Thesis for the Degree of Master of Science

Differences in virulence and drug sensitivity of different *Miamiensis avidus* isolates and effects of RNA interference-mediated knock-down of a cysteine proteinase on the ciliate growth

by

Chizuha Wakabayashi

Department of Aquatic life medicine

The Graduate School

Pukyong National University

February 23, 2018

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Advisor: Prof. Ki Hong Kim

by
Chizuha Wakabayashi

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Chizuha Wakabayashi

Approved by:

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(Chairman) Do Hyung Kim

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(Member) Hyun Do Jeong (Member) Ki Hong Kim

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Wakabayashi Chizuha

부경대학교 대학원 수산생명의학과

요약

양식 넙치 (*Paralichthys olivaceus*) 에서 발병하는 주요 기생충성 질병 중 하나인 스쿠티카증을 일으키는 *Miamiensis avidus* 는 최근 연구에서 혈청형에 따라 특이적인 병원성을 나타낼 뿐만 아니라 혈청형이 유사할 지라도 다른 장소에서 분리된 분리주에 따라서도 다른 병원성을 나타내는 것으로 보고되고 있다. 이번 실험에서는 각각 다른 양식장에서 분리한 *M. avidus* 의 3 가지 분리주 (분리주 1~3) 를 사용해 in vivo 병원성과 약물 감수성의 차이에 대해 분석하였다. 분리주 3 을 침지 감염시킨 넙치 치어는 분리주 1 혹은 2 를 침지 감염시킨 넙치보다 훨씬 높은 폐사율을 보였으며, 넙치 혈청에 대한 충의 저항성 분석에서도 분리주 3 이 가장 높은 저항성을 나타냈다. 또한 *M. avidus* 가 병원성을 나타내는데 결정적인 역할을 한다고 보고된 cysteine proteinase 의 분비 활성을 분석 하였을 때도 분리주 3 이 가장 높은 값을 나타냈다. 추가적으로 cysteine proteinase 억제제인 E-64 를 처리한 후 넙치 혈청에 대한 저항성을 분석하였을 때 모든 분리주의 저항성이 크게 감소된 것을 관찰하였으며, 이는 결과적으로 *M. avidus* 의 cysteine proteinase 는 충이 숙주에게 병원성을 나타내는데 중요한 역할을 하고 있다는 보고를 뒷받침하는 결과이다. 따라서 이 cysteine proteinase 를 억제시키는 것으로 *M. avidus* 의 병원성을 약화시킴으로 스쿠티카충에 의한 피해를 줄일 수 있다는 가정으로 다음 실험을 접근하게 되었다. 하지만, cysteine proteinase 분비 활성이 큰 차이가 없었던 분리주 1 과 분리주 2 가 혈청 저항성과 in vivo 병원성 분석에서 분리주 2 가

상대적으로 높은 저항성과 병원성을 가지는 결과를 관찰하였다. 이는 cysteine proteinase 가 병원성 결정에 중요한 역할을 하지만 그 이외에도 다른 인자들이 충의 병원성 결정에 관여함을 시사하였다. 또한 3 가지의 스쿠티카 분리주는 기생충성 질병치료제인 mebendazole 과 bithionol 에 대한 약물 감수성의 차이를 나타냈다. 비록 스쿠티카증에 대한 효과적인 약물 개발이 지금까지 전무하지만, 차후 효과적인 화학치료제를 개발하기 위해서는 분리주에 따른 약물 감수성 또한 고려해야 할 요소임을 알 수 있었다.

앞서 실험을 통해 관찰한 *M. avidus* 병원성과 cysteine proteinase 의 관계를 기반으로 RNA 간섭을 통해 충의 cysteine proteinase 의 발현을 억제시키고 이것이 충의 성장에 미치는 영향을 분석하였다. RNase III knock-out *Edwardsiella tarda* 영양요구성 돌연변이체를 cysteine proteinase 를 억제하는 긴 이중 가닥 RNA 를 생산하는데 사용하였고, 이를 *M. avidus* 분리주 3 에 먹이로 사용하여 RNA 를 충으로 전달하였다. 조작된 *E. tarda* 를 섭취한 충에서는 cysteine proteinase 유전자가 전사되는 양이 큰 폭으로 감소되었으며, cysteine proteinase 분비 활성 또한 큰 폭으로 감소되는 것을 확인하였다. 뿐만 아니라 RNA 간섭에 의한 cysteine proteinase 의 발현 억제는 충의 증식 또한 감소시키는 것을 관찰하였다. 이러한 결과들은 cysteine proteinase 가 *M. avidus* 에 대한 치료 및 예방을 위한 백신의 잠재적인 표적으로 사용될 수 있음을 나타냈다.

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Introduction

Scuticociliatosis is a disease caused by scuticociliates and recognized as one of the most important parasitological problems in aquaculture farms. Scuticociliates are facultative, histophagous parasites, and can invade into various internal organs such as liver, kidney, muscle and brain, in which they ingest host tissue components which resulted in the serious destruction of tissues.

Scuticociliates are in the subclass Scuticociliatia of the class Oligohymenophorea, and ciliates in the order Philasterida are recognized as marine scuticociliates (Feng et al., 2015). In the order Philasterida, 16 families are known, and, among them, ciliates in 4 families are known to cause scuticociliatosis in marine fish (Thompson and Moewus, 1964, Cheung et al., 1980, Evan and Thompson, 1964, Dragesco et al., 1995). Till now, 4 species have been reported to cause severe scuticoiliatosis in marine fish, *Pseudocohnilembus persalinus* in olive flounder (Kim et al, 2004) and rainbow trout (Jones et al., 2010), *Philasterides dicentrarchi* in sea bass (Dragesco et al., 1995) and turbot (Iglesias et al., 2001), *Miamiensis avidus* and *Uronema marinum* in olive flounder (Jung et al., 2007, Kim et al., 2004). Several studies have reported that *P. dicentrarchi* and *M. avidus* have similar characteristics, and *P. dicentrarchi* is thought to be the synonym of *M. avidus* (Jung et al. 2007). In general, identification of species is done through analysis of morphological characteristics and small subunit ribosomal RNA gene analysis. In a latest study (Fellipe et al., 2017), *P. dicentrarchi* was classified as a different species from *M. avidus* based on differences in morphology and 18S rRNA, α- and β-tubulin gene sequences. Therefore, for more accurate identification of scuticociliates, it would be better to combine morphological, biological, molecular and serological approaches (Fellipe et al., 2017). There are reports that ciliates harvested at

different places can have a difference in pathogenicity. Furthermore, different isolates harvested at different time did not show any cross protection (Piazzón et al., 2008), which suggests that different isolates can have different protective antigens.

In Korea, scuticociliates infect mainly olive flounder (*Paralichthys olivaceus*) that is one of the most important aquaculture fish. Formaldehyde (produced as a drug for aquatic animals) has been the only approved drug for the control of scuticociliatosis in Korea. However, as formaldehyde cannot be a therapeutic drug against scuticociliates parasitized in the internal organs of fish, other drugs that possess a therapeutic ability against internally infected scuticociliates have been required. In our in vitro preliminary experiments, we tested scuticocidal activity of 10 drugs, and selected 3 drugs (doxycycline, mebendazole and bithionol) that were proven to have higher scuticocidal ability than others. Previously, we reported the treatment potential of doxycycline or doxycycline plus CpG-ODN 1668 in olive flounder fingerlings infected with *M. avidus* (Kang and Kim, 2015). However, the treatment efficacy was severely reduced in heavily infected fish (not reported). Recently, The National Institute of Fisheries Science in Korea announced that mebendazole was effective for the treatment of *M. avidus* infection in olive flounder (not published). Iglesias et al. (2002) reported the strong in vitro scuticocidal activity of bithionol sulfoxide, and Madsen et al. (2000) reported a high treatment efficacy of bithionol against trichodiniais in eel (*Anguilla anguilla*). In the selection of effective drugs, the possibility of having a different sensitivity to drugs among different *M. avidus* strains or isolates cannot be excluded. Thus, in this study, we analyzed whether different isolates of *M. avidus* have a different sensitivity to doxycycline, mebendazole, and bithionol.

