metalloprotease protein respectively, were tested *in silico* by using the Basic Local Alignment Search Tool (BLAST). Those primers provided 100% specificity were utilized for further PCR application. After PCR, amplified products were purified by using NucleoSpin Gel & PCR clean-up kit (Macherey-Nagel, Germany) according to manufacturer's instruction. Finally, the purified PCR products were sequenced by 1st BASE DNA sequencing services (Malaysia). After doing sequencing, nucleotide sequence was aligned by using Vector NTI® Advance 10. Sequence alignments were compared with available sequence on the GenBank by using BLAST. The primers given amplified products showing high similarity (80-100%) to the corresponded target genes were (due to BLAST nucleic acid sequence analysis) were used in this study. The detail of primers using in this present work was indicated in table 2.

Table 2 Oligonucleotide primers used in this study

Primer name	Sequence 5'-3'	PCR products (bp)	Annealing temperature	Reference
cls-F	TAATTCACTCCTGCCCCTGC	798	54	This study
cls-R	TCATACACCTTCAAACTGGTGG			
m50-F	TTTACAGGCTATTCCAGAAGCA	250	54	This study
m50-R	AAGGGCARGTCCCCCATCTA			
sprA-F	GGCGCGGGCTGTTAAATG	626	54	This study
sprA-R	CGTTCTCCGTTAGCGACACT			
sprB-F	GCCTTCAGGTGCTACTGCGG	696	54	This study
sprB-R	GCCGCAGGTTGGGTACTTTA			
gldF-F	CCCGCTGTAACTATGAGGAGT	453	54	This study
gldF-R	GTCAATGACACCTCGGTCGAT			
ompA-F	CTGGTCGTTTGGAGTAATCG	674	54	This study
ompA-R	TAGGACCTGCTTGGTTAGGA			
FCISRFL	TGCGGCTGGATCACCTCCTTTC TAGAGACA	400–500	55	(Welker et al., 2005)
FCISRR1	TAATYRCTAAAGATGTTCTTTCT ACTTGTTTG			

3.1.3. DNA extraction

DNA template of bacteria using for PCR amplification was extracted by boiling method as described previously study (Welker et al., 2005). Briefly, bacterial stocks were subcultured on Anacker and Ordal (AO) agar medium and incubated at 28°C for 48 hours. One pure colony was transferred into AO broth and cultured at 28°C for 24 hours. Bacterial pellet collected by centrifugation (5000 rounds per minute (rpm) for 5 minutes) from 0.5 ml of bacterial suspension was mixed with sterile de-ionized water. Then bacterial mixture was boiled for 10 min at 100°C and keep at – 20°C for 5 minutes. Finally, the suspension was centrifuged at 8000 rpm for 5 minutes and the 200 µl of supernatant was transferred to a fresh sterile 1.5 ml microcentrifuge tube and used as template DNA.

3.1.4. Polymerase Chain Reaction (PCR) assay

The presence of the *cls, sprB, ompA, sprA, gldF* and *m50* in *F. columnare* genome were analyzed by using conventional PCR method. PCR was carried out in a total volume of 25 µl containing 12.5 µl of Master Mix 2X (Promega Corporation, Madison, WI, USA), 20 pmoles of each of forward and reverse primer (1.5 µl) and 3 µl of the DNA. The PCR amplification was performed in 1 cycle of denaturation at 94°C for 7 minutes; 35 cycles of melting at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1 minute; and then a final extension at 72°C for 10 minutes. PCR products were detected by agarose gel electrophoresis with 1.2% agarose containing 0.026 µl/ml volume of Redsafe™ Nucleic Acid Staining Solution (Intron Biotechnology, Korea) at 100V for 30 minutes and visualized under UV light of gel document system (VilberLourmat, France).

3.2. Experimental fish challenge

3.2.1. Bacterial isolates

In order to test for pathogenicity of *F. columnare* in the hybrid red tilapia, five isolates were randomly selected from each virulence gene pattern. Bacteria were cultured in AO agar and incubated at 28°C in 48 hours. One pure colonies were transferred into 200 ml of *F. columnare* growth medium (FCGM) broth according to Staroscik et al. (2008) and incubated at 28°C for 24 hours with shaking at 150 rpm. After incubation, the number of bacteria was determined spectropphometrically at 630 nm (OD_{630}) and plate counting (OD=0.14 ≈ 10^9CFU/ml). The purity of bacterial culture solution was tested on nutrient blood agar and the Gram stain for microscopic observation (*F. columnare* is long, rod shape, gram negative and cannot grow on nutrients sheep blood agar).

3.2.2. Experimental fish and water quality parameters

All healthy hybrid red tilapia with body weights of 5-7g were purchased from standard Thai tilapia farms. The experimental fish having no previous history of columnaris disease and no previous vaccination was acclimatized in tanks with dechlorinated water at 28°C for 14 days. One week before the experimental

challenge, 5% of total experimental fish were randomly selected for diagnoses whether the fish were contaminated by microscopic observation for ectoparasites and culture on AO agar for *F. columnare* by culture on AO agar. Besides, their internal organs (kidney, live) were subcultured on nutrients blood agar in order to determined infected fish by some bacterial pathogens.

Healthy hybrid red tilapia free of ectoparasites and *F. columnare* was used to experimental challenge. Water quality parameters including, pH (7.47 ± 0.1), dissolved oxygen (4-5 mg L^{-1}) the ammonia (<0.01 mg L^{-1}) were measured by commercial test kits (AQUA-VAB, Thailand). The temperature (25 ± 3°C) and dissolved oxygen were maintained by air-condition and aerator (Xu et al., 2009). Fish were fed about 3% body weight daily with commercial dry pelleted fish food (Aquarium Fish Food F-1, Plus Value enterprise Co., Ltd, Thailand). All procedures utilizing fish were performed according to the Chulalongkorn University Animal Care and Use Committee (Approval No. 1431031, August 2014).

3.2.3. Experimental challenge

The experimental fish challenge test was performed following the method of (Shoemaker and LaFrentz, 2014) by immersing challenge test with some modifications. In briefly, hybrid red tilapia, average weight 7-10 *g* was separated into eleven groups (twenty fish per group); ten groups for bacterial experimental challenge and one group for control. In each trial group, 20 fish were immersed with bacterial concentration of 10^{7}CFU/ml of rearing water; the total of water volume for challenge was three liters of rearing water. After one hour post immersion challenge, the 27 liters of clear rearing water were poured more into the tanks. In the control group, fish were treated similarly, but received pure FCGM medium (100 ml medium and 3000ml waters). The cumulative mortality rate was recorded every day for 14 days.

After 14 days post challenge, virulence isolates was group based on mortality of fish. Any isolates induced more than 80% fish mortality within 72 hours post-challenge was listed in the high virulence isolates. The isolates showed a mortality of fish from 20% to 80% was considered as moderate virulence isolates, while the

remainder isolates were assigned as low virulence isolates. Besides, three isolates, including one causing high percent mortality (100%), one causing moderate percent mortality (60%) and one causing no percent mortality (0%) were selected for determination of the median lethal dose (LD_{50}) by immersion challenge as previously described (Shoemaker and LaFrentz, 2014). The fish was separated into six tanks (10 fish/tank). Five tanks were challenged with different concentrations of bacteria approximately 10^8, 10^7,10^6, 10^5 and 10^4cfu/ml of the rearing water by immersion challenge. A tank without bacteria was served as negative control. Mortality of fish was observed during a period of 14 days. The LD_{50} of bacteria was calculated following the method of Reed and Muench (1938) after 14 days post-challenge. The three selected isolates representatives of in LD_{50} assay were used to investigate the virulence properties expression as well.

