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Different isolates of *Miamiensis avidus* showed differences in virulence to olive flounder (*Paralichthys olivaceus*) and in sensitivity to chemotherapeutics

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Differences in in vivo virulence and in sensitivity to drugs among different isolates of *Miamiensis avidus* were analyzed. The isolate III showed the highest resistance against the scuticocidal activity of olive flounder (*Paralichthys olivaceus*) serum, and induced the highest mortalities of olive flounder fingerlings. The isolate II showed significantly higher serum resistance than the isolate I, but in vivo virulence of isolate II was not significantly different from that of isolate I. The secreted proteinases activity of isolate III was significantly higher than that of isolate I and II, and the activity was significantly reduced by the addition of E-64, a cysteine proteinases inhibitor. There were no differences among isolates in the sensitivity to doxycycline, however, there were significant differences in sensitivities to mebendazole and bithionol. These results suggest that the different characteristics of different *M. avidus* isolates should be taken into consideration for the development of control measures against scuticociliatosis.

Key words: *Miamiensis avidus*, Different isolates, Virulence, Drug sensitivity

A scuticociliate *Miamiensis avidus* (or *Philasterides dicentrarchi*) has been a notorious pathogen in olive flounder (*Paralichthys olivaceus*) farms in Korea and other flat fish farms worldwide (Iglesias et al., 2001; Kim et al., 2004; Jung et al., 2005; Ramos et al., 2007). Although there have been many reports on scuticociliatosis, effective commercial countermeasures to control scuticociliatosis are not available. Furthermore, the presence of different serotypes (Piazzón et al., 2008; Song et al., 2009) have made it more difficult to develop vaccine-mediated controls.

Not only the presence of different serotypes but also the presence of various strains of *P. dicentrarchi* even in a same fish farm was demonstrated through morphological and genetic analyses (Budiño et al., 2011a, b). Furthermore, Alvarez-Pellitero et al. (2004) reported that *P. dicentrarchi* isolates sampled from different localities showed differences in virulence to turbot. Piazzón et al. (2011a,b) 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

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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.

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. 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.

Materials and methods

Scuticociliates and in vitro culture

M. avidus isolated from the brain of diseased olive flounders at 2007 (isolate I; Lee and Kim, 2008) and 2015 (isolate II and isolate III) in different farms were aseptically grown using Epithelioma papulosum cyprini (EPC) cells in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS, Gibco) at 20-25°C.

Sequencing of mitochondrial cytochrome c oxidase 1 (cox1) gene

Approximately 1×10^6 cells of each ciliate isolate were pelleted by centrifugation at 900 g for 5 min at 4°C and washed 3 times with Hank's balanced salt solution (HBSS, pH 7.4, Sigma). Genomic DNA was extracted using Accuprep® Genomic DNA Extraction Kit (Bioneer, Korea). Fifty ng of genomic DNA was used in 20 μl of PCR reaction mixture containing 10 pmoles of each primer (Forward, 5'-ATTAGATTAG AATTAGCTCATCCAG-3'; Reverse, 5'-AAAATCA AAAAATGTAGTTTGTCAATGTC-3') and 0.5 U of Taq DNA polymerase (Takara). The reaction was carried out for 30 cycles using an automated thermal cycler (iCycler, BioRad) at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, with an initial denaturation at 95°C for 2 min. The amplified PCR product was run on an agarose gel (0.8%), purified using a gel purification kit (GeneAll, Korea), subcloned into pGEM T-easy vector (Promega), and sequenced.

Resistance against serum killing 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.

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 20-21°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 1×10^5, 1×10^6, or 1×10^7/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.

Secretory proteinases activity

The secretory proteinases activity of each ciliate isolate was analyzed using azocasein (Sigma) as the substrate. Briefly, 1×10^8 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).

To know the effect of a cysteine proteinases inhibitor on the secretory proteinases activity, each supernatant was incubated with 10 μM of E-64 (Sigma) for 30 min at 25°C before mixing with azocasein.

Effect of E-64 on the resistance of M. avidus against serum killing 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 olive flounder sera in a 96-well plate.

Sensitivity to chemotherapeutics

Scuticociliates of each isolate were placed on 24-well plates with 1×10^3 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.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (SPSS, USA). Data on serum scuticocidal activity, proteinases activity and drugs sensitivity were analyzed by oneway ANOVA followed by Tukey HSD post-hoc test. The paired t-test was used to analyze the effect of E-64 on serum scuticocidal activity and proteinases activity in each isolate. The log-rank test of the Kaplan–Meier method was used to analyze the significance of cumulative mortalities among groups. A probability (P) value less than 0.05 was considered statistically significant.