It has been reported that parasites secrete some proteinases when they infect hosts. Proteinases play the role of digesting long protein chains by ripping the peptide bonds that link amino acid residues. Proteinases of parasites induces the host cell's apoptosis and involves the pathogenesis in several parasitic infections (Shin et al., 2014, Parama et al., 2007).

Moreover, cysteine proteinases of parasites are known the most intra and extracellular proteinases in ciliates and regulate several pathological actions that important for host-parasite interactions (Piazzon et al., 2011). From these, we thought that ciliate's proteinases would be useful for the control of scuticociliates. Alvarez-Pellitero et al. (2004) reported that *P. dicentrarchi* isolates sampled from different localities showed differences in virulence to turbot. Piazzón et al. (2011) reported that the virulence of *P. dicentrarchi* to turbot might partly be related to the resistance against host's complement-mediated killing, and the proteinases of *P. dicentrarchi* lysate could degrade turbot complement component and immunoglobulins. However, little information is available on the association of the secreted proteinases activity with in vivo virulence among different isolates of *M. avidus*. Thus, in the present study, we investigated on the differences among different isolates of *M. avidus* in the virulence to olive flounder, in the resistance against olive flounder serum scuticocidal activity, and in the activity of secreted proteinases.

RNA interference (RNAi) fulfilled by small non-coding RNAs (20-24 nt) is a biological phenomenon that regulates gene expression by either the inhibition of translation or the degradation of messenger RNAs that has sequences corresponding to the small RNA. Using this reaction, RNAi can decrease target gene expression by insert artificially prepared targeting siRNA or long double-stranded RNA (dsRNA). In this study, we investigated the effect of long dsRNA that has the sequence of a cysteine proteinase of *M. avidus* on the growth of the ciliates. To efficiently deliver the long dsRNA to *M. avidus*, RNaseIII gene knock-out auxotrophic *Edwardsiellar tarda* mutant (*ΔalrΔrncΔasd E. tarda*) that was developed in a previous study (Kim et al., 2017) was used. As this mutant *E. tarda* has no functional aspartate semialdehyde dehydrogenase gene (asd) and alanine racemase gene (alr), they cannot survive without the supplementation of D-alanine and DAP in culture medium. RNaseIII is an endoribonuclease that cleaves double-strand RNA molecules. Thus, RNaseIII knock-out mutant *E. tarda* cannot cleave long dsRNAs, which make it possible to be used as a long dsRNA delivery vehicle. In the present study, to know whether the

inhibition of the cysteine proteinase activity of *M. avidus* can be a way to control scuticociliatosis, ciliates were fed with *ΔalrΔrncΔasd E. tarda* that was transformed with plasmids that contain an expression cassette for long dsRNA corresponding to the cysteine proteinase of *M. avidus*, and analyzed the effect of RNAi-mediated knock-down of the cysteine proteinase on the replication of *M. avidus*.

Materials and methods

1. Ciliates and in vitro culture

M. avidus used in this study were isolated from the brain of olive flounder (*Paralichthys olivaceus)* at 2007 (isolate 1), 2014 (isolate 2) and 2015 (isolate 3) in different local farms in Korea. Ciliates were cultured by feeding *Epitheiloma papulosum cyprini* (EPC) cells grown with Leibovitz-15 Medium (L-15, Sigma) supplemented with penicillin-streptomycin (Pen Strep, 10000 U/ml, Gibco) and 4% fetal bovine serum (FBS, Young In Frontier) at 20℃.

2. Sequencing of mitochondrial cytochrome c oxidase 1 (*cox1*) gene

Approximately 1×10^6 cells of each ciliate isolate were pelleted by centrifugation at 3000 rpm for 5 min at 4℃ and washed three times with phosphate buffered saline (PBS, pH 7.0). Genomic DNA was collected using Exgene™ Clinic SV (GeneAll), and 50 ng of genomic DNA was used in 20 μl of PCR reaction mixture containing 10 pmoles of *M. avidus* cytochrome c oxidase 1 primer (Table 1) and Hipi plus × 5 PCR premix (Elpis) to perform PCR. The reaction followed for 30 cycles using an automated thermal cycler at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ 45 sec. The PCR products was runned on an agarose gel (0.8%), purified using Expin™ Gel SV (GeneAll), subcloned into pGEM T-easy vector (Promega),

and sequenced (Macrogen sequencing service).

3. Serum scuticocidal activity

For serum scuticocidal activity analysis, 3 olive flounder (about 200-250 g) were obtained from a fish farm and sera were isolated. After confirming no agglutination activity of each heat-inactivated serum against the 3 scuticociliate isolates, sera were serially diluted (1/2–1/256) using Hank's balanced salt solution (HBSS, Sigma) and 96-well flat-bottomed plates. Each ciliate isolate was added to the wells (1×10^2 ciliates/well) of the plate containing the serially diluted sera, incubated at 20°C, and observed for 3 h. The scuticocidal titer of each serum was the last dilution at which 100% of the ciliates were lysed or non-motile, which was observed under an inverted microscope at 40–100× magnification. In all assays, control wells containing heat-inactivated serum and HBSS alone were included.

4. In vivo virulence

Olive flounder fingerlings weighing approximately 1.5 g were obtained from a local fish hatchery in Korea, and were acclimated for 1 week at 20-22°C. During the acclimation period, 10 fish were randomly sampled and were confirmed free from scuticociliates by the microscopic observation of skin and internal organs. Fish were randomly divided into 10 groups with 10 fish, and were immersed in seawater containing 1 x 10^5, 1x 10^6, or 1 x 10^7/tank of each ciliate isolate for 3 days. The fish in the control group were not exposed to the ciliates. Mortality was recorded daily for 2 weeks post-challenge.

5. Secretory proteinase activity

The secretory proteinases activity of each ciliate isolate was analyzed using azocasein (Sigma) as the substrate. Briefly, 1 x 10^8 ciliates of each isolate were washed 3 times with HBSS at 3,000 rpm for 5 min, and incubated in HBSS for 12 h at 20°C, then, isolated each supernatant by centrifugation at 7000 rpm for 5 min at 4°C. The isolated supernatant was filtered with 0.2 μm filter, and kept at -80°C. The protein concentration in each supernatant was measured using bicinchoninic acid assay. The supernatant (100 μl; 2.5 μg protein/μl) was incubated with 100 μl of azocasein (10 mg/ml) at 25°C for 1 h, then, 0.75 ml of 5% trichloroacetic acid (TCA, Sigma) was added to terminate the reaction. After centrifugation at 13,000 g for 5 min at 4°C, the dye released was determined spectrophotometrically at 405 nm against the blank (the same incubation solution but with distilled water instead of crude extract).

6. Effect of E-64 on the resistance of *M. avidus* against serum scuticocidal activity

To know the effect of E-64 on the resistance of scuticociliates against serum killing activity, ciliates were incubated with 10 μm of E-64 for 30 min at 25°C, then, mixed with serially diluted (1/4~1/256) olive flounder sera in a 96-well plate.

7. Sensitivity to drugs

Scuticociliates of each isolate were placed on 24-well plates with 1 x 10^3 ciliates/well, and were exposed to 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ppm of doxycycline (Sigma) that was dissolved in phosphate buffered saline (PBS), or 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ppm of mebendazole (Sigma) that was dissolved in formic acid and then diluted with PBS, or 0.1, 0.2, 0.4, 0.6, 0.8, and 1 ppm of bithionol (Sigma) dissolved in dimethyl sulfoxide (DMSO, Sigma) then diluted with PBS. Control wells for mebendazole and bithionol contained formic acid or DMSO at the same concentration of each drug in the wells with the highest concentration. The plates were incubated at 20°C, and examined mortality for 24 h.