3.3. Determination of virulence properties

3.3.1. Adherence on gill tissue

The ability of bacterial adhesion was determined by using gill immersion model according to Kunttu et al. (2009) with slight modification. The gill arches were collected from healthy red tilapia (mean weight 20g). The fish gills collected from five healthy fish were washed three times in PBS in order to reduce bacterial contamination. After that, 0.5g of the intact gill was exposed to broth cultures of LV, MV and HV isolates (OD = 0.12 = 10^8 cfu/ml) and incubate at 28°C for two hours with 100 rpm of shaking. One control group was carried out similarly with sterile FCMG instead. After incubation, the infected gill was removed and rinsed slightly with PBS 1X for three times and then homogenized with PBS. Homogenized solution was diluted 10 times from 10^{-1} to 10^{-5}. One hundred µl of each dilution were spread on AO agar containing Tobramycin (Sigma, USA). The colony on AO agar was counted after 48 hours incubated at 28°. The number of colonies on AO agar were evaluated as number of bacterial cells attached to the gill.

3.3.2. Capsule formation

Capsule of bacteria was determined following the description of Klesius et al. (2010). Five µl of cultured bacterial cells were mixed with five of 5% skim milk on

the clean slide and then using a second clear slide streaked across the first slides in a swift motion. The slides were air-dried at room temperature and stained with crystal violet solution 0.1% for 1–2 min. Crystal violet was removed by 20% copper sulfate solution and air dried in the room temperature. The capsule formation of bacteria was observed using a 100X oil immersion lens with a microscope. The bacterial cells and skim milk were appeared a dark color, while the capsule remain colorless. The thickness of capsule were measured under microscope by Motic Images Plus 2 software.

3.3.3. Biofilm formation

Three isolates representing to low virulence, moderate and high virulence in red tilapia were compared for their biofilm formation according to Álvarez et al. (2006) . The bacterial culture (approximate 10^9 CFU/ml) was diluted 100 times in OA broth medium. Then 100 µl of each bacterial dilution were transferred into the U-shape 96-well microtiter polystyrene plates and incubate at 28°C in 48 hours, each dilution was replicated eight wells. The bacterial cells were removed by washing four times with distilled water. After rinsing, the plate was stained by 150µl of 1% crystal violet and incubated for 20 minutes at room temperature. Following this incubation, crystal violet was discarded by washing four times with distilled water. Finally, 200µl of 30% acetic acid or 96% ethanol were added into the wells and incubated at room temperature for ten minutes. Biofilm formation was measured by microplate reader at optical density (OD) at 600 nm. For positive controls, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 that have been confirmed well-characterized biofilm-forming isolates (Ceri et al., 1999; Naves et al., 2008; Cremet et al., 2013) were used in this experiment. Negative controls were performed with culture medium without bacteria.

3.3.4. Proteolytic activity

To determine protease activity, 5µl of bacterial cultures were dropped on Cytophaga agar containing 5% skim milk agar and incubated at 28°C, the zone of proteolysis was measured after 48 hours post incubation (Pandey et al., 2010; Amsaveni et al., 2014).

3.4. Data Analysis

In this study, putative virulence gene profiles, level of bacterial pathogenicity, level of virulence properties expression were determined by descriptive analysis. The relationship between virulence gene profiles and the pathogenicity of bacteria; between virulence properties expression and levels of pathogenicity were analyzed by one way - ANOVA in SPSS 20.

Chapter IV. Results

4.1. Determination of putative virulence gene profiles of *F. columnare* isolates

4.1.1. Primer design and sequence alignment

In this study, based on sequence of *F. columnare* strain ATCC 495125, six specific primers amplify for six putative virulence genes, including *cls, sprB, ompA, sprA, gldF* and *m50* have designed and applied by PCR. The strain CUVET1323 was positive for six putative virulence genes (figure 3) that were used for sequence alignment and nucleotide BLAST. The result of sequence alignment of these primers was presented in table 3. From identity percentages of sequence based on nucleotide – nucleotide alignment were revealed that homogeneous sequence ranged from 89% to 99% following *cls, sprB, ompA, sprA, gldF* and *m50* were 99%, 91%, 97%, 92% 89% and 94% respectively.

Table 3 Sequence alignment of primers for the detection of putative virulence genes of *F. columnare*

Primer	Target protein	Query cover	Nucleotide identity	Reference strain
cls	Chondroitin AC lyase	100%	99%	*F. columnare* strain ATCC 49512 (GenBank accession number CP003222)
sprB	adhesin SprB	99%	91%	
ompA	OmpA family outer membrane protein P60	100%	97%	
sprA	SprA protein	99%	92%	
gldF	Gliding motility protein GldF	99%	89%	
m50	M50 family membrane-associated zinc metalloprotease	100%	94%	

On the other hand, the results of nucleotide BLAST also showed that these primers were unique amplification for *F. columnare.* The putative virulence gene sequences of *cls, sprB, ompA, sprA* and *gldF* have been deposited to GeneBank with GenBank accession number of *cls, sprB, ompA, sprA* and *gldF* were KJ956998, KM197218, KP085640, KM107831 and KM065531 respectively. Besides, protein translate has been performed and demonstrated that our primer could amplify

genes that encode for expected protein (data unpublished). Therefore, these primers were used for PCR amplification of putative virulence gene of *F. columnare*.

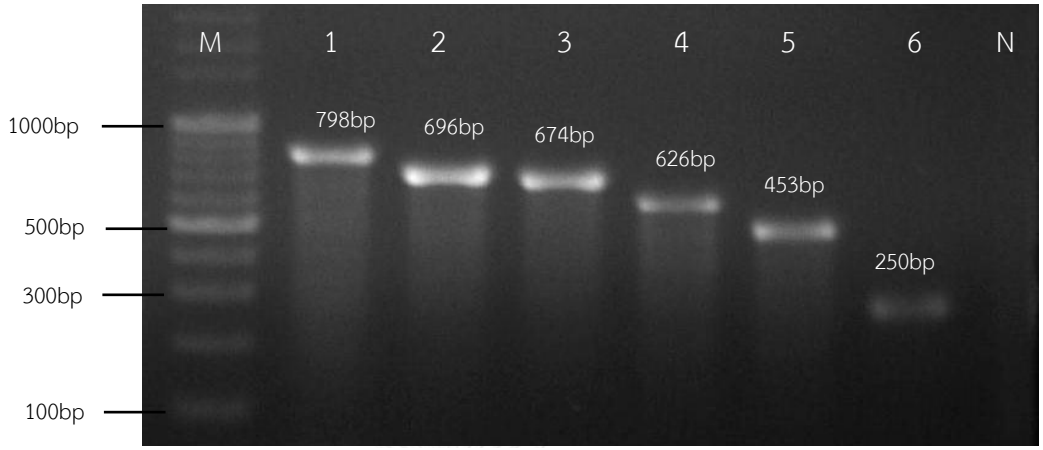


Figure 1 Agarose gel of six putative virulence genes of *F. columnare.* Lane M: 1 kb DNA ladder; lane 1-6 were *cls, sprB, ompA, sprA, gldF* and *m50* respectively; N: negative

4.1.2. PCR application

To determine putative virulence profiles of *F. columnare*, six associated virulence -genes were identified from 51 isolates by PCR method. The result of PCR application was reported in table 4. Overall, *cls, ompA, gldF* and *m50* genes were detected from all investigated *F. columnare* isolates. Among them eleven isolates possessed two more putative virulence genes, including *sprA* and *sprB*. From PCR results we found that there were the coexistence of two distinct putative virulence gene profiles among 51 isolates of *F. columnare* originated from diseased red tilapia in Thailand including P1 which contained six gene cls^{+}, $sprB^{+}$, $ompA^{+}$, $sprA^{+}$, $gldF^{+}$ and $m50^{+}$; P2 which consisted four genes cls^{+}, $ompA^{+}$, $gldF^{+}$ and $m50^{+}$. Among them, putative genetic virulence factors profiles P2 was dominative with 40 isolates (78%) when compared with P1 that contained only eleven isolates (22%).

