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Kinetics of infectious virus and viral RNA copy number in the blood of olive flounder infected with viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT

Viral hemorrhagic septicemia (VHS) is a cold-water disease caused by viral hemorrhagic septicemia virus (VHSV) at an optimal temperature of 9 °C–15 °C. VHSV isolation and detection have been accomplished by using a number of diagnostic methods such as cell culture and qRT-PCR. Spleen and kidney have been reported as the main target organs of VHSV-infection; however, how VHSV spreads throughout the fish body has not been clearly studied. The purpose of this study was 1) to investigate viral titer and viral RNA copy number in the blood of VHSV-infected olive flounder at 10 °C and 13 °C; 2) to compare VHSV titer and viral RNA copy numbers in blood from fish exposed to the virus by two different challenges. VHSV titer at 10 °C was higher than at 13 °C in blood samples of injection challenged group. Whereas, similar titer was observed at 10 °C and 13 °C in the blood samples of the immersion challenged group. At 10 °C, copy numbers of VHSV-N gene in blood of immersion challenged group increased slightly in comparison to injection challenged group. At 13 °C, similar patterns were observed between the injection and immersion challenged groups. Also, higher titer and copy number were observed in fish blood compared to tested organs from our previous study. Our results indicate that VHSV genome existed in fish blood at earlier time points after infection, and the blood may contribute to the spread of the virus in whole fish body. In addition, VHSV diagnosis by qRT-PCR from fish blood samples, not requiring sacrificing the host fish can be valuable to collect the kinetic information of viral infection.

Viral hemorrhagic septicemia (VHS) is one of the serious finfish viral diseases that adversely affects the cultured olive flounder (*Paralichthys olivaceus*) in East Asian countries including Korea (Kim et al., 2009). The etiological agent of VHS, an OIE (World Organization for Animal Health) listed disease, is a rhabdovirus known as viral hemorrhagic septicemia virus (VHSV). It is a member of the genus *Novirhabdovirus* in the family Rhabdoviridae (OIE, 2018). VHS is typically a cold-water disease that occurs within the temperature range of 9–15 °C (Kim et al., 2009; Smail, 1999). Cumulative mortality rates as high as 90% are recorded in many cold water species in the northern hemisphere and also among cultured olive flounder in East Asia including Korea (Isshiki et al., 2001; Kim et al., 2009; Smail, 1999; Snow et al., 2005). VHSV infectivity varies as it depends on the virus genotype, fish species, water temperature, and other environmental factors (Hawley and Garver, 2008; Isshiki et al., 2003; Kim and Faisal, 2010; Snow et al., 2005). Previously, we reported different titers in fish organs of VHSV-infected flounder at water temperature of 10 °C and 13 °C (Kim et al., 2016). The study highlighted that titer can vary within the susceptible seawater temperature, and VHSV spreads rapidly to other fish organs after infection. Additionally, earlier findings have reported spleen and kidney as the main target organs for VHSV (Brudeseth et al., 2002; Wolf, 1988), and heart acts as a key target site for VHSV replication (Iida et al., 2003; Marty et al., 1998). Iida et al., (2003) studied titer in organs and blood of VHSV-infected flounder, and the titer were found to be higher than $10^{9.0}$ $TCID_{50}$/mL in the heart and blood at 1 or 2 weeks post-immersion challenge. VHSV has been detected in myocardium and blood vessels, as well as in fish leukocytes (Al-Hussinee et al., 2011a,b; Brudeseth et al., 2005). Therefore, it can be presumed that blood can act as a medium for the spread of VHSV to target organs. In this context, the present study was undertaken to examine infectious viral titers and viral RNA copy numbers in the blood of olive flounder challenged with VHSV by intramuscular injection (IM) or immersion challenge at differential water temperature of 10 °C and 13 °C.