8. Construction of long dsRNA producing vector

A pUC57 plasmid (ThermoFisher Scientific)-based vector having two modified λ phage P_R promoters (pdpR-GFP SDM) was constructed in a previous study (Kim et al., 2017) to produce long dsRNAs. We cloned a partial region (307-786bp) of the cysteine proteinase mRNA of *M. avidus* using a primer pair (Table 1) that contains BglII or AvrII site. The amplified product was cloned into pGEM-T easy vector (Promega), and digested by BglII, AvrII (Fermentas) enzymes, then, inserted between the double P_R promoters in pdpR-GFP SDM plasmid that was also digested with the same enzymes, and named the plasmid as pdpR-CP SDM.

9. Expression of cysteine proteinase's long dsRNA in *ΔalrΔrncΔasd E. tarda*

The constructed pdpR-CP SDM plasmids were introduced into *ΔalrΔrncΔasd E. tarda* via electroporation (BioRad). Transformed colonies were grown in LB broth containing ampicillin (100 mg/ml), D-alanine (100 mg/ml), DAP (50 mg/ml) at 37℃ for overnight. Pellet was collected (10^{10}) by centrifugation at 5000 rpm 10 min and total RNA was purified using Trans Zol Up (Trans Genic) according to manufacturer's manual. The 50 μl of purified RNA (50 μg /ml) was treated with 1 μl DNase I (GeneAll) and 1 μl RNase A (GeneAll) at 37℃ for 30 min to remove genomic DNA and single-strand RNA, respectively, and dsRNA was isolated by Riboclear kit (GeneAll) according to manufacturer's manual. Isolated long dsRNA was loaded in 1% agarose gel and confirmed dsRNA bands by LS-4000. The *ΔalrΔrncΔasd E. tarda* that having no plasmid or expressing the long dsRNA of the green fluorescent protein (GFP) was used as control.

10. Feeding of ciliates with auxotrophic *Edwardsiella tarda* (*ΔalrΔrncΔasd E. tarda*)

RNase III knockout auxotrophic *E. tarda* mutant (*ΔalrΔrncΔasd E. tarda*) expressing GFP was subcultured with LB broth supplemented ampicilline (100mg/ml, affymetrix), D-alanine (100mg/ml, sigma), DAP (50mg/ml, sigma) overnight at 27℃, washed three times and diluted with PBS. After OD540 value of *ΔalrΔrncΔasd E. tarda* were matched for 1.0, ciliate isolate 3 (1×10^5) added in 6-well plate having each concentration of *ΔalrΔrncΔasd E. tarda* (1×10^5~1×10^9), and the number of bacteria fed by ciliates was monitored by a fluorescent microscope (Olympus IX70).

11. Feeding of ciliate with *ΔalrΔrncΔasd E. tarda* expressing long dsRNA

The effect of feeding ciliates with *ΔalrΔrncΔasd E. tarda* containing pdpR-CP SDM plasmids was investigated by analyzing the growth of ciliates, the quantification PCR of cysteine proteinase mRNA and cysteine protease activity. Three bacteria groups, *ΔalrΔrncΔasd E. tarda (E. tarda*), *ΔalrΔrncΔasd E. tarda* that expressing the long dsRNA of GFP (eGFP) and *ΔalrΔrncΔasd E. tarda* that expressing the long dsRNA of cysteine proteinase (cysteine) were used in this experiment.

11.1. Ciliate growth

M. avidus isolate 3 and *ΔalrΔrncΔasd E. tarda*, *ΔalrΔrncΔasd E. tarda* containing pdpR-GFP SDM, and *ΔalrΔrncΔasd E. tarda* containing pdpR-CP SDM were co-incubated in an e-tube to a final ratio of 10^7 bacteria versus 10^4 ciliates at 20℃. After 24, 48 and 72 h, the number of ciliates were counted using a hemocytometer.

11.2. Quantitative RT-PCR

5×10^5 *M. avidus* isolate 3 and 5×10^8 bacteria (*ΔalrΔrncΔasd E. tarda*, *ΔalrΔrncΔasd E. tarda* containing pdpR-GFP SDM, and *ΔalrΔrncΔasd E. tarda* containing pdpR-CP SDM) were put in T-25 culture flasks containing 10 ml of HBSS at 20℃ for 24 hours, then, ciliates were collected by centrifugation at 3000 rpm for 5 min. The total RNA was purified by Trans Zol Up (Trans Genic) following manufactural protocol, treated DNase I to remove genomic DNA by Riboclear™ (GeneAll) and synthesized cDNA by Hyperscript RT premix with Oligo dT (GeneAll). Quantification RT-PCR for analyzing the expression of the cysteine proteinase gene and elongation factor gene as a reference (GeneBank: KF952262.1, used primers presented in Table1) were conducted using LC480 (Roche).

11.3. Secretory proteinase activity

5×10^5 *M. avidus* isolate 3 and 5×10^8 bacteria (*ΔalrΔrncΔasd E. tarda*, *ΔalrΔrncΔasd E. tarda* containing pdpR-GFP SDM, and *ΔalrΔrncΔasd E. tarda* containing pdpR-CP SDM) were put in T-25 culture flasks containing 10 ml of HBSS at 20℃ for 24 hours, then, ciliates were collected by centrifugation at 3000 rpm for 5 min. The number of ciliates in each group was adjusted to 5×10^5, and 100 μl Azocasein (sigma, 10 mg/ml) was added to the ciliates pellet, then, incubated at 20℃. After 1 h incubation, 5% TCA (sigma) was added, and centrifuged 13,000 rpm for 5 min at 4℃, then, the supernatant absorbance was measured at 405 nm by spectrophotometer.

Table 1. Summary of primers used in this study

1- 1. For sequence of *Miamiensis avidus* cytochrome c oxidase 1 gene

Name of primer	Sequence (5' to 3')
Scu-COX-F	ATTAGATTAGAATTAGCTCATCCAG (25mer)
Scu-COX-R	AAAATCAAAAAATGTAGTTTGTCAATGTC (29mer)

1- 2. For construction of plasmid

Name of primer	Sequence (5' to 3')
BglII-cys307-F	AGATCTGTTTCCGGAATTACCCACCA (26mer)
Cys786-AvrII-R	CCTAGGGATGGTGGCGGGGTTGTTTTA (27mer)

1- 3. For quantitative RT-PCR

Name of primer	Sequence (5' to 3')
scuEF-RT-F	GTACAAAGGACCCACCCTTATT (22mer)
scuEF-RT-R	GGTTCCGATTCCTCCGATTT (20mer)
Scucys894-RT-F	CACCGCTGGATCTGAACCTT (20mer)
Scucys1033-RT-R	AGAGGGGGTAGGCAACGTAT (20mer)

Results

1. *cox1* gene sequence and differences in resistance against serum killing activity

The cytochrome oxidase 1 gene sequences of 3 isolates were completely matched and 100% agreement with *Miamiensis avidus* strain WD4 cytochrome oxidase subunit 1 gene (sequence ID: EU831213.1) by confirmation using BLAST (Basic Local Alignment Search Tool, U.S. National Library of Medicine) service.

The result of serum scuticocidal activity showed differences in resistance among 3 isolates. Isolate 3 had the highest resistance for serum scuticocidal activity and the difference was more than double of isolate 1. The serum killing activity against scuticociliates was significantly enhanced by pre-incubation of the ciliates with E-64, a cysteine proteinase inhibitor. The resistance of each isolate compared with scuticocidal activity using E-64, isolate 3 might have much cysteine proteinases than other isolates. The scuticocidal activity of isolate 2 was higher than isolate 1, the activity treated with E-64 was also higher than isolate 1 (Fig. 1).