Table 4 Putative virulence gene profiles of *F. columnare* isolates

Putative virulence gene profiles	Geographical source					Total
	Chachoengsao (14)	Kachannaburi (23)	Ratchaburi (4)	Ayutthaya (6)	Phetchaburi (4)	52
P1	0	11	0	0	0	11
P2	14	12	4	6	4	40

Note: P1 (*cls*$^{+}$, *sprB*$^{+}$, *ompA*$^{+}$, *sprA*$^{+}$, *gldF*$^{+}$ and *m50*$^{+}$), P2 (*cls*$^{+}$, *ompA*$^{+}$, *gldF*$^{+}$ and *m50*$^{+}$)

4.2. Pathogenicity investigation

In order to evaluate the pathogenic ability of *F. columnare* isolates, based on putative virulence gene profiles, five *F. columnare* isolates in each virulence gene profile were randomly selected for pathogenicity determination in red tilapia by immersion challenge. Results of the virulence evaluation were presented at figure 2.

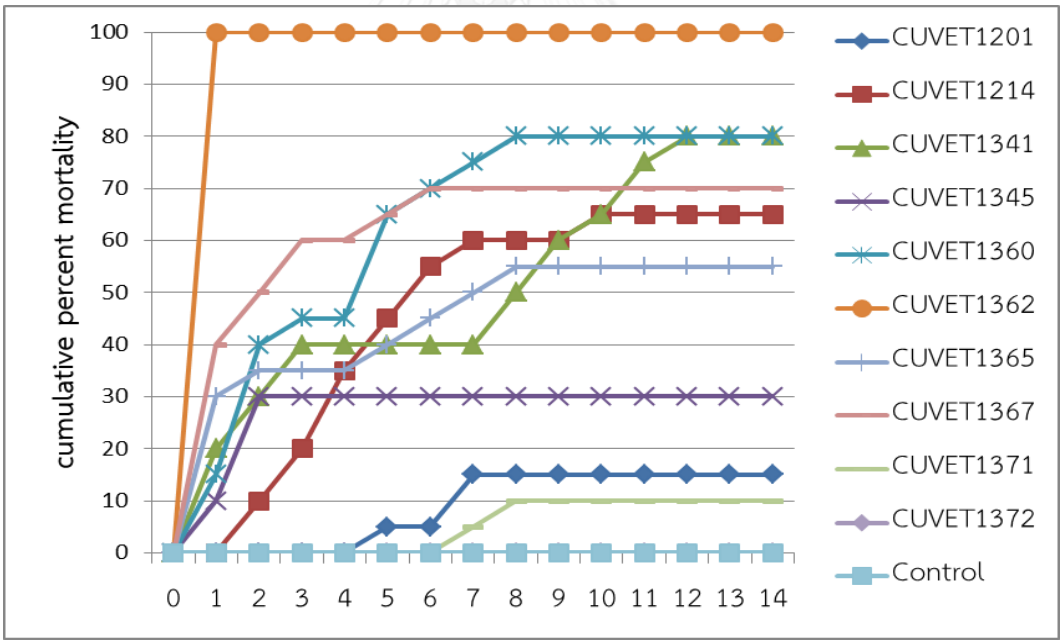


Figure 2 Percent mortality of red tilapia during 14 days post challenge. Experimental dose was approximate $7\log_{10}$CFU ml^{-1}

Profile 1 (CUVET1360, CUVET1362, CUVET1365, CUVET1371, CUVET1372)

Profile 2 (CUVET1201, CUVET1214, CUVET1341, CUVET1345, CUVET1367)

Our result indicated that variation in levels of pathogenicity was observed among ten trialed isolates. The cumulative percent mortality (CPM) of experimental fish fluctuated from 0% to 100% depend on individual isolate. During the experimental challenge, our observation revealed that fish died after 24 hours post - challenge and remained the death until 12 late days. Typical symptoms of columnaris disease were absent in dead fish within 24 hours. However, some clinical signs were observed in diseased fish after 24 hours post-challenge such as lesion on the skin, abrasion of tail (figure 4). Before dying the fish showed lost movement, listlessness and swimming slowly near the surface of the water. All dead fish were positive for *F. columnare* from gill and skin by culture and specific PCR (figure 3), but rarely in internal organs (liver, brain and kidney). Neither extraordinary signs nor *F. columnare* were identified in fish of control groups

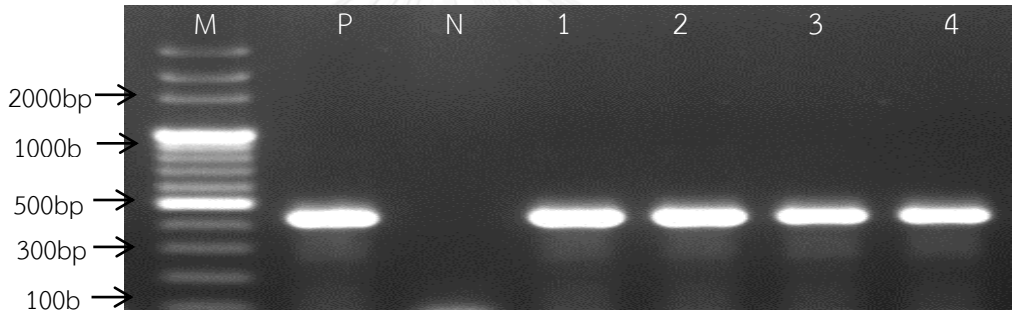


Figure 3 Specific PCR for *F. columnare* with specific primers FCISRR and FCISRR1 (PCR products ~ 500 bp) from fish gill tissue. Lane M (DNA F ladder), P (positive control; DNA of F. columnare), N (fish of control group); lanes 1-4 (dead fish challenger with *F.columnare*

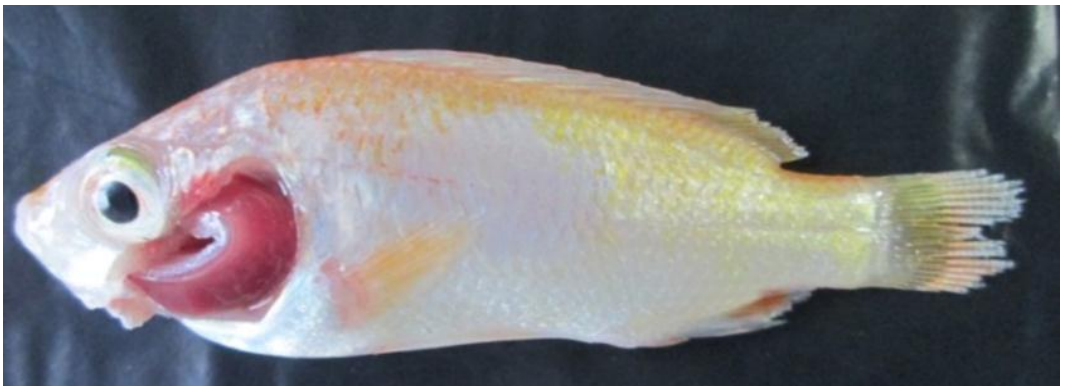


Figure 4 Columnaris disease cause by *F. columnare* with tail fin erosion, skin

In this experiment, the mortality rate after challenge with virulence gene profile P1 isolates were reached from 20 to 80%, meanwhile P2 were 0 to 100 %. Nevertheless, it was not an association between genetic virulence profiles and levels of pathogenicity in red tilapia based on cumulative percent mortality after 14 days post challenge (P>0.05). Among tested isolates, CUVET1362 was predominant high virulence in red tilapia, which was responsible for 100% mortality of experimental fish within 24 hours after post challenge. This isolate was assigned as high virulence isolate. Six isolates (CUVET1214, CUVET1341, CUVET1360, CUVET1365, CUVET1345, CUVET1367) that caused 20-80% mortality of challenged fish was considered as moderate virulence isolates. In addition, three isolates (CUVET 1210, CUVET1372, CUVET1372) were referred as the low virulence isolates which not due to death of tested fish (CUVET1372) or low mortality (CUVET1371, CUVET 1210). Interestingly, in this study, it has been found that all isolates in low virulence group were non-rhizoid colonies (CUVET1372, CUVET1371 and CUVET 1210).