A total of 240 olive flounders with mean body weight of 10 g were procured from a private fish farm at Tae-an, South Korea. The juvenile fish used in the present experiment was slightly larger in size than the fish used in our previous study (Kim et al., 2016). The fish were reared in eight aquaria (n = 30 each) and prior to start of experiment, spleen and kidney samples from five fish were inspected for presence of VHSV

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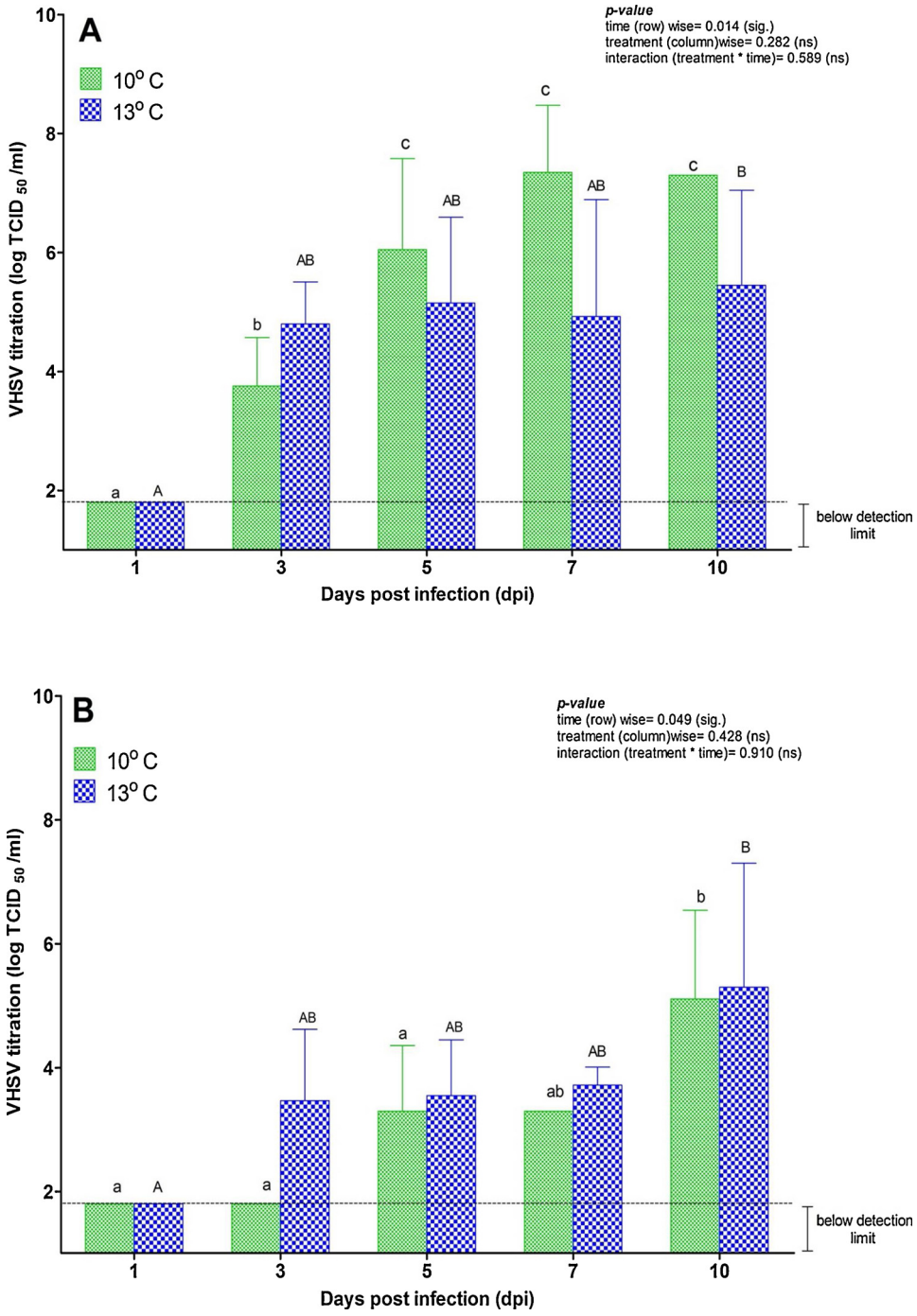
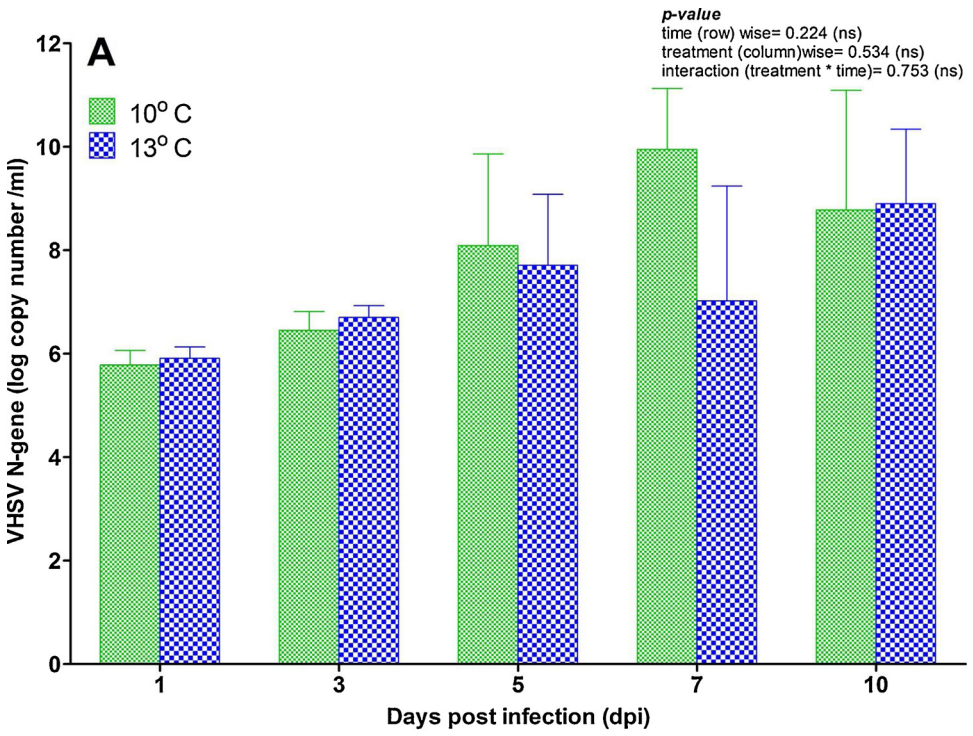