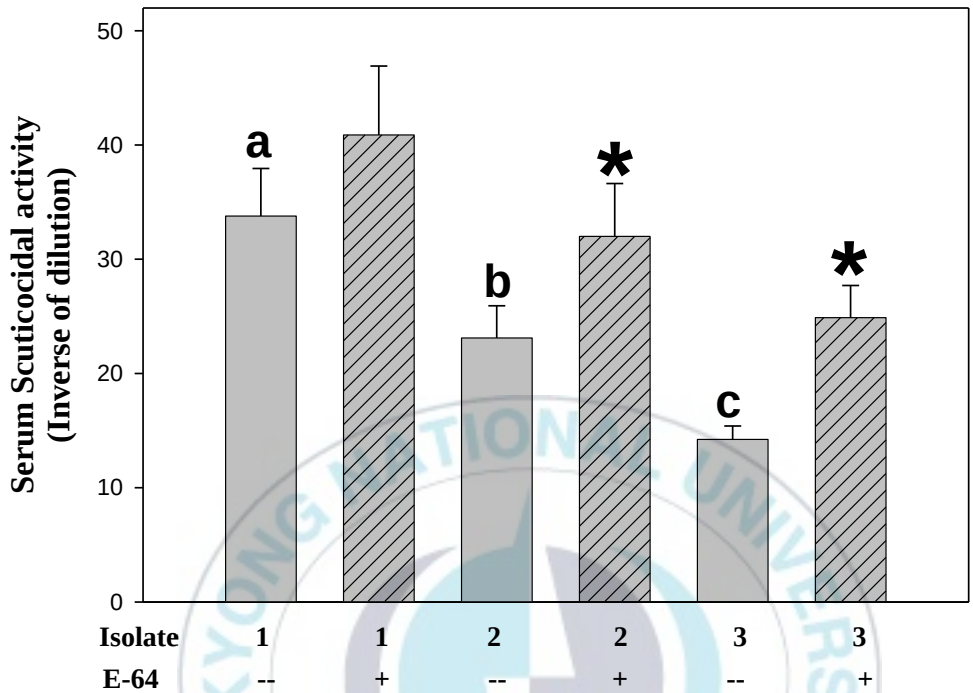

Figure 1. The serum scuticocidal activity of olive flounder against 3 isolates and compared with each group that treated by E-64. Values (inverse of dilution) are means and T-bars indicate standard error.

2. in vivo virulence

Olive flounder fingerlings challenged with isolate 3 showed significantly higher mortalities than the fish challenged with isolate 1 and 2 (Fig. 2). Fish even challenged with the lowest number of isolate 3 showed a higher mortality than fish challenged with the highest number of isolate 1 and 2. Fish challenged with isolate 2 showed slightly higher mortality than fish challenged with isolate 1, but there were no significant differences.

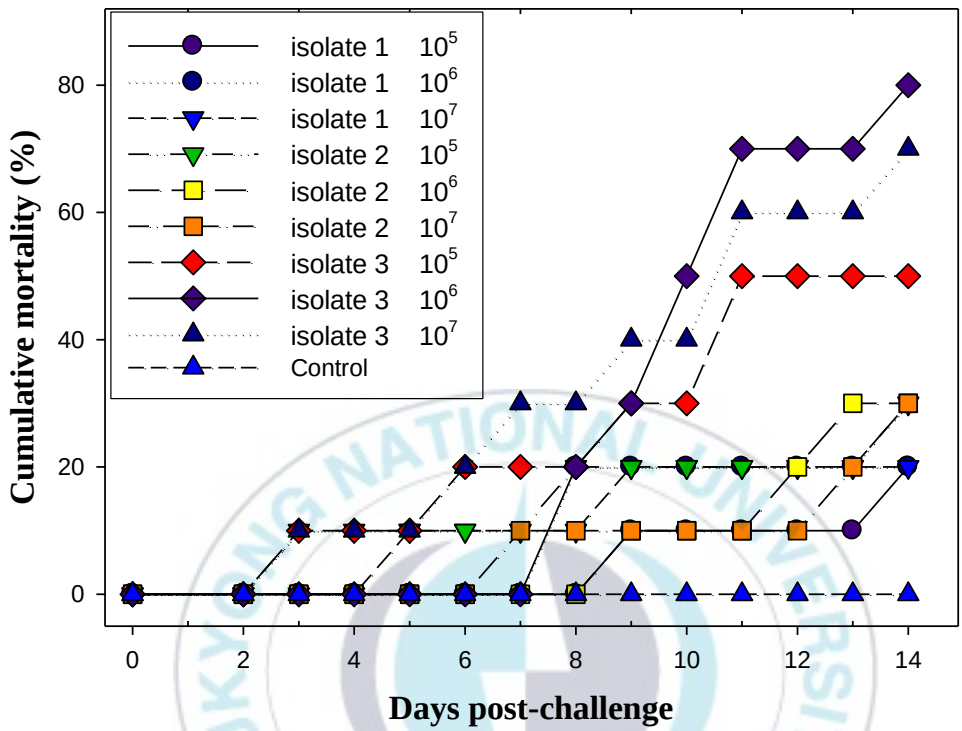

Figure 2. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerlings by immersion in seawater containing 1 x 10^5, 1x 10^6, or 1 x 10^7/tank of each isolate of *Miamiensis avidus* for 3 days. The fish in the control group were not exposed to the ciliates. The mortality was recorded for 14 days.

3. Secretory cysteine proteinase activity

Isolate 3 showed the highest secreted proteinase activity among 3 isolates, and isolate 1 and 2 showed a similar activity. The proteinases activities of the three isolates were markedly decreased by incubation with E-64 (Fig. 3).

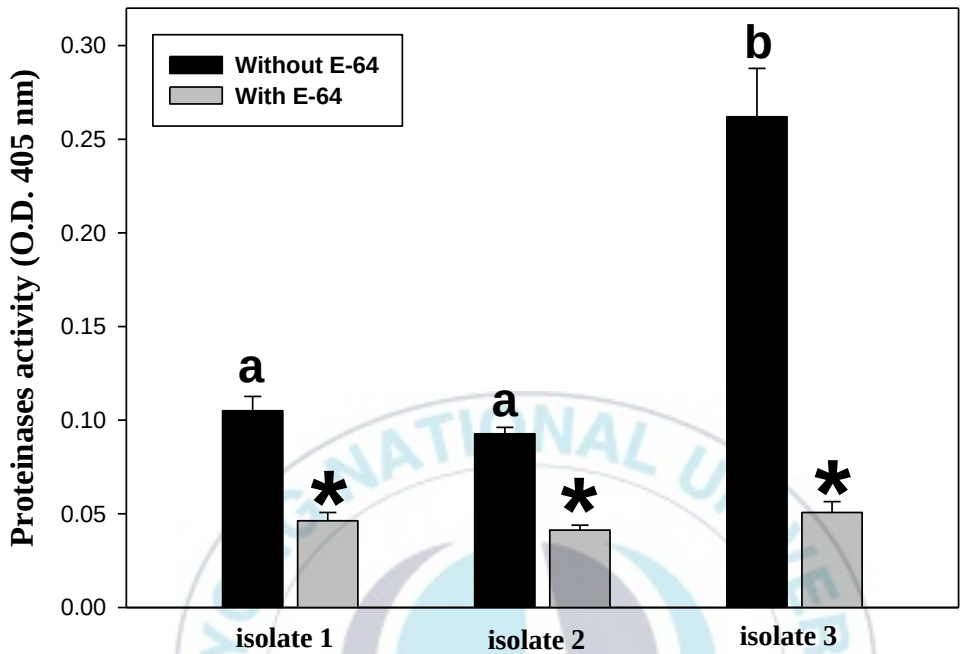

Figure 3. The difference of cysteine proteinase activity among 3 isolates.

The proteinase activity of each isolate was assayed using azocasein as a substrate. And then used E-64 that cysteine proteinase inhibitor to measure inhibitory effects.