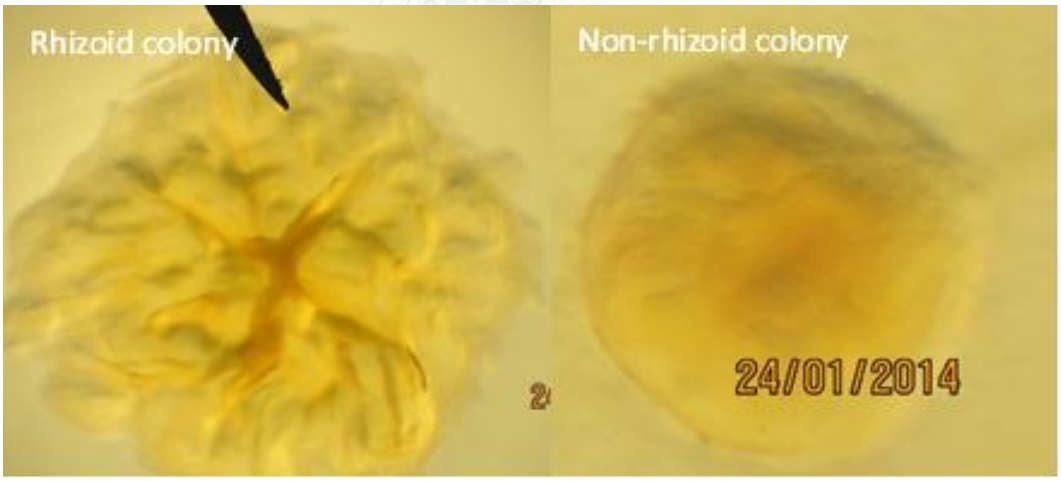


Figure 5 Rhizoid colony and non-rhizoid colony of *F. columnare*

Base on screening of bacterial virulence test *in vivo*, three isolates in different levels of pathogenicity were selected for LD_{50} determination including CUVET1362, CUVET1360 and CUVET1372 that corresponded to high virulence (HV), moderate virulence (MV) and low virulence (LV) respectively. The value of LD_{50} of three isolates representing HV, MV and LV isolates were 6.10lg_{10} CFU, 6.65lg_{10}CFU and more than 8lg_{10} CFU ml^{-1} of rearing water respectively after 14 days post-immersion challenge.

This result also indicated that the LD_{50} of HV isolate was higher than MV and LV isolates approximately 3.5 times and more than 100 times respectively. These isolates were also used for comparison of virulence properties expression *in vitro*, including adherence capability, biofilm formation, capsule formation and proteolytic activity.

4.3. Bacterial adhesion and biofilm formation ability

The adherence capability of *F. columnare* was evaluated by determining number of bacteria attach to the fish gill *in vitro*. The result of bacterial adherence of three representative virulence isolates of *F. columnare* was presented in figure 6. In particular, the number of bacterial cells attached to the gill after two hours of HV, MV and LV isolates were 7.72 ± 0.08, 7.46 ± 0.24 and 6.86 ± 0.16 (log_{10}CFU g^{-1}; mean ± 2SD) respectively. From these results indicated that the adhesion ability of HV and MV isolates was significant higher than LV isolate ($p<0.05$). However, no significant difference was found between MV and HV isolates ($P>0.05$). Besides, there have been no any *F. columnare* cells in control groups.

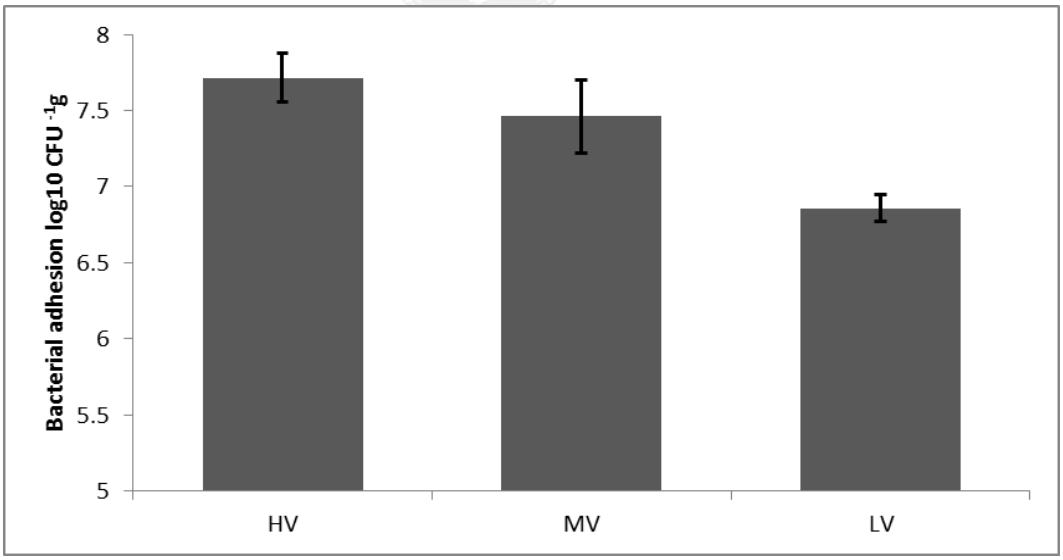


Figure 6 Comparison of *F. columnare* adhesion ability to the gill of *F. columnare* among HV, MD and LV isolates after two hours gill exposed with culture bacteria. The number of bacteria attached to gill tissue (colony forming unit /g of gill tissue; log CFU g^{-1}) of HV and MV isolates were significant higher than LV isolates ($P<0.05$). No significant difference ($p>0.05$) was found between HV isolate and MV.

The biofilm formation ability of *F. columnare* was compared based on optical density (OD) value at 600 nm in wavelength. Our results showed that all investigated isolates could form biofilm in the wells of the microtiter plate including positive control (*E. coli and P. aeruginosa).* However, OD values were significant different among HV, MV and LV isolates ($p<0.05$) (figure 7). Herein, the OD values of biofilm products of HV, MV and LV isolates were 0.594 ± 0.0837, 0.271 ± 0.021 and 0.134 ± 0.021 respectively. These results demonstrated that the biofilm formation capacity of HV isolate was highest and LV isolate was lowest.