Fig. 1. Log_{10} infectious VHSV titration in blood samples from VHSV-infected olive flounder at 10 °C and 13 °C by intramuscular injection and immersion challenges. As a negative control, fish were injected with $DMEM_0$ medium at 100 µL/fish and also immersed with $DMEM_0$ medium diluted with 1000 times per liter. For injection challenge, flounder were injected with 100 µL of VHSV ($10^{4.5}$ $TCID_{50}$/100 µL/fish) and afterwards reared at 10 °C and 13 °C for 10 days. For immersion challenge, flounder were immersed with VHSV at a dose of $10^{5.5}$ $TCID_{50}$/mL for 1h at 10°C and 13°C and afterwards maintained at respective water temperatures. (A) VHSV infectivity in blood samples at 10°C and 13°C by injection challenge (B) VHSV infectivity in blood samples at 10°C and 13°C by immersion challenge. The dotted line demarcates the viral titer below detection limit. A two − way ANOVA was followed by Duncan's multiple range test, and, an unpaired *t*-test was used to determine the significant differences (if any) at different time points. Mean with different superscript letter per factor indicates significant difference while mean without any superscript letter per factor indicates no significant difference (Abbreviation: *sig.* = significant difference; *ns* = non-significant difference).

by cell culture and reverse transcriptase polymerase chain reaction (RT-PCR) to confirm VHSV-free status. The fish were acclimatized for 3 days at each temperature (10 °C and 13 °C) and then exposed to VHSV by injection (IM) or immersion challenge and reared at two different water temperatures of 10 °C and 13 °C. The VHSV IVa genotype (FYeosu05 strain) was used in the present study and cultured as described previously (Kim et al., 2016). For immersion challenge, 60 flounders were placed in static sea water containing $10^{5.5}$ $TCID_{50}$ (50% tissue culture infective dose) /mL VHSV for 1 h at 10 °C and 13 °C, and afterwards 30 fish each were maintained at their respective water temperatures of 10 °C and 13 °C. Whereas, injection challenge (IM) was performed at a dose of $10^{4.5}$ $TCID_{50}$/100 µL/fish by injecting 60 fish, and afterwards 30 fish were maintained at 10 °C while remaining 30 fish were maintained at 13 °C. As a negative control for immersion challenge group, 60 fish were immersed in sea water diluted with control medium viz., 10^{-3} Dulbecco's minimum essential medium without FBS ($DMEM_0$) /mL and fish were then divided into two groups with 30 fish in each group and maintained at 10 °C and 13 °C, respectively. Similarly, as a negative control for the injection challenge group, 60 fish were injected with control medium viz., $DMEM_0$/100 µL/fish and then divided into two groups with 30 fish in each group and maintained at 10 °C and 13 °C, respectively. To gain an insight on the viral kinetics, we exploited the use of quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and $TCID_{50}$ to quantify viral copy number and to measure VHSV titer, respectively, in the blood samples. To this cause, six fish from each experimental group at 10 °C and 13 °C were sampled at 1st, 3rd, 5th, 7th and 10th day post-infection (dpi). Blood (70–100 µL) was collected from the caudal vein of each fish using a 1 ml syringe and then