4. Sensitivity to drugs

Doxycycline and Mebendazole showed similar scuticocidal activity against 3 isolates at similar concentrations, however, bithionol showed scuticocidal activity at much lower concentrations compared to doxycycline and mebendazole. There were no significant differences in the sensitivity to doxycycline among isolates (Fig. 4). However, in mebendazole, all three isolates showed significant differences, and isolate 2 showed significantly higher sensitivity than other isolates. In bithionol, isolate 1 showed significantly lower sensitivity than other isolates (Fig. 4).

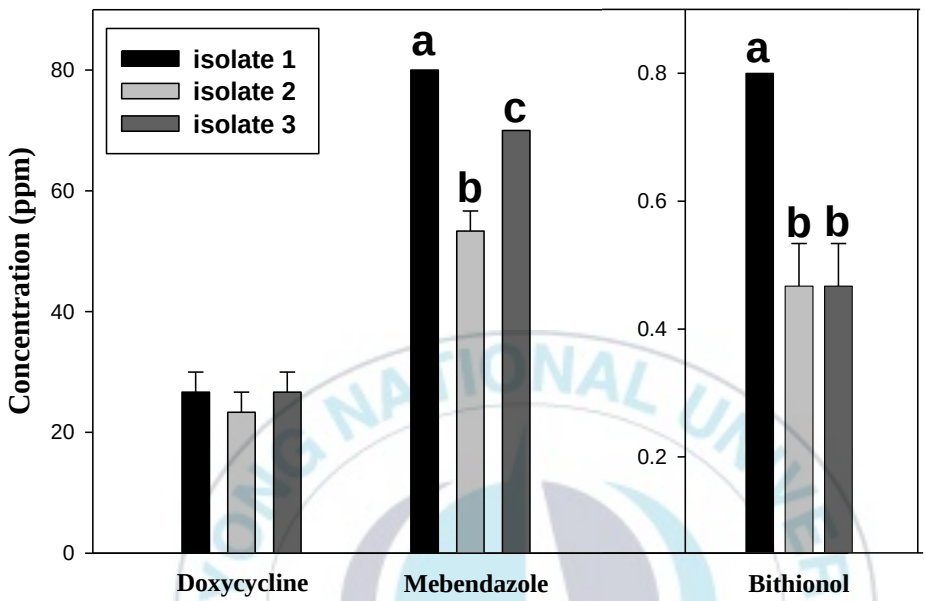

Figure 4. In vitro killing activity of doxycycline, mebendazole, and bithionol against three isolates of *Miamiensis avidus*. Different letters on the bars indicate statistical differences ($p < 0.05$) among the ciliate isolates.

5. Expression of long dsRNA

The long dsRNA band corresponding to GFP and cysteine proteinase was confirmed with 1% agarose gel. The size of each gene that insert into pUC57 vector was 714 bp in GFP and 500 bp in cysteine proteinase, and the gel analysis showed the same size of each long dsRNA, which indicated that double P_R promoter acted to produce dsRNA in *ΔalrΔrncΔasd E. tarda*. (Fig. 5).

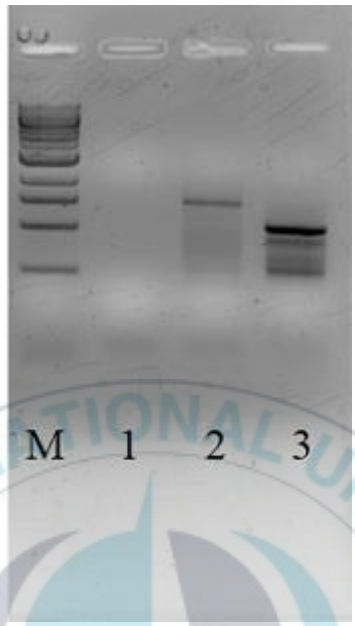

Figure 5. Long dsRNAs produced in *ΔalrΔrncΔasd E. tarda*.

1: negative control is *ΔalrΔrncΔasd E. tarda*, 2: the long dsRNA band of eGFP that expressed in *ΔalrΔrncΔasd E. tarda*, 3: the long dsRNA band of cysteine proteinase that expressed in *ΔalrΔrncΔasd E. tarda*.

6. Effect of feeding ciliates with *ΔalrΔrncΔasd E. tarda* expressing long dsRNA

6.1. Feeding ciliates with *ΔalrΔrncΔasd E. tarda* expressing GFP

To determine the optimal feeding concentration of *E. tarda*, ciliates were fed with *ΔalrΔrncΔasd E. tarda* that expressing GFP. After coincubation of ciliates and *E. tarda*-GFP, the fluorescence from the cytoplasm of ciliates was observed, and any detrimental effect of *ΔalrΔrncΔasd E. tarda* to ciliates was not observed. In the groups that ingested higher numbers of *ΔalrΔrncΔasd E. tarda*-GFP, the fluorescence observed from 2 h post-incubation. Ciliates that ingested lower numbers of *ΔalrΔrncΔasd E. tarda*-GFP showed the fluorescence from 96 h post-incubation and control group that feed ciliates with *ΔalrΔrncΔasd E. tarda* showed no fluorescence at all. From these results, the group that coincubated 10^5 ciliates with 10^8 *ΔalrΔrncΔasd E. tarda*-GFP was considered as the best ratio (Fig. 6).