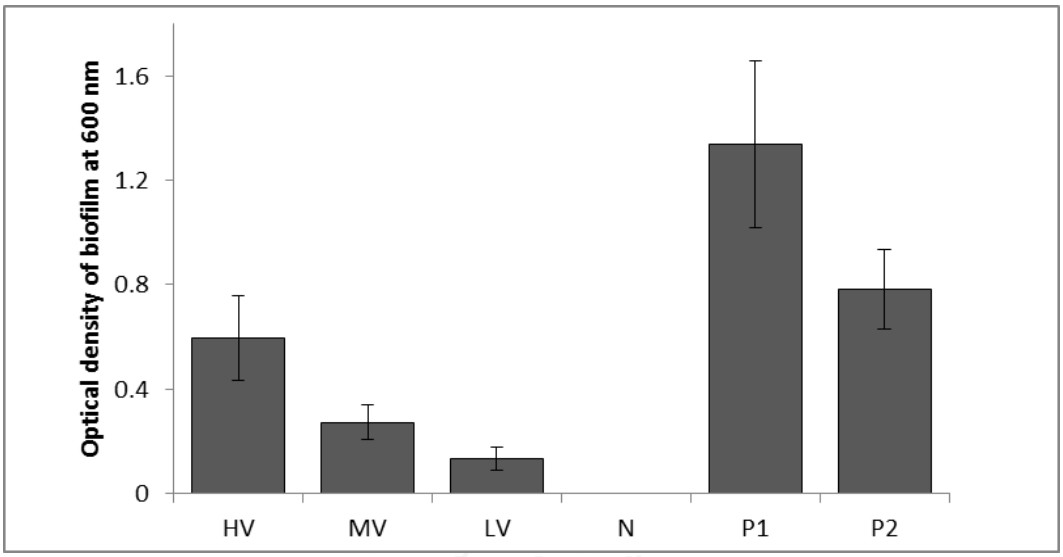


Figure 7 Optical density (OD) of biofilm products were produced by *F. columnare*. It was significant difference among LV, MV and LV isolates. HV isolate was highest follow MV and LV isolates. P1, P2 (positive control) were the biofilm products of *E. coli* and *P.aeruginosa* respectively. No biofilm products were found in control group (medium)

4.4. Determination of *F. columnare* capsule

The polysaccharide capsule of HV, MV and LV *F. columnare* isolates were detected by microscopic observation and evaluated by Motic Images Plus 2 software. Our observation indicated that capsule layer covered all three isolates. However, thickness and density of them were different. Here, the capsule thickness of HV and MV and LV were 0.61± 0.18, 0.62 ± 0.14 and 0.39 ± 0.21 (μm)

respectively. From these results, it showed that capsule of HV and MV and LV were isolates were thicker and denser than LV virulence (Figure 8, 9).

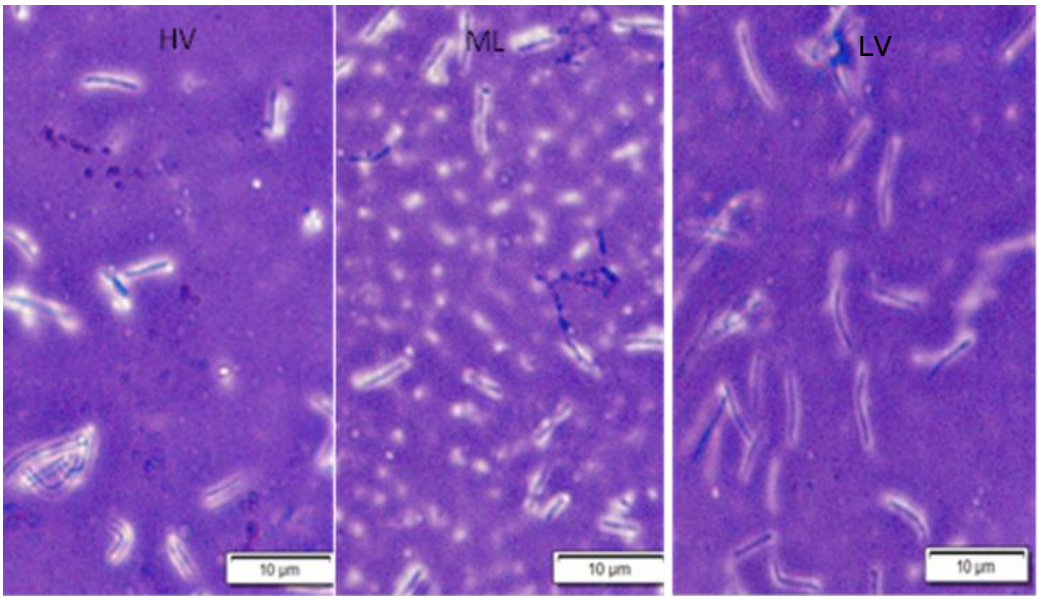


Figure 9 Capsule thickness of LV, M, HV *F. columnare* isolates were 0.61± 0.18, 0.62 ± 0.14 and 0.39 ± 0.21 (µm).

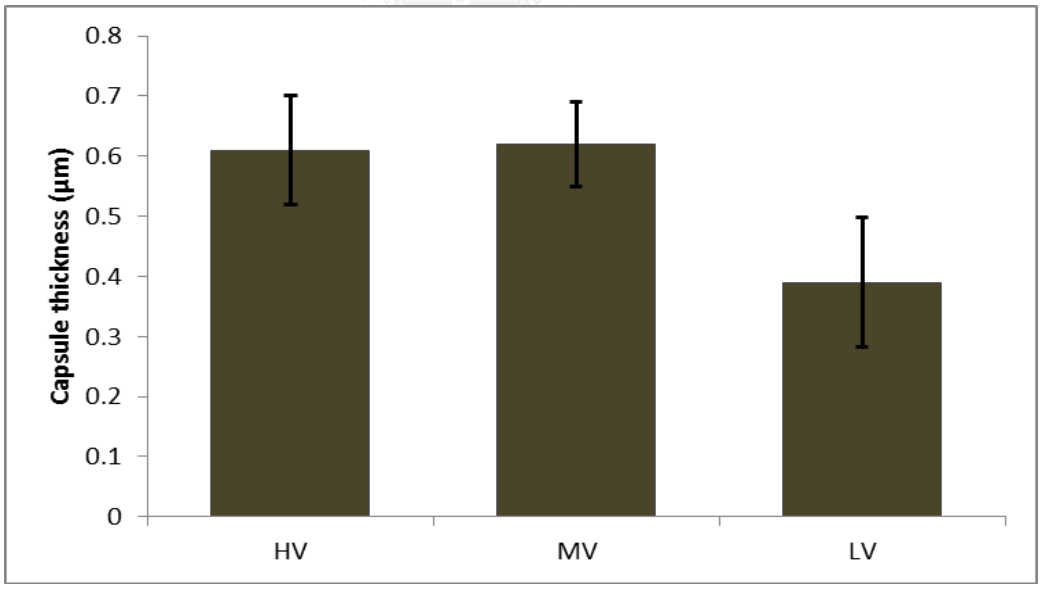


Figure 8 Measurement of capsule thickness by Motic Images Plus 2. Thickness of capsule of HV and MV isolates were significant higher than LV isolate a *P* value of <0.05

4.5. Proteolytic activity

In this study, proteolytic activity of *F. columnare* was determined on skim milk. The sizes of the clear zone surround colony were measured after 48 hours post – incubation. The results showed that, three representative virulence isolates were strong to produce protease enzymes on skim milk agar. However, the sizes of the diameter of the clear zone of proteolytic activity were different (figure 10). In particular, the diameters of clear zones of LV, MV and HV isolates were 22.25 ± 0.85 mm, 23 ± 0.71 mm and 18.25 ± 0.73 mm respectively.

