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Note

The safety of live VHSV immersion vaccine at a temperature-controlled culture condition in juvenile olive flounder, *Paralichthys olivaceus*

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Viral hemorrhagic septicemia (VHS) is one of the most serious viral diseases affecting farmed olive flounder (*Paralichthys olivaceus*) in Asian countries. VHS, caused by viral hemorrhagic septicemia virus (VHSV), occurs in over 80 different cultured and wild fish species worldwide. Our previous study demonstrated that VHSV infection can be restricted by adjusting the water temperature to over 17°C from the host optima. We confirmed that the effective VHSV immersion vaccine treatment was a tissue culture infection dose (TCID) of $10^{5.5}$ $TCID_{50}$/mL at 17°C. However, the safety of live VHSV immersion vaccines remains unclear. The objectives of this study were to 1) demonstrate the safety of the live VHSV immersion vaccine under co-habitant conditions and 2) estimate the pathogenicity of VHSV in live VHSV-vaccinated flounder at 10°C. No mortality was observed in olive flounder treated with the live VHSV immersion vaccine, and the vaccinated flounder challenged with VHSV did not transfer VHSV to naïve fish at 10°C through cohabitation. VHSV titration was below the detection limit (< 1.3 log $TCID_{50}$/mL) in live VHSV immersion vaccine-treated flounder challenged with VHSV at 10°C. This study demonstrated that flounder treated with the live VHSV immersion vaccine were resistant to VHSV infection, and the live vaccine was also safe for naïve fish even at a water temperature known to be VHS infectious.

Key words: VHSV, olive flounder, live vaccine, horizontal transmission

Introduction

Viral hemorrhagic septicemia virus (VHSV) is a globally important pathogenic virus that infects a wide range of fish species. VHSV is a member of the family *Rhabdoviridae* and genus *Novirhabdovirus*. Viral hemorrhagic septicemia (VHS) often occurs during the winter and spring seasons, when the water temperature is low, particularly in the range of 9–15 °C (Kim et al., 2009). However, the mortality caused by VHS was reduced when the rearing water temperature was increased to 21°C (Nishizawa et al., 2011). Our research group has explored the potential of live VHSV immersion vaccination at a controlled water temperature of 17°C (Kim et al., 2019; Kim et al., 2020). Our results suggest that live VHSV immersion at refractive water temperatures confers resistance to VHS outbreaks in fish. Live vaccination has been reported to induce a protective immune response against target pathogens in fish (Nishizawa et al., 2012; Oh

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et al., 2014); however, the safety of this vaccine remains unevaluated. Water temperature is a crucial factor in the transmission of VHSV to the olive flounder (Kim et al., 2016; Sano et al., 2009). Similarly, the current study showed that water temperature plays an important role in live VHSV immersion vaccine treatment.

VHSV is a systemic viral disease that infects a wide range of fish species in aquaculture systems and the wild. It can be transferred via horizontal transmission through the environment. Contagious VHSV can be shed into the environment where it might transfer to susceptible species (Hershberger et al., 2010a, 2013; Wargo et al., 2017). The live VHSV vaccine conferred more effective protection than formalin-killed vaccines. (Kim et al., 2019). Although the live VHSV immersion vaccine protected the fish from infection, the safety of the vaccination protocol was not investigated. Based on our previous findings, we conducted the present study to demonstrate the safety of the live VHSV immersion vaccine and estimate VHSV pathogenicity in vaccinated olive flounder challenged with VHSV at 10°C.

Materials and Methods

Virus and cells

The VHSV strain (FYeosu05) used as the model virus was propagated in the fathead minnow (FHM) cell line. The cells were cultured at 20°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco™, Life Technologies, Carlsbad, CA, USA) supplemented with 1% penicillin-streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (Gibco™). Semi-confluent cell monolayers in a Nunc™ EasYFlask™ 75 cm^2 (Thermo Fisher Scientific, Waltham, MA, USA) were inoculated with 100 µL of a 10^{-4} dilution (in DMEM) of the viral stock, incubated at 15°C, and maintained until cytopathic effects (CPE) were observed in > 75% of the cells.