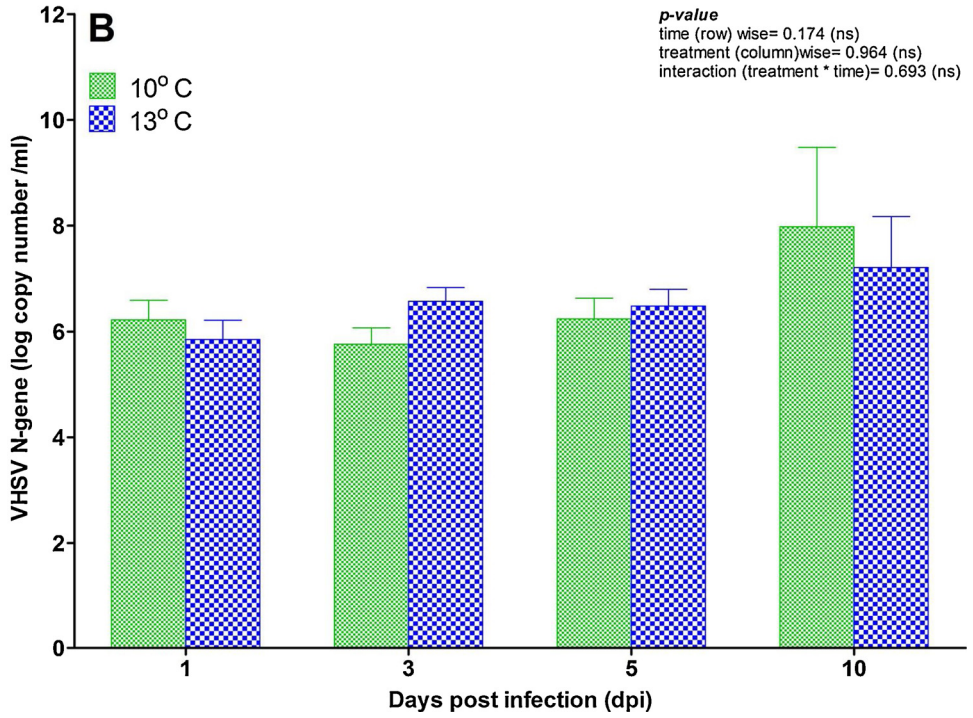


Fig. 2. Log_{10} VHSV N-gene copy numbers in blood samples from VHSV-infected olive flounder at 10 °C and 13 °C by intramuscular injection and immersion challenges. As a negative control, fish were injected with $DMEM_0$ medium at 100 µL/fish and also immersed with $DMEM_0$ medium diluted with 1000 times per liter. For injection challenge, flounder were injected with 100 µL of VHSV ($10^{4.5}$ $TCID_{50}$/100 µL/fish) and afterwards reared at 10 °C and 13 °C for 10 days. For immersion challenge, flounder were immersed with VHSV at a dose of $10^{5.5}$ $TCID_{50}$/mL for 1h at 10°C and 13°C and afterwards maintained at respective water temperatures. (A) VHSV N-gene distribution in blood samples at 10°C and 13°C by injection challenge (B) VHSV N-gene distribution in blood samples at 10°C and 13°C by immersion challenge. A two-way ANOVA was followed by Duncan's multiple range test, and, an unpaired *t*-test was used to determine the significant differences (if any) at different time points. Mean with different superscript letter per factor indicates significant difference while mean without any superscript letter per factor indicates no significant difference (Abbreviation: *sig.* = significant difference; *ns* = non-significant difference).

stored at -80 °C until use. To determine VHSV infectivity in blood samples, 96-well microtiter plate were seeded with FHM cells and incubated at 15 ± 0.5 °C. Afterwards, blood samples were serially diluted (10^{-1}–10^{-8}) with nine volumes of $DMEM_0$. Fifty microliter of each serial dilution was then added to each well and incubated at 15 ± 0.5 °C for 10 days to monitor viral cytopathic effect (CPE) microscopically. For each dilution, the number of wells with positive CPE was scored. Reed and Muench (1938) calculation method was used to determine dose of infectious virus in units of $TCID_{50}$/mL of blood. In order to determine viral copy number, RNA was extracted from blood samples using RNAiso Plus (Takara Bio Inc., Shiga, Japan) following manufacturer's instructions. Dried RNA pellet was dissolved in RNase-DNase free water (Sigma-Aldrich, St. Louis, Mo, USA) and cDNA was synthesized using M-MLV reverse transcriptase (Bioneer, Daejeon, South Korea) following manufacturer's protocol. The details about primer pair and qRT-PCR procedure to quantify viral copy number are outlined in previously published report (Kim et al., 2014). Briefly, reaction conditions were set as follows: 10 min pre-denaturation cycle at 95 °C, 40 cycles of denaturation at 95 °C for 20 s, and extension at 58 °C for 40 s. The quantitative data for the viral titer and copy number in the