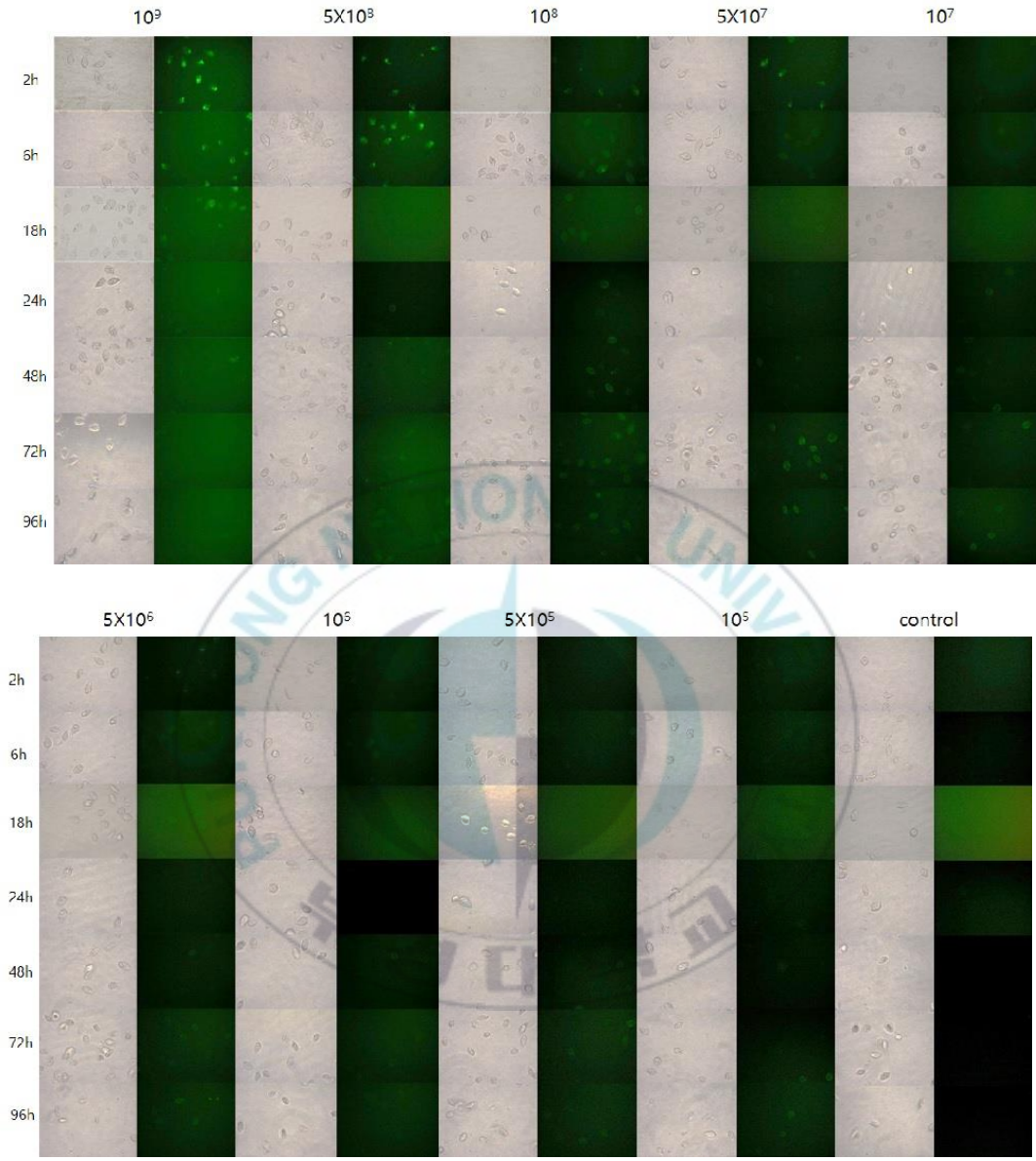

Figure 6. The fluorescence of *M. avidus* that ingest *ΔalrΔrncΔasd E. tarda*-GFP. The upper side of the figure shows the concentration of ciliates and the left side shows the time.

6.2. Growth of ciliates

In all groups, ciliates proliferated more than the first inoculated number (10^4). The groups that fed ciliates with *ΔalrΔrncΔasd E. tarda* grew more than 200 times after 24 hours, but they were reduced 100 times after 48 hours and maintained the numbers until 72 hours. This phenomenon occurred similarly in other 2 groups, the *ΔalrΔrncΔasd E. tarda*-dsRNA-GFP group showed slightly lower numbers than *ΔalrΔrncΔasd E. tarda* group. The *ΔalrΔrncΔasd E. tarda*-dsRNA-CP group showed significantly lower numbers than other groups (Fig. 7).

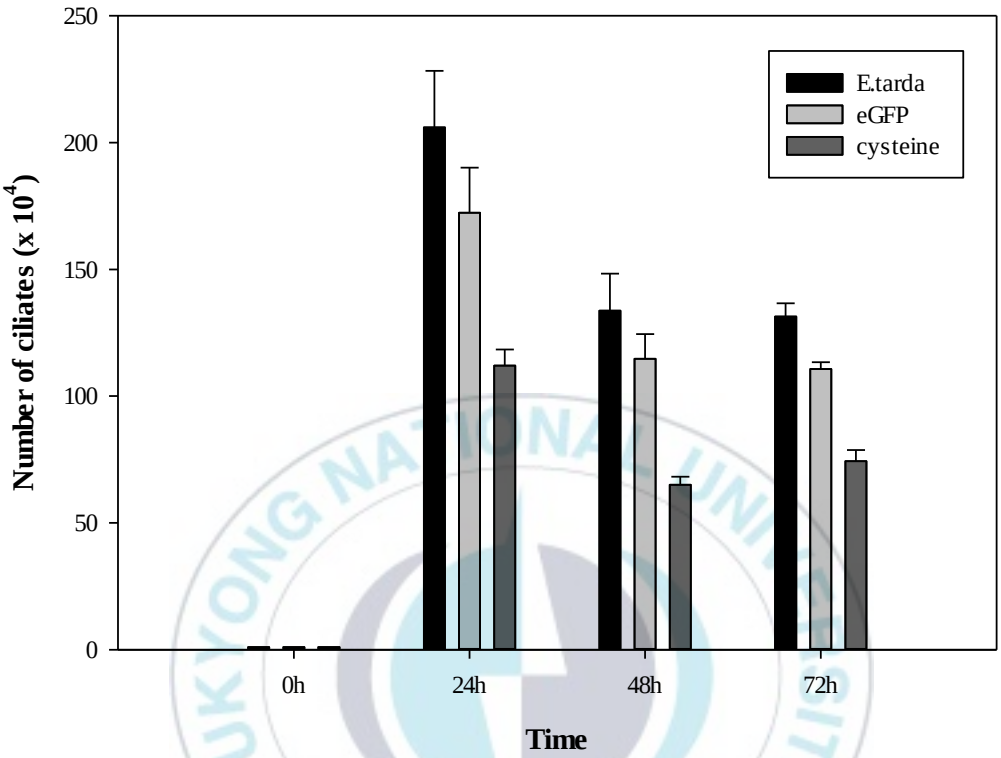

Figure 7. The number of ciliates counted for 3 days (every 24 hours) after ingesting each *ΔalrΔrncΔasd E. tarda* group. E. tarda: *ΔalrΔrncΔasd E. tarda,* eGFP: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of GFP, cysteine: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of cysteine proteinase.

6.3. Quantitative RT-PCR

Relative quantitative RT-PCR was performed using elongation factor gene as a reference gene to compare the expression level of the cysteine proteinase gene in ciliates that ingested each *ΔalrΔrncΔasd E. tarda* group. As a result, the group of ciliates fed *ΔalrΔrncΔasd E. tarda*-dsRNA-GFP showed a slightly lower expression of the cysteine proteinase gene than the ciliates fed *ΔalrΔrncΔasd E. tarda*, however, the cysteine gene expression level in the ciliates fed *ΔalrΔrncΔasd E. tarda*-dsRNA-CP was significantly decreased than other groups (Fig. 8).

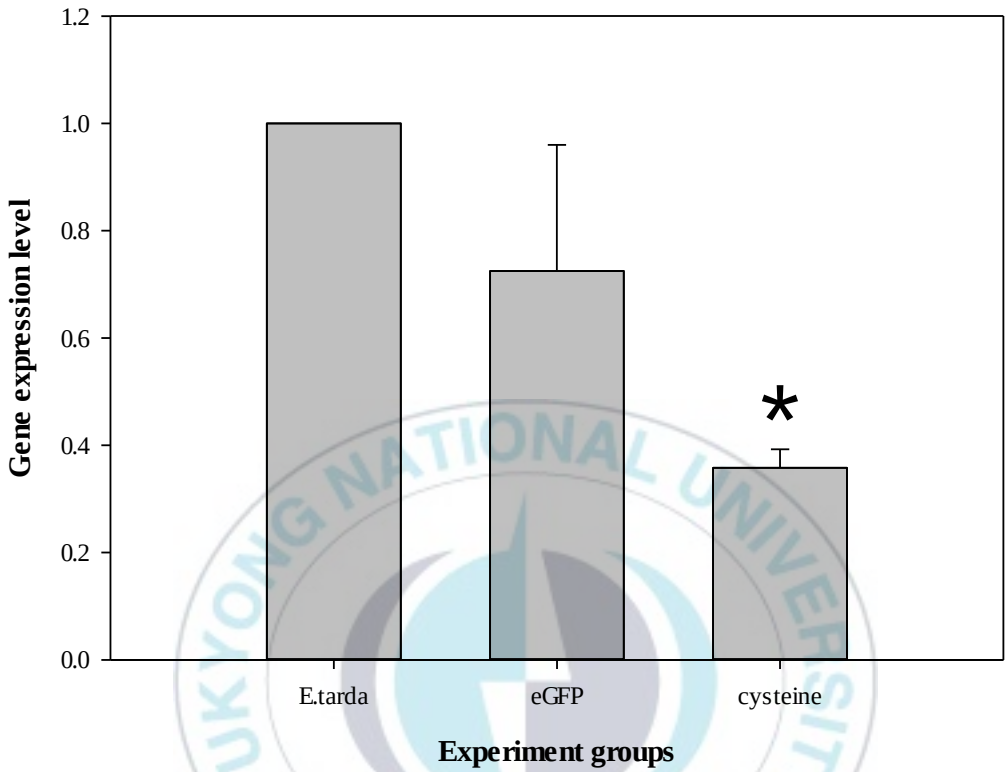

Figure 8. Relative quantification analysis of the expression of ciliate cysteine proteinase gene. E. tarda: *ΔalrΔrncΔasd E. tarda,* eGFP: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of GFP, cysteine: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of cysteine proteinase.