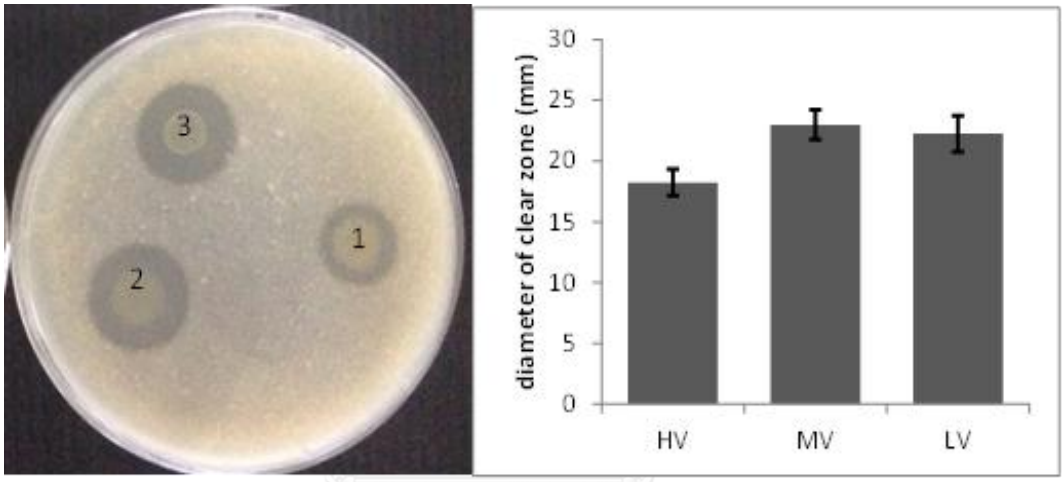


Figure 10 Diameter (mm) of clear zone surround colony of proteloytic activity on skim milk agar after 48 hours of incubation. 1 (HV), 2 (MV), 3 (LV)

Chapter V. Discussion and conclusion

5.1. Discussion

F. columnare, the causative agent of columnaris disease was regarded as an important bacterial pathogen with broad host in freshwater fish and have been becoming a major problem of cultural fish in the world that inducing significant economic losses (Declercq et al., 2013; Mohammed and Arias, 2014). During many last decades, several studies have performed in order to interpret the pathogenesis of columnaris disease. However, many related questions with the pathogenesis of this disease still are uncomprehensible. Therefore, the investigation of the virulence properties of *F. columnare* pathogen is necessary.

In this study, based on complete genome sequence of *F. columnare* strain ATCC49512 (GenBank accession number CP003222), six specific primers have been designed to amplify six putative virulence genes of *F. columnare* and their virulence gene profiling. Fifty-one *F. columnare* isolates were divided in two putative virulence gene patterns P1 and P2; this is also the first time some genetic virulence profiles of *F. columnare* were documented and reported in *F. columnare*. Among tested putative virulence genes, *cls* gene (detected from all isolates) that encode to Chondroitin AC lyase, an enzyme digests the complex polysaccharides of connective tissue chondroitin sulfates A and C and hyaluronic acid was deeply considered *in vitro*. According to Suomalainen et al. (2006) all tested *F. columnare* isolates could produce Chondroitin AC either low virulence or high virulence isolates and their activity related to temperature (25°) being high temperature is higher than low temperature (20°). Besides, *gldF, m50* and *opm*A genes, which encode for gliding protein F, M50 family membrane-associated zinc metalloprotease and OmpA family outer membrane protein P60 respectively were also amplified from all isolates. Although virulent functions of these genes in *F. columnare* are still unclearly, however virulent contribution of them was demonstrated in other species, such as *F. johnsoniaea, A. hydrophila and Burkholderia cepacia* (Hunnicutt et al., 2002; Corbett et al., 2003; Khushiramani et al., 2012). Interestingly, in this study *sprA* and *sprB* genes were just detected from 11 isolates of 51 isolates. While SprA are responsible for

secretion of the Adhesins SprB, one of cell surface gliding motility adhesins. Out of contribution to gliding, SprA and SprB were considered as virulence factors that participate to bacterial adherence (Shrivastava et al., 2013). However, until now, there were about of ~20 various proteins related to gliding motility, which were encoded by *gld* and *spr* genes have been found in genus *Flavobacterium* (Braun et al., 2005; Shrivastava et al., 2013). Therefore, further study in individual gene expression will be performed in order to determine the virulence function of each gene.

On the other hand, in the present work, the relationship between the putative virulence gene profiles and geographical distribution of isolates has revealed; whereas the *F. columnare* isolates were recovered from Kachannaburi province were existed both putative virulence gene profiles (P1 and P2). Contrary to that, *F. columnare* isolates obtained from Chachoengsao, Ratchaburi, Ayuthaya and Phetchaburi only carried putative virulence gene profile P2. This suggested that genetic virulence diversity may relate to the geographical originality of isolates.

From experimental immersive challenge, in agreement with other publications (Decostere and Haesebrouck, 1999; Shoemaker et al., 2008; Shoemaker and LaFrentz, 2014) our result found that the different isolates exhibited different levels of pathogenicity. Although, all isolates of this experiment were isolated from diseased fish, however virulence of them was various. These interpreted in the natural outbreak of columnaris disease, fish mortality rate fluctuated variously from 10 to70% (Dong et al., 2014), sometimes was 100% (Plumb and Hanson, 2011). Interestingly, in this study, *F. columnare* can be recovered from the external organ (skin. gill, tail) of infected fish, however, it was not found or rarely from internal organs (brain, kidney, samples). On the other hand, from our experiment in channel catfish (data unpublished), *F. columnare* could re-isolate from internal organs. Therefore, it is suggested that the different host species may be have different specific mechanism of the bacterium host interaction. Therefore, further study in different host will interpret specific host mechanisms in columnaris pathogenesis.

Previously, the relationship between virulence and genomovar of *F. columnare* has addressed (Shoemaker et al., 2008; Li et al., 2010; Shoemaker and LaFrentz,

2014). Unfortunately, no information of on the relationship between virulence genes and pathogenicity of *F. columnare* has been addressed so far. In this study, two putative virulence gene profiles have been grouped. However, virulence between two putative virulence gene profiles in red tilapia were not significant difference based on cumulative percent mortality ($p>0.05$) after 14 days post- infection. These outcomes demonstrated that the pathogenicity of *F. columnare* did not depend on the presence of the investigated putative virulence genes in this study. Our finding suggested that the pathogenic ability of *F. columnare* isolates maybe depend on putative virulence gene expression during bacterial infection or *in vivo*. This is suitable with Méndez et al. (2012), the capacity of pathogens to cause disease depends on a large level of the expression of virulence genes, which help them to invade the host, produce pathological effects and evade host defense. Further investigation of the relationship between putative virulence genes expression *in vivo* and virulence of *F. columnare* may find virulence function of these genes. Besides, virulence of bacteria is multifactor and complex and may involve the products of a number of different genes acting either alone or in concerned factors (Sha et al., 2009). Therefore, it was believed that the virulence of *F. columnare* may not only depend on our tested virulence genes, but its virulence also will combine with other genes, which were undetected in this study such as *Fur* (Guan et al., 2013) *, gldB* and *gldH* (Klesius et al., 2010) etc..

Until now, virulence of different *F. columnare* morphology colonies has been also investigated by some previous researches; however, it still is a controversial issue. A majority of studies believed that rhizoid colony morphology was more virulent than non-rhizoid colony morphotype (Kunttu et al., 2011; Laanto et al., 2014). In the present work, as an enjoyable accidentally, different colony morphologies of *F. columnare* induced different mortality rate of fish. Here, bacteria in non-rhizoid colony morphology (CUVET1201, CUVET1771 and CUVET1772) were less virulence than rhizoid colony form (residual isolates) significantly. According to Laanto et al. (2014) differences in the cell surface structures and extracellular protein profiles between the rhizoid and non – rhizoid colony have been found. Among them OmpA and SprF linked with bacterial adhesion and invasion were

dominant in rhizoid colony (virulent). Therefore, it was suggested that the adhesion ability of rhizoid colony was higher than non-rhizoid colony and they are potentially to connect with the virulence of *F. columnare*.