Results

cox1 gene sequence and differences in resistance against serum killing activity

There were no differences in the sequences of *cox1* gene among the 3 isolates. However, the resistance

of each isolate against serum killing activity was markedly different (Fig. 1). The isolate I showed the lowest resistance and the resistance of isolate II was significantly lower than that of isolate III.

Differences in in vivo virulence

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

Extracellular proteinases activity and the effect of E-64 on the resistance against serum killing activity

The secretory proteinases activity of isolate III was significantly higher than that of isolate I and II, and the proteinases activities of the three isolates were markedly decreased by incubation with E-64 (Fig. 3).

The serum killing activity against scuticociliates was significantly enhanced by pre-incubation of the ciliates with E-64 (Fig. 1).

Sensitivity to drugs

There were no significant differences in the sensi-

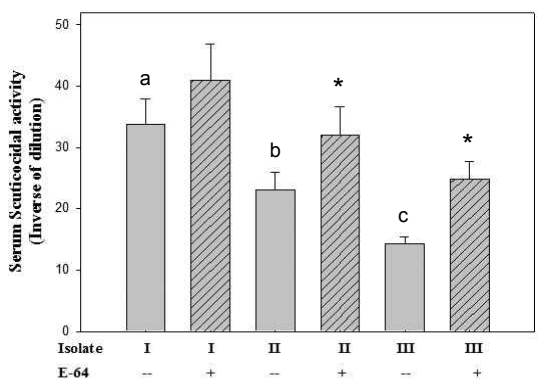

Fig. 1. Scuticocidal activity of olive flounder (*Paralichthys olivaceus*) sera against three isolates (isolate I, II, and III) of *Miamiensis avidus*, and the effect of the pre-incubation of ciliates with E-64 for 30 min on the serum scuticocidal activity. Values (inverse of serum dilution) are means and T-bars indicate the standard error. Different alphabet letters on the bars indicate statistical differences ($p<0.05$) among the ciliate isolates, and bars with an asterisk indicate statistical differences at $p<0.05$, when compared to the same isolate without an E-64 exposure.

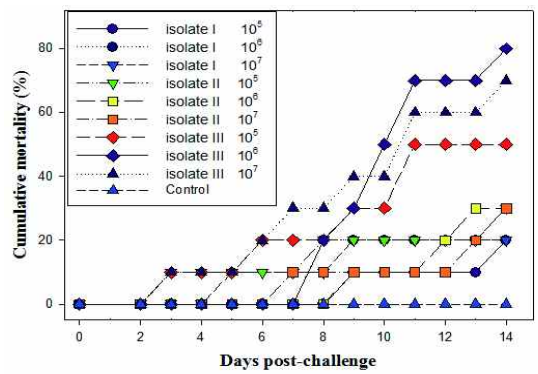

Fig. 2. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerlings by immersion in seawater containing 1×10^5, 1×10^6, or 1×10^7/tank of each isolate of *Miamiensis avidus* for 3 days. The fish in the control group were not exposed to the ciliates.

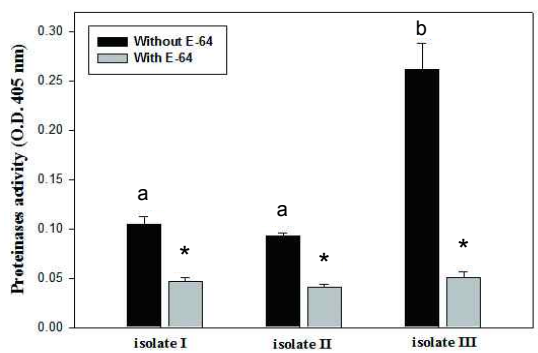

Fig. 3. The secretory proteinases activity of each isolate of *Miamiensis avidus*. The proteolytic activity was estimated using azocasein as a substrate. Different alphabet letters on the bars indicate statistical differences ($p<0.05$) among the ciliate isolates, and bars with an asterisk indicate statistical differences at $p<0.05$, when compared to the same isolate without an E-64 addition.

