**Effects of Dexamethasone on the Burden of Marine Birnavirus (MABV) in Olive Flounder, *Paralichthys olivaceus***

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The effect of dexamethasone injection on the burden of marine birnavirus (MABV) in asymptomatically infected olive flounder (*Paralichthys olivaceus*) fingerlings was investigated. In real time PCR analysis, the threshold cycle (Ct) value of the fish injected with dexamethasone was significantly lower than that of the fish in the PBS-injected and no-handling groups. The higher amplification of the MABV gene in the dexamethasone-injected group than the 2 control groups was confirmed also by semi-quantitative RT-PCR. The results indicate an increase of MABV burden in olive flounder fingerlings after a single injection with dexamethasone.

**Key words:** marine birnavirus, dexamethasone, viral titer, olive flounder

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**Introduction**

Aquabirnaviruses belong to the family Birnaviridae which is characterized by an unenveloped, icosahedral capsid approximately 60 nm in diameter, containing a bisegmented, double-stranded RNA genome. The type species of genus Aquabirnavirus is infectious pancreatic necrosis virus (IPNV), a causative agent of an acute, contagious disease in salmonid fishes. Since yellowtail ascites virus (YTAV) had been reported from yellowtail, *Seriola quinqueradiata*, in Japan (Sorimachi and Hara, 1985), YTAV-like aquabirnaviruses were isolated from various marine fishes, mollusks and crustaceans (Reno, 1999). These aquabirnaviruses from marine organisms differ antigenically and genomically from IPNV and hence have been referred to as marine birnavirus (MABV) distinguish it from IPNV (Hosono et al., 1994, 1996; Zhang and Suzuki, 2003).

It has been known that MABV can infect various fish species without clinical signs with relatively low virus titers in the infected fish (Ishiki et al., 2001). Through broad survey of MABV in marine fish, Ishiki et al. (2004) concluded that flatfish including olive flounder would be the main reservoirs of MABV throughout the year and might be responsible for establishing the infection cycle in aquaculture environments.

Recently, it has been reported that olive flounder coinfected with MABV and pathogenic bacteria showed higher mortality than fish independently infected with a single pathogen (Pakingking et al. 2003; Oh et al., 2006). Similarly, Chou et al. (1994, 1998) reported that MABV in hard clam (*Meretrix lusoria*) became highly virulent by exposure to temperature stress or heavy metal. However, little information is available about the effect of immunosuppression caused by coinfected bacteria or stress on the burden of MABV in hosts. In the present study, to evaluate the effect of immunosuppression on MABV burden in fish, fingerlings of olive flounder naturally infected with MABV were injected with dexamethasone, a potential immunosuppressing agent, and the change in MABV titer was analyzed using real time PCR and semi-quantitative RT-
Materials and Methods

1. Fish and experimental regime

Fingerlings of olive flounder, Paralichthys olivaceus (mean body weight about 8 g), were obtained from a culture facility located in southern coastal area of Korea. The fish were naturally infected with MABV which was confirmed by nested reverse transcriptase polymerase chain reaction (RT-PCR). Fish were divided into 3 nested groups of 10 fish in a 100 L tank, and each group was separated by small net-pens. Fish were acclimated 2 weeks prior to initiating the experiment, and the water temperature was maintained at 17~18°C throughout the experiment. No handling stress was imposed on the fish in group I. Fish in group II were intraperitoneally injected with 50 µL phosphate buffer saline (PBS), while fish in group III were injected with dexamethasone at a dose of 10 µg/g body weight in 50 µL PBS. After 25 days of the injection, fish were dissected and removed spleen to evaluate MABV burden.

2. Detection of MABV by nested reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from spleen using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. Concentration and purity of the extracted RNA was measured spectrophotometrically at 260 and 280 nm. First strand cDNA was synthesized from 1 µg of total RNA using recombinant reverse transcriptase (Promega) and random hexamer primers (1.25 mM; Promega). Reaction volume was 20 µL. RT reaction was carried out at 42°C for 1 h, and the RTase was inactivated by heating the reaction at 95°C for 10 min. One µL of cDNA was directly used as a template for PCR amplification including 10 pmole of each primer, dNTP mix, and 0.5 U of Taq DNA polymerase (TaKaRa). The primers used for MABV detection were forward (5′-AGCACCCATGGTGAAGG-3′) and reverse (5′-CAGGAAAGCTCAAGAC-3′). PCR was performed with 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min using an automated thermal cycler (iCycler, BioRad). In nested PCR, 1 µL of the first PCR product and the nest PCR primer set (forward 5′-GCTGTTAAAAGCACGAAGT-3′ and reverse 5′-ACCGCAATTCCCTGTTATAG-3′) were used for amplification. The PCR product was separated on 0.7% agarose gel and visualized by ethidium bromide staining.