6.4. Secretory proteinase activity

The secretory proteinase activity of ciliates fed with each *ΔalrΔrncΔasd E. tarda* group measured using azocasein method. The groups of ciliates fed with *ΔalrΔrncΔasd E. tarda* and *ΔalrΔrncΔasd E. tarda*-dsRNA-GFP showed similar cysteine proteinase activity, however, the ciliates fed with *ΔalrΔrncΔasd E. tarda*-dsRNA-CP showed significantly lower proteinase activity than other 2 groups (Fig. 9).

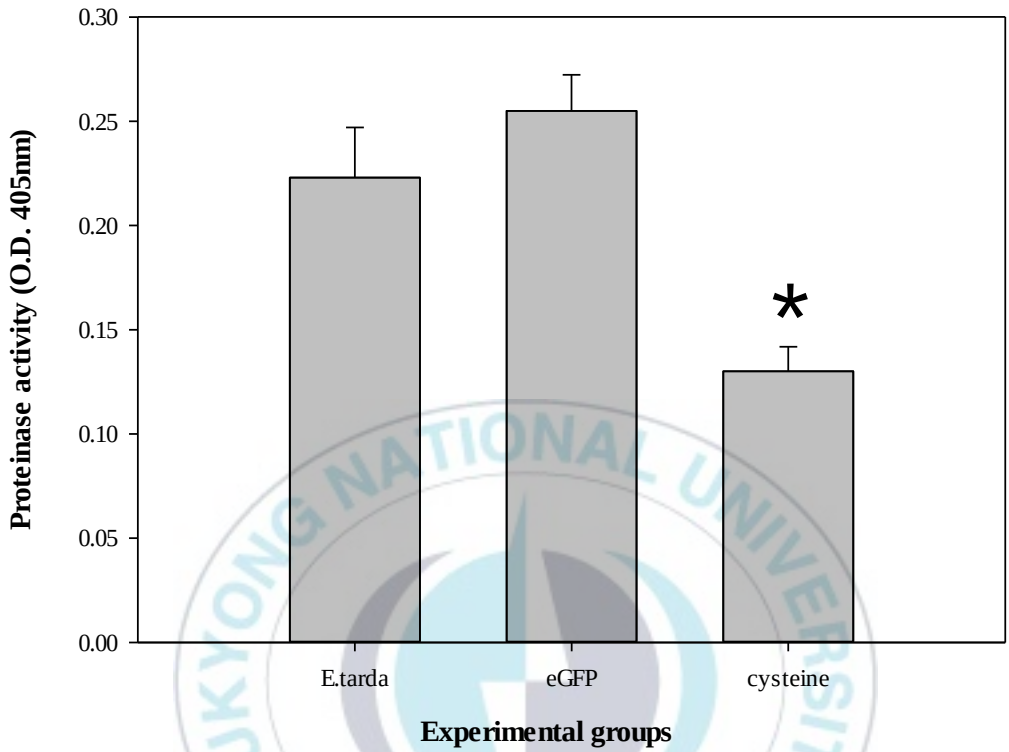

Figure 9. The secretory proteinase activity of each ciliate group.

E. tarda: *ΔalrΔrncΔasd E. tarda,* eGFP: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of GFP, cysteine: *ΔalrΔrncΔasd E. tarda* expressing long dsRNA of cysteine proteinase.

Discussion

Scuticociliatosis is a serious disease caused by scuticociliates that lead to huge economic losses in aquaculture particularly olive flounder culture in Korea. Several studies have reported that scuticociliates possess different characteristics even among the same species depending on the period of in vitro culture. Furthermore, they can possess different virulence (Alvarez et al., 2004). In this study, the *cox1* gene sequence among isolates was identical. Jung et al. (2011) reported *cox1* type do not reflect host species or virulence but match serotypes of the strains well. However, the difference of virulence by gene information in scuticociliates has not been clarified, it is difficult to correlate the match of genotypic with the virulence. Therefore, further studies about genomic composition of *M. avidus* would be needed for the clarification of virulence factors.

In this study, we analyzed virulence of 3 different isolates of *M. avidus* based on the resistance against serum scuticocidal activity, in vivo virulence and the secreted proteinase activity. It turned out that the 3 isolates have different characteristics. Olive founder fingerlings challenged with isolate 3 showed the highest mortality, and the fish challenged with isolate 1 and 2 showed almost the same mortality. According to scuticocidal activity using olive flounder serum, isolate 3 showed the highest serum resistance, the difference of serum resistance between isolate 1 and 3 was almost double. Furthermore, we analyzed the serum scuticocidal activity of 3 isolates that treated with E-64, a cysteine proteinase inhibitor, which significantly lowered serum resistance than without E-64 in all isolates. Isolate 3 showed the highest serum resistance and had the most difference of serum resistance before and after treatment of E-64 among 3 isolates. In proteinase activity analysis, isolate 3 showed the highest secreted proteinase activity among 3 isolates and isolate 1 also showed slightly higher secreted proteinase activity than isolate 2. In addition, we analyzed secreted

proteinase activity using ciliates treated E-64, through which it was found that cysteine proteinase was the major secreted proteinases. According to several studies, parasites produce a large amount of proteinase to induce systemic infection in the host. Cysteine proteinase can inactive self-protecting immune responses and activate the host inflammatory response (Mallo et al., 2017), and these can give systemic damage to the host. In the present results, isolate 3 had the highest pathogenicity and the highest cysteine proteinase activity among 3 isolates, suggesting that cysteine proteinase may play a major role in infection and pathogenicity of *M. avidus*. In addition, in this study, isolate 1 has been in vitro cultured for 8 years and showed the lowest virulence, although isolate 2 and 3 have been cultured for approximately similar period, they showed clearly different virulence. Thus, the period of in vitro culture may not be the crucial factor for the virulence of *M. avidus*. Whereas, isolate 2 showed higher serum resistance and the in vivo virulence than isolate 1, secreted proteinase activity of isolate 2 was not significantly differenced from that of isolate 1, suggesting that not only proteinase but also other factors of *M. avidus* related with the in vivo virulence.

In aquaculture, the drug sensitivity of pathogen is important for the determination of treatment doses. In this study, we investigated whether not only virulence but also drug sensitivity is different among 3 isolates. As a result, 3 isolates showed almost similar sensitivity to doxycycline, isolate 1 showed the lowest sensitivity to mebendazole and bithionol. Also, isolate 2 and 3 were same sensitivity to bithionol, although isolate 3 showed lower sensitivity to mebendazole than isolate 2. Following these, it is suggested that the drug sensitivity of *M. avidus* may be changed depending on isolates and drugs.