The pathogenesis of columnaris disease is implicated multiple virulence properties (Beaz-Hidalgo and Figueras, 2013). Among them adhesion to the host is primary and most important factors of many bacterial pathogens during infection process (Decostere et al., 1999b; Shoemaker et al., 2008). In agreement with their researches, our finding showed that the adherence capability of HV and MV isolates were significant higher than LV isolate of *F. columnare* after two hours exposed with gill. Attaching to the host tissue is necessary factor for *F. columnare* colonization and then successful invasion, however, the most of studies have focused only in phenotypic level. Therefore, the specific molecular of the adhesion of *F. columnare* have not elucidated yet. Some studies believed that gliding protein contributed to adherent ability of *F. columnare*. In this present work, some gliding genes were detected such as *gldF, sprA* and *sprB* but it was seemed be present genes were not related to bacterial adhesion ability. Therefore, further investigation of other gliding protein and their expression may elucidate this relationship.

Bacterial adherence is an involution with many processes, which can be separated into the stages of attraction, adhesion, and aggregation. Successful attachment to tissue or surface is first step to form biofilm by bacteria that was also considered as a virulence factor of many pathogens (O'Toole et al., 2000; Myszka and Czaczyk, 2011). Previous study was indicated that biofilm formation maintained the adherent capacity of *F. columnare* during bacterial infection in channel catfish (Cai et al., 2013). As an agreement with Laanto et al. (2014), our finding revealed that capacity of biofilm formation of *F. columnare* was related to bacterial virulence follow HV isolate was significant higher than LV isolate. The consequence of adherence and biofilm formation were not only help bacteria to maintain infection, but also lead to creating numerous clumps that cover the gill and its outcome due to fish could not uptake oxygen from the environment and causing death of the fish (Declercq et al., 2013).

As well as bacterial infection, many factors participate in biofilm formation, for example gliding protein, outer membrane protein (Basson et al., 2008; Sundell and Wiklund, 2011; Laanto et al., 2014). Among them was bacterial capsule; out of important virulence functions such as resistance to specific and nonspecific host immunity, prevention of desiccations, the capsule also related to form biofilm of bacteria (Costerton et al., 1987; Declercq et al., 2013). Capsule polysaccharides are one of the materials that attach some of the cells to the surface and to each other. Consequently, polysaccharide capsule is able to forward adherence of bacterial cell to cell and cell to surface, these may lead to form biofilm and persistence of the pathogens during colonization (Costerton et al., 1987; Dzul et al., 2011). In *F. columnare* pathogen, lectin–like carbohydrate receptors combine with capsule is involved in chemotactic responses and the adhesion ability (Decostere et al., 1999b; Klesius et al., 2010). On the other hand, by using transmission electron microscopy Decostere et al. (1999b) have also revealed that the capsule products of low virulence had a thinner and less dense than high virulence isolates. In this study, the capsule thickness seemed to relate among adhesion, biofilm and virulence of *F. columnare*. In particular, HV possessed thicker and denser capsule, high adhesion ability and biofilm formation. While LV isolate was opposite. The capsule products together biofilm formation become important barrier that help bacterial resist the host immunity defense and protect pathogen from environmental factors. These maintain bacterial infection and invasion (Costerton et al., 1987). From obvious evident, polysaccharide capsule plays as an important factors in columnaris disease as well.

As previous discussion, the successful biofilm formation not only helps pathogens to resist the severe environments but also it helps bacteria to maintain for bacterial infection and promote the production of extracellular products. Definitely, extracellular products play potential function in bacterial pathogenesis. Among many extracellular products chondroitin AC lyase , protease together collagenase have been proposed as the virulence factors of *F. columnare* (Newton et al., 1997; Suomalainen et al., 2006; Kunttu et al., 2011; Declercq et al., 2013). Chondroitin AC lyase, an enzyme digests the complex polysaccharides of connective tissue

chondroitin sulfates A and C and hyaluronic acid was deeply considered *in vitro*. Out of chondroitin AC lyase, other extracellular product has been mentioned as a bacterial virulence factor in columnaris disease is protease enzyme. However, relation between virulence of *F. columnare* and proteolytic activity is still unknown. In the present work, the proteolytic activity on skim milk agar of three representative levels of *F. columnare* virulence was not correlation with their virulence in red tilapia. Although three representative virulence isolates were strong to produce protease in skim milk agar. However, the MV and LV isolates were higher than HV isolate did. This was interpreted below, many factors contributed to cause columnaris disease, their activity was interdependent (Declercq et al., 2013). Besides, there are many kinds of protease, which digest many various proteins such as casein, collage and keratin. Therefore, it was suggested that the proteolytic activity on skim milk agar was not enough to demonstrate relationship between protease and virulence of *F. columnare*. Further study, individual investigation of the role of protease maybe clarified this relation.

Previously, the relationship between protease and zinc metalloprotease gene (*zmpA*) have investigated in *Burkholderia cepacia,* it demonstrated that the *zmpA* mutation isolate created less protease than the wild strain (Corbett et al., 2003). In our work gene *m50* encode to membrane-associated zinc metalloprotease have detected from all isolates. Thus in further study we will investigated the relationship between *m50* gene and protease enzyme. In particular, functional protease enzyme in columnaris pathogenesis is unclear and need to investigate in further study.

5.2. Conclusion

From studied result we can conclude that there have at least two putative virulence associated gene profiles among 51 isolates of *F. columnare* recovered diseased red tilapia but no significant correlation between bacterial virulence and two genetic virulence profiles. Our results believe that pathogenicity of *F. columnare* is a combination of many virulence factors, among them, adhesion ability, biofilm formation, polysaccharide capsule play important virulence function of *F. columnare*.

5.3. Advantage of study

This study has provided the information of some relationship among virulence properties and pathogenicity of *F. columnare*. These are the foundation to elucidate the pathogenesis of columnaris disease. Our results provide knowledge for the development of further study such as virulence gene expression of *F. columnare*. Besides, this study will orient to develop the effective vaccines and the identification of compounds to treat columnaris disease. Finally, it will be the academic reference source for other study.