3. Real-time polymerase chain reaction and semi-quantitative RT-PCR

One set of primers of MABV (F 5′-ATTCCCGGACACCTTCTGTTG-3′ and R 5′-CAGGGAAGCAGACAAG-3′) were redesigned to amplify a 103 bp fragment corresponding to the region encoding the VP2 site of MABV. For normalizing the MABV RNA, a 123 bp fragment of olive flounder 18S rRNA was amplified as an internal control using two oligonucleotide primers (F 5′-AGTTGCTGAGTATTAAACAAG-3′ and R 5′-ACTCAGGTAACGAG-3′). PCR was performed using the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad). Each reaction had a volume of 20 µL including 10 µL of reaction mixture containing 2 × SYBR Green I Mix (Bio-Rad), 0.5 µM of each primer, 1 µL of cDNA and 8 µL of distilled water. An initial denaturation step at 95°C for 3 min was followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s. Fluorescence unit was measured after each elongation step to calculate the average threshold cycles of both target and control genes. At the last amplification cycle, dissociation curves were monitored within 55 to 95°C to examine the specificity of PCR amplification.

In addition to the real time PCR detection, endpoint semi-quantitative RT-PCR was carried out to confirm the result. MABV segment was amplified with 31 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, while 18S ribosomal RNA gene with 20 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. Primer pairs were same as described above. The amplified products were separated by electrophoresis on a 1% agarose gel. Et-Br stained bands were analyzed with image analysis software (Quantity-One, BioRad) implemented in Gel Doc XR system (BioRad).

4. Statistical analysis

Statistical analysis was performed using the Student's t-test, and significant differences were determined at P < 0.05.
Results and Discussion

In real time PCR, the PCR amplification was readily successful in all groups as evidenced by the specific dissociation curve showing the same melting point (Fig. 1a). The average Ct values in the amplification of control 18S rRNA was relatively steady-state among treatments (Fig. 1b). However, the Ct value in MABV RNA from the fish injected with dexamethasone was significantly lower than that from the fish belonging to either PBS-injected or no-handling groups (Fig. 1b). The higher amount of MABV RNA in the dexamethasone-injected group compared to the 2 control groups was also confirmed by semi-quantitative RT-PCR (Fig. 1b). Based on the normalization against 18S rRNA bands, the estimated fold increase of MABV RNA in dexamethasone-injected group was more than 2 fold relative to the no handling control group (Fig. 1c).

Administration of glucocorticoids has various immunosuppressive effects on mammals (Goulding and Flower, 1997) and fish (Balm, 1997), and the suppressed immune response could lead to increased viral titers. The present results indi-
cate an increase of MABV titer in olive flounder fingerlings after a single i.p. injection with dexamethasone at 10 µg/g body weight of fish. Similarly, van Nieuwstadt et al. (2001) reported that shedding of eel herpesvirus Herpesvirus anguillae (HVA) was much increased by dexamethasone treatment.

Although the synergistic effect of MABV and coinfected pathogenic bacteria in mortality of olive flounder has been reported (Pakingking et al., 2003; Oh et al., 2006), the mechanism of the increased mortality is unknown. Further studies 1) whether the present increased MABV titer by dexamethasone injection reflect the process under natural stresses including coinfection with other pathogens and 2) whether the increased MABV particles are related to virulence or defective interfering particles—should be conducted.

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**References**


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Dexamethasone 투여가 넙치(Paralichthys olivaceus)의 marine birnavirus (MABV) 감염강도에 미치는 영향
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Marine birnavirus (MABV)에 무증상적으로 감염된 넙치 (Paralichthys olivaceus)에서 면역 억제제의 일종인 Dexamathasone을 투여하였을 때 MABV의 감염강도에 영향을 미치는가를 조사하였다. Real time PCR 분석결과 dexamethasone을 투여한 그룹이 생리식염수를 주사한 그룹 및 no handling 그룹에 비해 유의적으로 낮은 Ct 값을 나타냈으며, 또한 semi-quantitative RT-PCR 분석결과에 있어서도 dexamethasone을 주사한 그룹이 대조구 그룹들에 비해 MABV 유전자 유의적으로 높게 증폭되는 것으로 나타났다. 이러한 결과로부터 dexamethasone 투여가 넙치 치어에 감염된 MABV의 복제를 증가시킴을 확인하였다.