Experimental design

A total of 128 juvenile olive flounder (8.5 ± 0.5 g) were acclimated to 17°C for 7 days. During the acclimation period, three experimental fish were sacrificed, the kidneys were extracted and inoculated into FHM cells for inspection of VHSV, and the virus was not isolated from any fish examined. We conducted a cohabitation experiment with juvenile olive flounder (mean body weight 8.5 g). We immersed 48 fish were immersed in live VHSV at a dose of $10^{5.5}$ $TCID_{50}$/mL and 17°C for 1 h. As a negative control, 80 fish were immersed in $DMEM_0$ for 1 h. Fish were reared at 17°C for 60 days to promote an immune response. The rearing water temperature was decreased from 17 to 10°C at a rate of 1°C/h. As viral donors, juvenile olive flounder, including both live VHSV vaccine-treated and untreated flounder (naïve fish), were challenged intramuscularly with VHSV at a dose of $10^{4.5}$ $TCID_{50}$/100µL/fish and divided into four tanks (Fig. 1). Ten donor fish were placed in a 20 L tank and then the same number of uninfected fish (recipients) with fin-cut marks were introduced into the same tank to enable direct contact with the donor fish. Group A included VHSV-infected naïve fish as donors, and uninfected naïve fish as recipients. (Fig. 1A). Group B included untreated naïve fish challenged with VHSV as donors, and live VHSV immersion vaccine-treated fish as recipients (Fig. 1B). Group C included live VHSV immersion vaccine-treated fish and uninfected naïve fish as the recipients (Fig. 1C). Group D included live VHSV immersion vaccine-treated fish challenged with VHSV as donors, and uninfected live VHSV immersion vaccine-treated fish as recipients (Fig. 1D). Fish in all tanks were reared in seawater with aeration for 30 days at 10°C. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Chonnam National University (CNU IACUC-YS-2020-13). When any donor or recipient fish died, it was immediately removed to titrate the virus from the kidney.

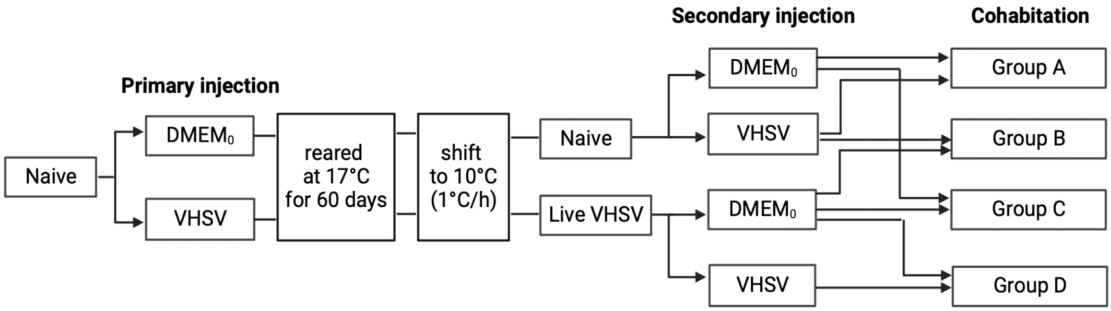

Fig. 1. Experimental layout of VHSV injection and cohabitation to study cohabitation challanege.

VHSV infectivity in kidney

VHSV titrations were evaluated using 50% tissue culture infection dose ($TCID_{50}$). Briefly, 96-well microplates seeded with an FHM cell line were incubated with a homogenate of infected tissues at 15 ± 0.5°C for 10 days. After 10 days of culturing, CPE was monitored to determine the $TCID_{50}$. The kidney samples were serially diluted ($10^{-1} - 10^{-8}$). We then added 50 μL of each serial dilution to each well and incubated the microplates at 15 ± 0.5°C for 10 days to monitor the CPE microscopically. The $TCID_{50}$ was determined based on the number of wells displaying positive CPE. For each dilution, the number of wells with positive CPE was recorded. The Reed and Muench calculation method (Reed and Muench, 1938) was used to determine a 50% infection dose.

