Original Article

Virulence properties and pathogenicity of *Flavobacterium columnare* in hybrid red tilapia (*Oreochromis* sp.)

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Abstract

Flavobacterium columnare is the causative organism of columnaris disease in freshwater fish, which may exhibit differences in pathogenicity. The virulence properties and the pathogenicity of *F. columnare* in juvenile hybrid red tilapia were investigated using a collection of *F. columnare* isolates recovered from diseased fish. Ten isolates were selected to perform immersion challenge assay in juvenile tilapia. The pathogenicity was highly diverse. Subsequently, three bacterial isolates exhibited different pathogenicity levels, consisting of high, moderate and low virulence (100%, 80% and 0% mortality respectively) and were subjected to virulence properties evaluation e.g. the median lethal dose (LD_{50}), bacterial adhesion, capsular production and biofilm formation. Interestingly, the adhesion ability to the gill surface, biofilm formation and the production of capsular polysaccharide are significantly associated with the highly pathogenic traits of *F. columnare*. These findings suggest that efficient columnaris disease control in farmed fish should target these pivotal virulence properties.

Keywords: *Flavobacterium columnare*, pathogenicity, hybrid red tilapia, virulence properties

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Introduction

Flavobacterium columnare, a non-flagellum, Gram-negative, bacillus bacterium, ubiquitous in freshwater habitats, is the causative agent of columnaris disease, one of the most severe diseases in both wild and cultured freshwater fish (Starliper and Schill, 2011). Several cultured fish species e.g. channel catfish, *Ictalurus punctatus* (Shoemaker *et al*., 2008), striped catfish, *Pangasianodon hypophthalmus* (Tien *et al*., 2012; Dong *et al*., 2015c), rainbow trout, *Oncorhynchus mykiss* (Lafrentz *et al*., 2012), hybrid red tilapia, *Oreochromis* sp. (Dong *et al*., 2015a) and Nile tilapia, *Oreochromis niloticus* (Dong *et al*., 2015b), are known to be susceptible to columnaris disease. To date, the bacterium is distributed worldwide causing tremendous economic loss to the global aquaculture business annually. The clinical signs and pathological changes associated with this disease are usually restricted to external lesions such as skin damage, gill necrosis and fin erosion (Davis, 1922; Declercq *et al*., 2013). The degree of disease severity can be highly diverse due to the variability of bacterial strains, environmental conditions and host species (Declercq *et al*. 2013).

Although *F. columnare* can cause disease at every growth stage of the fish, outbreaks in farms have been predominantly found in small fish (fry to fingerling). In our previous study (Dong *et al*., 2016), we demonstrated that red tilapia fry were highly susceptible *to F. columnare* as 100% cumulative mortality can occur within 24 hours of infection. However, in the case of red tilapia, the juvenile stage is presumed to be the most vulnerable stage for columnaris disease because the animals are subject to stressful conditions during handling and transportation from husbandry sites to the grow-out farms. Unfortunately, information regarding the virulence of *F. columnare* in juvenile tilapia has not been available hitherto. Therefore, the objective of this study is to elucidate the pathogenicity of *F. columnare* in juvenile hybrid red tilapia. In this experiment, the Thailand originated strains (isolated and characterized by Dong *et al*., 2015) underwent further genotypic characterization using virulence gene profiling assays. Representative strains of distinct genetic profiles were used in the experimental challenge assay. In addition, the comparison of *in vitro* virulence properties between highly virulent and low virulent strains of *F. columnare* were also investigated in our study.

Materials and Methods

Bacterial isolates: Ten bacterial isolates of *F. columnare* isolated from diseased red tilapia (*Oreochromis* sp.) in Thailand from 2012 to 2013 were used in this study (Table 1). The bacteria were phenotypically and genotypically characterized from our previous publication (Dong *et al*., 2015a).

Prior to the experiments, the bacterial isolates were recovered from vial stocks preserved in a -80 °C freezer. A loopful of stock solution was streaked directly on Anacker and Ordal (AO) agar supplemented with neomycin (Sigma) 0.5 mg/ml and polymyxin B (Sigma-Aldrich) 200 UI/ml (Anacker & Ordal, 1955). The bacterial culture plates were incubated at 28 °C for 48 hours then a single colony was transferred to AO broth and incubated in the same manner. Subsequently, *F. columnare* inoculum was subjected to genomic DNA extraction.