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APPENDIX

Appendix A: PCR results

Table 5 Result of PCR determination of putative virulence genes of *F.* ***columnare***

Order	Isolates	*cls*	*M50*	*gldF*	*sprA*	*sprB*	*ompA*
1	CUVET1201	+	+	+	-	-	+
2	CUVET1202	+	+	+	-	-	+
3	CUVET1203	+	+	+	-	-	+
4	CUVET1204	+	+	+	-	-	+
5	CUVET1212	+	+	+	-	-	+
6	CUVET1213	+	+	+	-	-	+
7	CUVET1214	+	+	+	-	-	+
8	CUVET1215	+	+	+	-	-	+
9	CUVET1336	+	+	+	-	-	+
10	CUVET1338	+	+	+	-	-	+
11	CUVET1340	+	+	+	-	-	+
12	CUVET1341	+	+	+	-	-	+
13	CUVET1343	+	+	+	-	-	+
14	CUVET1344	+	+	+	-	-	+
15	CUVET1337	+	+	+	-	-	+
16	CUVET1339	+	+	+	-	-	+
17	CUVET1342	+	+	+	-	-	+
18	CUVET1345	+	+	+	-	-	+
19	CUVET1346	+	+	+	-	-	+
20	CUVET1347	+	+	+	-	-	+
21	CUVET1348	+	+	+	-	-	+
22	CUVET1349	+	+	+	-	-	+
23	CUVET1350	+	+	+	-	-	+
24	CUVET1351	+	+	+	-	-	+
25	CUVET1352	+	+	+	-	-	+
26	CUVET1353	+	+	+	-	-	+

27	CUVET1354	+	+	+	-	-	+
28	CUVET1355	+	+	+	-	-	+
29	CUVET1357	+	+	+	-	-	+
30	CUVET1358	+	+	+	-	-	+
31	CUVET1359	+	+	+	+	+	+
32	CUVET1360	+	+	+	+	+	+
33	CUVET1361	+	+	+	-	-	+
34	CUVET1362	+	+	+	+	+	+
34	CUVET1363	+	+	+	+	+	+
36	CUVET1364	+	+	+	+	+	+
37	CUVET1365	+	+	+	+	+	+
38	CUVET1367	+	+	+	-	-	+
39	CUVET1368	+	+	+	+	+	+
40	CUVET1369	+	+	+	-	-	+
41	CUVET1370	+	+	+	+	+	+
42	CUVET1371	+	+	+	+	+	+
43	CUVET1372	+	+	+	+	+	+
44	CUVET1373	+	+	+	+	+	+
45	CUVET1374	+	+	+	-	-	+
46	CUVET1375	+	+	+	-	-	+
47	CUVET1376	+	+	+	-	-	+
49	CUVET1377	+	+	+	-	-	+
49	CUVET1378	+	+	+	-	-	+
50	CUVET1379	+	+	+	-	-	+
51	BU1	+	+	+	-	-	+

Appendix B: Adhesion, biofilm formation and proteolytic activity

Table 6 the number of bacterial cells attached to gill tissue

Isolates	cfu g^{-1}		Average (cfu g^{-1})
Low virulence	5,905,000	8,571,249	7,238,125
Moderate virulence	36,672,720	21,176,471	28,924,596
High virulence	57,500,000	46,785,714	52,142,857

Table 7 the value of optical density of biofilm formation (at 600 nm)

Order	CUVET1372	CUVET1360	CUVET1362	Positive control *(E. coli)*	Positive control *(P.aeruginosa)*	Negative control
1	0.254	0.394	0.63	1.616	1.000	0.142
2	0.265	0.35	0.677	1.536	0.782	0.121
3	0.251	0.427	0.67	1.467	0.851	0.132
4	0.248	0.363	0.79	1.323	0.865	0.102
5	0.252	0.354	0.834	1.379	0.825	0.123
6	0.33	0.363	0.751	1.375	0.95	0.104
7	0.263	0.351	0.797	1.217	0.928	0.124
8	0.243	0.339	0.844	1.75	1.000	0.112
9	0.222	0.413	0.81	1.366	1.02	0.132
10	0.245	0.454	0.657	1.365	0.809	0.124
11	0.254	0.407	0.55	1.487	0.909	0.122
12	0.248	0.399	0.67	1.745	0.809	0.112
13	0.251	0.402	0.714	1.354	1.017	0.123
14	0.252	0.399	0.631	1.298	0.876	0.104
15	0.242	0.432	0.677	1.368	0.863	0.134
16	0.236	0.416	0.724	1.555	1.006	0.101
Average	0.253	0.391	0.714	1.45	0.907	0.1195

Table 8 diameters of proteolytic activity zone surround colony

Isolates	Diameter of clear zone (mm)				Average (mm)
Low virulence	24	20	22	23	22.25
Moderate virulence	22	22	23	25	23
High virulence	18	17	18	20	18.25

Appendix C: Nucleotide sequence alignment

cls (GenBank accession number KJ956998)

1 aattcactcc tgcccctgca taatagttat aattccctga attattacct gcttcagcac
61 ctactgttct agttgaagtc attctaactg tagataagaa atttgatctt ttatgtgcca
121 tataatctcc tctccaaaac attttatttc cttctaaatc ctttttttgt cctgacattc
181 tatcatgggc atcttgtaat gcggatttat tagctgtatt taaggcaacc attttagtta
241 caatattttt tagatcatcc atgtactcga aagtattatt atatcttcct gttgggttag
301 gatcgtaatt tcctgcatga aaaaggtatt gaacatgatc aattaccatt ttttctaaat
361 taactaacgt ttgaacattg tattgtgttc cttttgtaaa tacgaagtaa tcaagtactg
421 actttaaata ttcttttcca tatccaaaag tgtatatctg tctaccgtta ccactgtggc
481 tataaaatcc ataatctttt tcaatacctt ctcctccttc ttgaatagat gttaatctct
541 tcatcaaatc actaaaatct gttagtaatt ttgcatcttt ttctgctact gctgcaacaa
601 atgcccctat ggttttatct gcaccattag ctccttccat gtggtcttgt tgtaaataag
661 cccattcttg ataatcaata attttcctat aaagagttgg atttttggtt ttgatttctt
721 caattacaaa aacaagtcct ttattcatct ctttaggata tgcaatatgt ctataccacc
781 agtttgaagg tgtatga

sprB (GenBank accession number KM197218)

1 cagggtgcta ctgtggaatt aaataaatag gtttttattt ctttgggttt tctattattt
61 tttgtagagg gaagttttat tatagttttg tgtgtagtat ctgtatctat tggatttaga
121 atctttttttt gtttagatat ttgtagattg ttttgtgtaa ttggatgagt gtttgatgaa
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361 tgaca

Appendix D: Medium and reagent

Media using for *F. columnare*

1. **Anacked and Ordal's medium (AO medium)**

Tryptone	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
Agar	11.0 g
Distilled water	1.0 L

Adjust pH to 7.2. Autoclave at 121°C for 15 minutes.

2. **Flavobacterium columnare growth medium (FCGM)**

Tryptone	8.00g
Yeast extract	0.80g
$MgSO_4(7H_2O)$	1.00g
$CaCl_2(2H2O)$	0.74g
Nacl	5.00g
Sodium Citrate	1.50g
Agar	9.00g

Adjust pH to 7.2, autoclave at 121°C for 15 minutes.

3. Skim milk agar

Tryptone	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
skim milk powder	50 g
Agar	11.0 g
Distilled water	1.0 L

Reagents

1. **Copper (II) sulfate 20%**

Copper (II) sulfate powder	20g
Distilled water	80ml

2. **Crystal violet 1%**

Crystal violet powder	1 g
Distilled water	100ml

3. **Phosphate Buffered Saline 1X (PBS 1X)**

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Distilled water	Adjust volume to 1L

   Adjust pH to 7.4 with HCl or NaOH and sterilize by autoclaving.

VITA

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ACADEMIC PRESENTATION

1. Oral presentation in the topic “Putative virulence gene profiles and pathogenicity of Flavobacterium columnare isolated from red tilapia (Oreochromis sp.) at the 9th Symposium on Diseases in Asian Aquaculture (DAA9) in Ho Chi Minh City, Vietnam from 24-28 November 2014

2. Poster presentation in topic “Relationship between virulence and biofilm formation of Flavobacterium columnare recovered from red tilapia (Oreochromis sp.) at the 39th International Conference on Veterinary Science (39th ICVS 2014) in Bangkok, Thailand during 16th – 18th December 2014.

3. Poster presentation in topic "Virulence factors comparison of high, moderate and low virulence isolates of F. columnare recovered from diseased red tilapia" at CUVC2015 Bangkok, Thailand from April 20-22, 2015.