Results and Discussion

No mortality was observed in the negative control (naïve) or live VHSV immersion vaccine-treated flounder reared at 17°C for 60 days (data not shown). Our results indicated that live VHSV immersion vaccination to olive flounder did not occur mortality at 17°C. The cumulative mortality of olive flounder challenged with VHSV is shown in Fig. 2. In group A (negative control (naïve) fish challenged with VHSV), mortality was first observed at 10 days post-infection (dpi) and then rapidly increased to 90% at 16 dpi. Naïve fish were reared with a VHSV-infected negative control fish at 10°C. Mortality was first observed at 13 days and then gradually increased. The cumulative mortality in naïve fish reared with VHSV-infected negative control fish was 80% at the end of the experiment (Fig. 2A). These results showed that horizontal transmission of VHSV from negative control fish to naïve fish was established at 10°C, suggesting that the infected fish shed VHSV into the rearing water, which directly infected the naïve fish. In contrast, no mortality was observed in live VHSV immersion vaccine-treated fish reared with VHSV-infected fish in this study (Fig. 2B, D). Additionally, none of the fish in group C showed mortality during the 30 days of group C (Fig. 2C), suggesting that live VHSV immersion vaccine-treated fish do not affect naïve fish, although the rearing temperature is shifted to a temperature susceptible to VHSV infection.

The results of this study showed that live VHSV immersion vaccine-treated flounder acquired resistance to VHSV at 10°C. Figure 3 shows the log VHSV titrations in the kidneys of olive flounder challenged with VHSV at 10°C. VHSV titration ranged from 1.8 to 7.8 log $TCID_{50}$/mL in naïve fish and cohabitant naïve fish challenged with VHSV (Fig. 3A). However, VHSV titration was below the detection limit (<1.3 log $TCID_{50}$/mL) in live VHSV immersion vaccine-treated flounder, whereas cohabitant naïve fish challenged with VHSV showed a VHSV titration greater than 4.2 log $TCID_{50}$/mL on all sampling dates, with a peak titration of 7.2 log $TCID_{50}$/mL at 10 dpi (Fig. 3B).