Chemotherapy is the most frequently used method for the treatment of parasitic diseases in aquaculture, but side effects of chemotherapy cannot be excluded. Thus, the development of control measures using immunological methods can be useful. In this study, we analyzed the effect of RNA interference against *M. avidus* and suggested the control method using RNA interference as a new treatment for scuticociliatosis In *Ichthyophthirius multifiliis*, it

was suggested that cysteine proteinases play an important role in the infection process, and in the other ciliate, it acts as virulence determinants (Jousson et al., 2007). For that reasons, cysteine proteinase may also be an important virulence factor in *M. avidus*, and we produced long double-strand RNA (dsRNA) corresponding to cysteine proteinase gene by dual P_R promoters. To deliver dsRNA corresponding to cysteine proteinase into *M. avidus*, RNase III knock-out auxotrophic *Edwardsiella tarda* mutant (*ΔalrΔrncΔasd E. tarda*) was produced, because auxotrophic mutant *E. tarda* has the advantage of safety and economical aspect. In this study, we fed ciliates with *ΔalrΔrncΔasd E. tarda* expressing the long dsRNA corresponding to cysteine proteinase (*ΔalrΔrncΔasd E. tarda*-dsRNA-CP) as a target or the green fluorescent protein (*ΔalrΔrncΔasd E. tarda*-dsRNA-GFP) as a control and analyze the effect. The group that fed ciliates with *ΔalrΔrncΔasd E. tarda*-dsRNA-CP showed the lowest transcriptional expression level and proteinase activity, suggesting that RNA interference by the long dsRNA of cysteine proteinase has occurred in ciliates. Furthermore, the ciliate growth in the same group was decreased, thus the down-regulation of cysteine proteinase by RNA interference brought the suppression of *M. avidus* proliferation.

In conclusion, *M. avidus* showed different characteristics according to different isolates, which suggested that this phenomenon should be considered when developing control and prevention methods. The present results demonstrated that cysteine proteinase can act as a strong virulence factor in *M. avidus* and shows the potential as a target for therapeutics and vaccines. Furthermore, RNA interference-mediated knock-down of cysteine proteinase induced the suppression of transcriptional expression and ciliate proliferation, which suggested that the possible application of RNA interference for the control of scuticociliatosis.

Differences in virulence and drug sensitivity of different *Miamiensis avidus* isolates and effects of RNA interference-mediated knock-down of a cysteine proteinase on the ciliate growth

Chizuha Wakabayashi

Department of Aquatic Life Medicine, The Graduate School,

Pukyong National University

Abstract

Scuticociliatosis caused by opportunistic scuticociliates, *Miamiensis avidus*, has been recognized as one of the important parasitic diseases in the culture of flat fish including olive flounder (*Paralichthys olivaceus*). Recently, the presence of not only different serotypes but also isolates with different virulence has been reported in *M. avidus*, and the critical role of a *M. avidus* cysteine proteinase in the determination of virulence was demonstrated. In the present study, differences in in vivo virulence and in sensitivity to drugs among different isolates (isolate 1, 2, and 3) of *M. avidus* were analyzed. Olive flounder fingerlings challenged with isolate 3 showed significantly higher mortalities than the fish challenged with isolate 1 and 2. Furthermore, isolate 3 showed the highest resistance against olive flounder serum and the highest secreted proteinases activity, both of which were significantly decreased by E-64, suggesting that the secreted cysteine proteinases of *M. avidus* might play an important role in the virulence, and measures that can inhibit the cysteine proteinases might be a way to diminish damages caused by scuticociliatosis. Although the isolate 2 showed significantly higher serum resistance and in vivo virulence than the isolate 1, the extracellular proteinase activity of the isolate 2 was not significantly different from that of isolate 1, suggesting that not only proteinases but also other factors of *M. avidus* might be involved in the in vivo virulence.

The three isolates of *M. avidus* showed different sensitivities to mebendazole and bithionol. Although effective drugs against scuticociliatosis so far have not been developed, the present results suggest that the varying sensitivity to drugs in scuticociliate isolates should be taken into consideration for the development of chemotherapeutics against scuticociliatosis.

Considering the important role of a cysteine proteinase in *M. avidus* virulence, we analyzed the effect of RNA interference (RNAi)-mediated knock-down of the cysteine proteinase on the growth of *M. avidus*. To deliver long double-stranded RNA (dsRNA) corresponding to the cysteine proteinase into *M. avidus*, RNase III knock-out auxotrophic *Edwardsiella tarda* mutant (*ΔalrΔrncΔasd E. tarda*) was produced, and *M. avidus* isolate 3 was fed with the mutant *E. tarda* that expressing long dsRNA corresponding to the cysteine proteinase or the green fluorescent protein (GFP; as a control). After feeding ciliates with auxotrophic mutant *E. tarda* (*ΔalrΔrncΔasd E. tarda*) that expressing the long dsRNA of the cysteine proteinase, the transcriptional expression of the cysteine proteinase was significantly reduced, and the cysteine proteinase activity was also significantly reduced. The down-regulation of the cysteine proteinase by RNAi brought the significant decrease of *M. avidus* proliferation. These results indicate that the cysteine proteinase is a potential target for the therapeutics and prophylactic vaccines against *M. avidus*.

謝辞

魚病を学ぶため、単身韓国に降り立った日から7年が過ぎ、この修士学位論文を提出するまでにあたり、多くの方々のご指導とご助力をいただきました。そんな私の力になっていただいた方々に謝意の辞を述べます。

먼저 물고기 의사라는 꿈을 가지고 부경대에 입학한 후 기생충에게 관심을 가지게 되어 그것에 관한 연구를 하고 싶다는 마음 하나만으로 실험실의 문을 두드렸을 때부터 지금까지 늘 따뜻하게 때로는 엄격하게, 연구의 하나부터 석사논문 작성까지 변함없고 한결같이 지도를 해 주신 저의 지도 교수님이신 김기홍 교수님께 감사의 마음을 전하고 싶습니다. 언제나 열정적으로 연구를 하시고 많은 애정으로 학생들을 대하시면서 문제와 고민에 대해서는 진지하게 해결책을 생각해 주시고 지도를 해주시는 선생님, 이러한 선생님의 모습을 보면서 저도 이런 사람이 되고 싶다는 생각이 들었고, 항상 선생님을 따라갈 수 있도록 열심히 할 수 있었습니다. 제가 다른 나라에서 학사, 석사과정을 무사히 마칠 수 있었던 것은 모두 선생님 덕분이라고 생각합니다. 앞으로 저는 다른 길에서부터 물고기 질병 또는 기생충에 다가갈 생각이지만 늘 선생님의 제자로서 부끄럽지 않게 계속 노력하겠습니다. 그리고 선생님께 좋은 소식을 들릴 수 있게 더 열심히 하겠습니다.

또한 연구와 업무로 바쁘신 와중에도 관심을 주시고 논문을 심사해주신 정현도 교수님과 김도형 교수님께도 감사드립니다. 그리고 석사까지의 6년간 많은 전문 지식과 학문에 대한 흥미를 전해주신 정준기 교수님, 허민도 교수님, 강주찬 교수님께도 감사 말씀 드립니다.

4년동안 함께 지낸 분자질병공학 실험실원분들께도 감사드리고 싶습니다. 막 들어왔던 실험실에서 처음에 실험의 기초부터 차근차근 자상하게 다양한 지식을 가르쳐 주신 예재선배, 벽에 부딪히면 어떤 사소한 것에도 도움을 주셨던 승혁선배, 늘 신경을 써 주시고 좋은 방안책을 함께 생각해 주셨던 민선이언니, 같은 외국인이라는 것만으로 든든하고 안심할 수 있는 존재였던 나집, 조교를 하셨을 때부터 늘 걱정해주시고 늘 저를 아껴 주신 선영이언니, 언제나 좋은 실험실을 위해

애쓰시고 희생하시는 준성선배, 누구보다 편하게 이야기할 수 있었고 옆에서 위로를 나눌 수 있었던 정인이, 다양한 지식을 가져 문제가 있을 때마다 도와 주신 민준선배, 모든 일에 대해 꼼꼼히 열심히 하는 모습이 인상적인 명광이, 우리 동기라는 생각으로 편한 존재였던 미정이, 옆에서 도와주면 언제나 든든했던 현주, 열심히 실험을 배우는 모습에 보람이 컸던 수연이, 경민이, 현옥이, 학부생 시절에 함께 있으면서 저의 힘이 되었던 소리, 영현선배에게도 감사의 마음을 드립니다. 그리고 다른 업무도 많은 와중에 신경 써 주시면서 논문심사 준비를 도와 주신 정서경 조교님, 허재영 조교님께도 감사드립니다.

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