Table 1 Phenotypic, genotypic characteristics and virulence of 10 *F. columnare* isolates used in immersion challenge assay

Isolate	Colony morphotype[a]	Challenge dose (cfu/ml water)	Cumulative mortality (n=20 fish)	Time reaching 50% cumulative mortality
CUVET1371	Non-rhizoid	5.7×10^8	10%	NA
CUVET1372	Non-rhizoid	4.2×10^8	0%	NA
CUVET1201	Non-rhizoid	5.6×10^7	20%	NA
CUVET1360	Rhizoid	1.8×10^7	80%	6 days
CUVET1362	Rhizoid	0.9×10^7	100%	Less than 2 days
CUVET1365	Rhizoid	1.2×10^7	50%	9 days
CUVET1214	Rhizoid	1.6×10^7	60%	7 days
CUVET1341	Rhizoid	0.7×10^7	80%	9 days
CUVET1345	Rhizoid	1.2×10^7	30%	NA
CUVET1367	Rhizoid	2.8×10^7	70%	3 days
control	-	0	0%	NA

Underlined bold-faced codes were isolates subjected to virulence properties investigation

In vivo pathogenicity of F. columnare in juvenile hybrid red tilapia

Fish and husbandry: Apparently healthy juvenile red tilapia fingerlings (6 ± 1 g body weight) were purchased from a commercial husbandry. The fish were diagnosed to be free from ecto-parasitic infestation and flavobacterium infection based on 5% of the animals (~15 fish) being randomly selected and yielding negative results for standard wet-mount investigation, and *F. columnare*-specific PCR from the gill specimen (Welker *et al*., 2005). The fish were acclimatized in 100 L tanks with dechlorinated water at 25 ± 3 °C for 14 days. Other parameters including pH, dissolved oxygen (DO) and ammonia were measured daily using commercial test kits (AQUA-VAB, Thailand), which were averaged to pH 7.4 ± 0.1; DO of 4-5 mg L^{-1} and ammonia <0.01 mg L^{-1}, respectively. The fish were fed daily (3% body weight) with commercial dry pelleted fish feed (Aquarium Fish Food F-1, Plus Value Enterprise Co., Ltd, Thailand). All procedures involving the fish were performed in accordance with the Chulalongkorn University Animal Care and Use Committee rules (Approval No. 1431031).

Experimental immersion challenge: In total, ten bacterial isolates from different geographical origins and genotypes were chosen for examination of their pathogenicity using immersion challenge assay (Table 1). The genotypes of bacteria were categorized using current virulence gene profiling incorporated with 16S-23S rRNA intergenic spacer region (ISR) sequencing which had been carried out in our previous publication (Dong *et al*., 2015a). To prepare the bacteria, one pure colony of *F. columnare* grown on AO agar was inoculated in 300 mL of AO broth at 28°C with 150 rpm shaking for 24 hrs. Bacterial density was adjusted by spectrophotometry to OD_{540} = 0.8 prior to the challenge, while the actual concentration of flavobacterium inoculum was determined by the standard plate count method on AO agar supplemented with 1 µg mL^{-1}Tobramycin (Sigma, Germany). The number of bacterial colonies on plates was counted after 48 hrs of incubation at 28 °C.

Two hundred and twenty red tilapia fingerlings were assigned into 11 groups consisting of ten challenge groups and one control group. To challenge the fish, 300 mL of flavobacterium inoculum was poured into 2.7 L of water to reach a final volume of 3 L. The accurate challenging concentration was between 0.7 x 10^7 to 5.7 x 10^8cfu/mL water (Table 1). A single group of ten fish was immersed in a tank containing a given quantity of bacteria for 1 hr. Subsequently, 27 litres of *F. columnare* –free water was gently added to the tanks. For the control group, sterile AO broth was used instead of bacterial suspension. The cumulative mortality was recorded for 14 days and all experimental challenges were performed in duplicate.

Comparison of bacterial virulence properties: For the further investigation of virulence properties, three bacterial isolates, i.e. CUVET1362, CUVET1360 and CUVET1372, representing high, moderate and low virulence, respectively, were selected for further investigation of their virulence properties.

Median lethal dose (LD_{50}): Five different concentrations of bacteria (10^8, 10^7,10^6, 10^5 and 10^4 CFU/mL water) were used for the immersion challenges described above. The LD_{50} of bacteria was calculated following the method of Reed and Muench (1938) after 14 days post-challenge**.**

Bacterial adhesion ability: The adhesion ability was determined using the *ex vivo* fish gill model according to Kunttu *et al*. (2009). Briefly, the gill arches were collected from healthy red tilapia fingerlings and washed three times in sterile PBS. Then, 30 mg of the gill specimen was immersed in 10 mL of the 24-hr-grown bacteria (OD_{540}=0.8) in AO broth, followed by incubation at 28 °C for two hours with 100 RPM shaking. After incubation, the gills were rinsed slightly with PBS three times and homogenized in a microcentrifuge tube using plastic polypropylene pestle. The number of bacteria attached to the gills was determined by the standard plate count method. The number of bacteria attached to the gills was demonstrated as CFU/g fish gills.