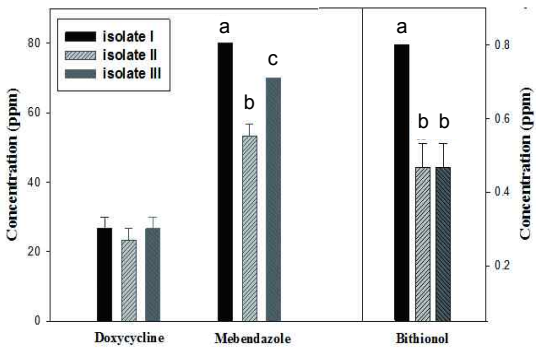

Fig. 4. In vitro killing activity of doxycycline, mebendazole, and bithionol against three isolates of *Miamiensis avidus*. Different letters on the bars indicate statistical differences ($p<0.05$) among the ciliate isolates.

tivity to doxycycline among isolates (Fig. 4). However, in mebendazole, all three isolates showed significant differences, and isolate II showed significantly higher sensitivity than other isolates. In bithionol, isolate I showed significantly lower sensitivity than other isolates (Fig. 4).

Discussion

The presence of different isolates with different virulence has been reported in *Ichthyophthirius multifiliis*, a notorious ciliate pathogen to freshwater fish (Wang et al., 2002), and the length of time to complete the life cycle of *I. multifiliis* was supposed to be a main factor that determine in vivo virulence (Swennes et al., 2006). In *P. dicentrarchi*, Alvarez-Pellitero et al. (2004) reported the differences in virulence to turbot between two isolates sampled from different localities. Later, Piazzón et al. (2011a) reported the differences in susceptibility to complement activity might partly related to the differences in the virulence of different isolates. Similarly, in the present results, the *M. avidus* isolate showing the highest resistance against olive flounder serum induced the highest mortality of olive flounder fingerlings. A long-term in vitro culture can be a factor for attenuation of scuticociliates as Alvarez-Pellitero et al. (2004) mentioned. In this study, isolate I has been in vitro cultured for 8 years and showed the lowest virulence, however, although isolate II and III 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*. In this study, the *cox1* gene sequence among isolates was identical. However, due to lack of a broad investigation on the genomic differences among *M. avidus* isolates, it is difficult to correlate the differences of virulence with genotypic differences, and further investigations on the genomic composition of *M. avidus* would give information needed for the determination of factors involved in virulence differences.

The important roles of proteinases in the infection of pathogenic protozoans have been well demonstrated (Rosenthal, 1999). Paramá et al. (2004a) reported that *P. dicentrarchi* secreted several cysteine proteinases that might participate in the invasion into host tissues. Later, Paramá et al. (2007) also showed that the cysteine proteinases of *P. dicentrarchi* lysate could induce apoptosis in turbot pronephric leucocytes. In the present study, the secreted proteinases activity of each isolate was greatly reduced by a cysteine proteinase inhibitor (E-64), suggesting that much of the secreted proteinases were cysteine proteinases. The high susceptibility of scuticociliates to humoral immunity rather than cellular immunity has been reported in several papers (Leiro et al., 2008; Piazzon et al., 2011a). The activation of complement cascade via alternative pathway in naïve fish or classical pathway in immunized fish plays a main role in killing of scuticociliates (Leiro et al., 2008). Piazzón et al. (2011b) reported that the proteinases of *P. dicentrarchi* lysate can degrade turbot complement component and immunoglobulins. In the present study, the isolate having the highest secreted proteinases activity showed the highest in vivo virulence and the highest resistance against serum scuticocidal activity. Furthermore, the resistance to serum scuticocidal activity was

significantly decreased by E-64. These results suggest 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. However, although the isolate II showed significantly higher serum resistance and extracellular proteinase activity than the isolate I, the in vivo virulence of the isolate II was not significantly different from that of isolate I, suggesting that not only proteinases but also other factors of *M. avidus* might be involved in the in vivo virulence.

Although there are several reports on in vitro scuticocidal activity of various chemotherapeutics (Iglesias et al., 2002; Quintela et al., 2003; Paramá et al., 2004b), as far as we know, there is only one paper on the different sensitivities of scuticociliate isolates to drugs (Budiño et al., 2012), in which differences in the in vitro susceptibility among different isolates of *P. dicentrarchi* to formalin and hydrogen peroxide were reported. In the present study, the three isolates of *M. avidus* showed a similar sensitivity to doxycycline, but showed different sensitivities to mebendazole and bithionol. Although effective chemotherapeutics against scuticociliatosis so far have not been developed, the present results suggest that the different sensitivity to drugs among scuticociliate isolates should be taken into consideration for the development of chemotherapeutics against scuticociliatosis.

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