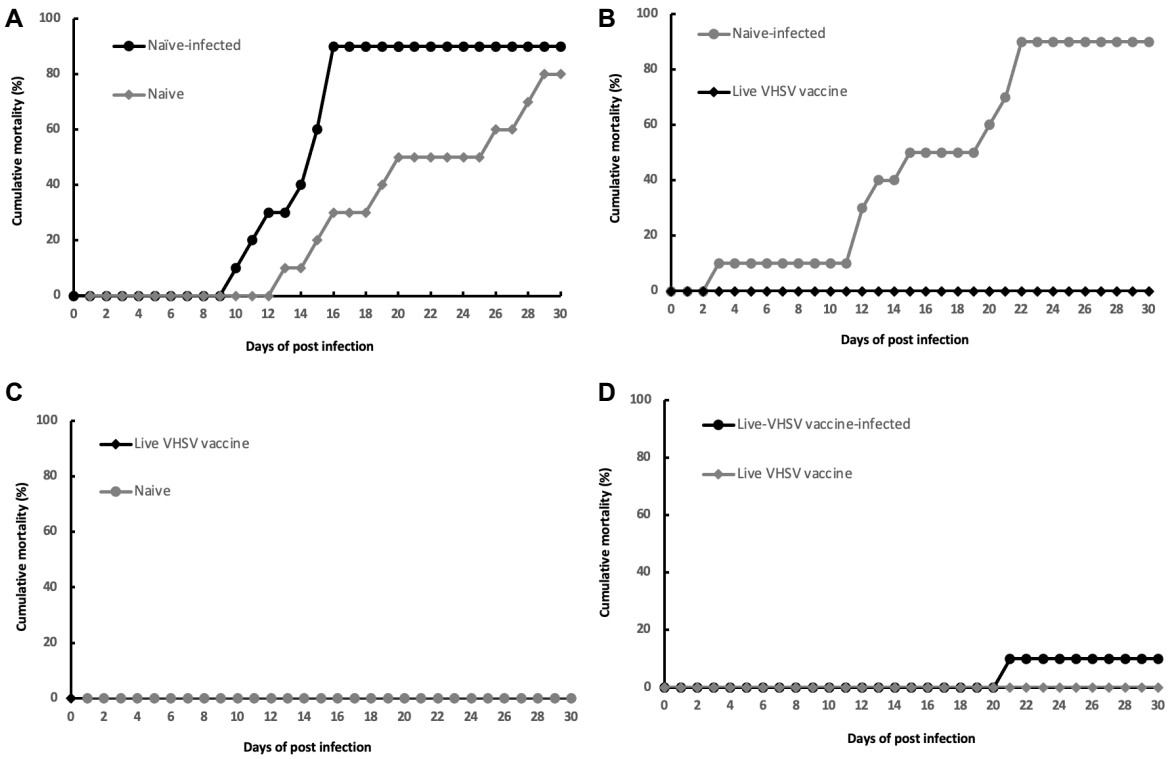

Fig. 2. Cumulative mortality under the cohabitant condition at 10˚C. (A) Naïve flounder challenged with $10^{4.5}$ $TCID_{50}$/100μL/fish VHSV (black circle) and naïve flounder (grey diamond). (B) Naïve fish challenged with $10^{4.5}$ $TCID_{50}$/100μL/fish VHSV (grey circle) and live VHSV immersion-vaccinated flounder (black diamond). (C) Live VHSV immersion vaccinated flounder (black diamond) and naïve flounder (grey circle). (D) Live VHSV immersion vaccinated flounder challenged with $10^{4.5}$ $TCID_{50}$/100μL/fish VHSV (black circle) and live VHSV immersion vaccinated flounder (grey diamond).

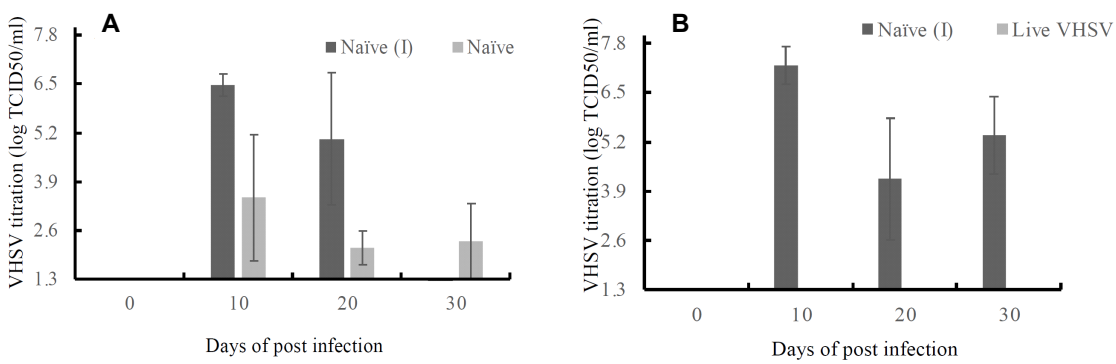

Fig. 3. Log VHSV titration (log $TCID_{50}$/mL) in kidney of olive flounder challenged with VHSV at 10˚C. (A) Negative controls (Naïve flounder, Black bar) were challenged with VHSV then negative control were reared with naïve flounder (Grey bar) at 10˚C. (B) Negative controls (Naïve flounder, black bar) were challenged with VHSV, then reared with live VHSV immersion vaccine-treated flounder at 10˚C. The horizontal axis represents the days of post infection at 10˚C. The vertical axis represents the log VHSV titration in kidney of challenged flounder and co-habitant flounder in this study.

A previous study reported that a live vaccine could stimulate humoral and cellular immunity (Adams., 2019). For example, Pacific herring (*Clupea pallasii*) survivors with prior exposure develop acquired immunity to diseases, including VHSV infection (Hershberger et al., 2007, 2010b, 2011; Kim et al., 2020; Kocan et al., 2001). Our cohabitation challenge study showed that live VHSV-vaccinated flounder followed by VHSV rechallenge did not transfer the virus to live VHSV-vaccinated flounder even at VHSV-susceptible water temperature. This study confirmed that 1) live VHSV immersion vaccine-treated flounder did not pose a risk of infection to naïve fish, even at a VHS-infectious water temperature; and 2) live VHSV immersion vaccine-treated flounder were resistant to VHSV infection at a susceptible water temperature.

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