Bacterial polysaccharide capsule: The thickness of a polysaccharide capsule of *F. columnare* isolates was measured according to the method described by Klesius *et al*. (2010). Five microlitres of 24-hr-grown bacteria in AO broth was mixed with two drops of 5% skimmed milk on a clean glass slide and a thin smear was then prepared afterwards. The slide was air-dried at room temperature and stained with 0.1% cyrstal violet solution for 1 min. The slide was rinsed with 20% copper sulphate to remove the excess crystal violet. The bacteria were observed using a light microscope. Bacterial capsule thickness was measured from five bacterial cells by Motic images plus 2.0 software (Motic China Group Co., Ltd) applied to the photograph which was recorded digitally under 100X microscopic filed.

Bacterial biofilm production: The biofilm production capacity of *F. columnare* was evaluated by the methodology described by Alvarez *et al*. (2006). Overnight grown bacteria in AO broth were diluted 100 times with new AO broth. One-hundred microlitres of bacterial dilution was transferred to the 96-well U-shaped microtiter polystyrene plates (Nunc, Wiesbaden, Germany brand) and incubated at 28°C for 48 hrs (8 replicates per bacterial isolate). Bacterial cells which were unattached to the plate were then removed by gently washing the plate four times with sterile distilled water. Later, the plate was stained with 150µl of 1% crystal violet and incubated for 20 mins at room temperature followed by four times of washing with sterile distilled water. Finally, 200µl of 30% acetic acid was added to the wells and incubated at room temperature for ten minutes. Biofilm formation was measured by microplate reader (BioTek Instruments Inc, Winooski, VT) at OD_{600}. For the positive control, biofilm-producing bacteria, namely *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, were used instead of *F. columnare* (Cremet *et al*., 2013), while a suspension without bacteria was used as the negative control.

Proteolytic activity: To determine the protease activity, 5µL of overnight grown bacterial cultures (OD_{540}=0.8) was applied to AO agar containing 1.5% skimmed milk, followed by 28°C incubation for 48 hrs. After incubation, the clear zone was measured to determine bacterial proteolysis capability (Pandey *et al*., 2010).

Data Analysis: All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., IL, USA). The one-way ANOVA statistics followed by LSD multiple comparison were used to compare virulence properties among different *F. columnare* isolates.

Results

Pathogenicity of F. columnare in juvenile hybrid red tilapia: The immersion challenge assay indicated diverse pathogenicity among *F. columnare* isolates. In general, the isolate with non-rhizoid morphotype exhibited non- to low-pathogenicity (0 to 20% cumulative mortality). On the other hand, challenging with the isolates of rhizoid morphotype caused moderate to high mortality (30 to 100%) (Table 1). There was no correlation between genetic profiles and the virulence of bacteria as each genotype of bacteria consisted of both virulent and avirulent strains. Herein, three bacterial isolates showing high (CUVET1362; 100% mortality), moderate (CUVET1360; 80% mortality), and low virulence (CUVET1372; no mortality) were selected for a comparison of bacterial virulence properties.

Virulence properties of high-, moderate- and low-virulent isolates

Median lethal dose (LD_{50}): The high-virulent (HV), moderate-virulent (MV) and low-virulent (LV) isolates were further evaluated for their LD_{50} value in order to determine the accurate pathogenicity of the bacterial isolates. In this instance, the LD_{50} of HV, MV and LV isolates were 1.23 x 10^6, 4.45 x 10^6 and higher than 10^8 CFU mL^{-1}, respectively (Table 2).

Bacterial adhesion ability: The result for bacterial adherence of the three isolates of HV, MV and LV of *F. columnare* to the fish gills is presented in Table 2. In particular, the numbers of bacterial cells that adhered to the fish gills of the HV and MV isolates were significantly higher than the LV isolate (p<0.05).

Bacterial polysaccharide capsule: By using the capsule staining method, it was obvious that all three isolates of *F. columnare* were able to produce capsules. However, the thickness of the capsule in HV and MV isolates was relatively higher than that of LV isolate (p<0.05). The capsule thickness of each bacterial isolate is included in Table 2.

Biofilm formation: The result shows that the HV, MV and LV isolates were capable of forming the biofilm on the polystyrene surface, as shown in Table 2. The amount of biofilm as determined spectrophotometrically clearly indicated the highest biofilm production capability of HV isolate (p<0.05), followed by MV and LV isolates, respectively.

Bacterial proteolytic activity: The digestion on casein-agar plate indicated protease activity in the HV, MV and LV isolates. However, the proteolytic activity of these bacteria was not significantly different (Table 2).

Table 2 Comparison of virulence properties among high, moderate and low virulent isolates of *F. columnare*.

Bacterial virulence properties	High virulent (CUVET1362)	Moderate virulent (CUVET1360)	Low virulent (CUVET1372)
LD_{50} (CFU mL^{-1})	1.23 x 10^6	4.45 x 10^6	>10^8
Bacterial adhesion (CFU g^{-1} fish gills)	52×10^6 ± 7×10^6 [a]	29×10^6 ± 11×10^6 [b]	7×10^6 ± 1×10^6 [c]
Biofilm production (OD_{600})	0.59 ± 0.08 [a]	0.27 ± 0.02 [b]	0.13 ± 0.02 [c]
Capsule thickness (µm)	0.6 ± 0.2 [a]	0.6 ± 0.2 [a]	0.4 ± 0.2 [b]
Proteolytic activity (mm)	18 ± 0.7 [a]	23 ± 0.7 [a]	22 ± 0.8 [a]

[*] LD_{50} was not used for statistical analysis

[a,b,c]Dissimilarity of alphabet superscript indicates the statistical difference (p<0.05)

Discussion

Due to the phenotypic comparison, HV, MV and LV isolates were clearly distinguished according to colony morphologies and some virulence properties, e.g. adhesion and biofilm production. The LV is the only isolate which exhibited a non-rhizoid colony morphotype. In accordance with previous publications, the rhizoid morphotype exhibited higher virulence than its non-rhizoid counterparts (Kunttu *et al*., 2011; Laanto *et al*., 2014; Dong *et al*., 2016). For adhesion ability, our findings were consistent with previous studies suggesting a significant relationship between *Flavobacterium* virulence and its capability to adhere to gill tissue (Decostere *et al*., 1999; Shoemaker *et al*., 2008). The adhesion ability of *F. columnare* has been reported to be associated with other bacterial virulence properties, such as gliding motility and biofilm formation (Cai *et al*., 2013; Shrivastava *et al*., 2013).

The biofilm of *F. columnare* was proven to be one of the most important virulence factors as it could enchance the augmentation of bacteria on the gill surface and consequently interfere with oxygen uptake (Declercq *et al*., 2013). Moreover, biofilm formation also helps the bacteria to evade host-mediated complement immunity and phagocytosis (Domenech *et al*., 2013). Likewise, the high biofilm-producing trait

observed in HV isolates emphasizes the importance of *Flavobacterium*'s biofilm in bacterial pathogenesis. The HV and MV isolates also possessed capsule production capability superior to the LV isolate. The bacterial capsule is a polysaccharide layer commonly known as a significant virulence factor promoting the immune evasion potency of several pathogenic bacteria, including *Flavobacterium* sp. (Decostere *et al*., 1999). Additionally, the *Flavobacterium* capsule was able to elicit host-bacterial surface adhesion. Capsular formation was able to intensify adherence of the pathogen, which may mediate cell-to-cell and cell-to-surface attachment, inducing biofilm formation and the persistence of bacteria on gill surface (Kim *et al*., 1986). It is possible that the significantly higher adhesion ability of HV isolate to *ex vivo* gill tissue could be linked to the high capsule production phenotype of certain bacteria.

Of note, the bacterial protease activity was included in the *in vitro* virulence assays because the proteases have been recognized, in several publications, as the significant virulence factor of *F. columnare* causing lesions in the infected fish due to connective tissue degradation (Kunttu *et al*., 2011; Newton *et al*., 1997; Suomalainen *et al*., 2006). In the current study, the bacterial proteolytic activity on skimmed milk supplemented agar seems to be unrelated to the virulence of *F. columnare*. However, evaluation of proteolytic activity using the skimmed milk agar method may lead to a false positive outcome since the bacteria-derived hydrolases and acidic metabolites could also produce clear zones (Jones *et al*., 2007). Therefore, whether the HV, MV and LV isolates exhibited different protease activity is still uncertain.

In conclusion, analyses of virulence properties of *F. columnare* isolates revealed that the adhesion ability to the gill tissue surface, biofilm formation and the production of capsular polysaccharide are highly related to the high-pathogenic traits of *F. columnare*. Thus, the design of potential countermeasures which target these pivotal virulence properties may provide more efficient protection against columnaris disease in farmed fish.

Acknowledgements

This study was supported by the Scholarship Program for ASEAN Countries, the 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund no. GCUGR1125582086M), a grant for Fish Infectious Diseases Research Unit (FID RU), Faculty of Veterinary Science, Chulalongkorn University, The In-depth Research Cluster Project of Chulalongkorn University (Grant number: CU-59-023-FW), and Chulalongkorn University Ratchadaphiseksomphot Endowment Fund (Grant number: CU-GR_61_013_31_003).

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