fish blood was statistically analyzed using two-way ANOVA (analysis of variance) to determine significant differences in viral titer or copy number at 10 °C and 13 °C. The level of significance within the group (time wise), between the groups (group wise), and the interaction effect was determined at $P < 0.05$. Post hoc analysis was followed by Duncan's multiple range test, and, an unpaired *t*-test was used to determine the significant differences (if any) at different time points. All the statistical analysis was carried out using statistical package SPSS version 16 (SPSS Inc., USA).

VHSV titer in blood samples of infected flounder at 10 °C and 13 °C by injection challenge (Fig.1A) and immersion challenge (Fig.1B) are presented in Fig.1. VHSV titer showed a significant difference (time-wise) at 10 °C and 13 °C in both injection and immersion challenged groups. However, the titer showed a non-significant higher trend (treatment-wise) at 10 °C than at 13 °C in blood samples of injection (IM) challenged group. Whereas, similar VHSV titer was observed between 10 °C and 13 °C in the blood samples of the immersion challenged group. The disparity in infectious viral loads within the host susceptible water temperature of 10 °C and 13 °C suggests that even a subtle difference in water temperature can affect the VHSV infectivity pattern in fish blood. Usually, intramuscular injection is the most common method for artificial infection as it ensures an approximately equal amount of virus delivered to fish, but, it has some limitations as the method is time consuming, stressful and laborious. On the other side, immersion challenge is a preferred mode as it mimics natural course of infection and is less stressful to fish. However, the method requires a large amount of virus and also virus uptake varies from fish to fish. Having said that, both methods offer their own advantages and disadvantages and have been used either alone or in combination to study VHSV infection in fishes. In our study, virus titer was under detection limit (< 1.8 log $TCID_{50}$/mL) at 1st dpi (days post infection) at 10 °C and 13 °C for injection challenged group. Mean VHSV titer was around 3.8–7.3 and 4.8–5.5 log $TCID_{50}$/mL at 10 °C and 13 °C, respectively. For the immersion challenge, $TCID_{50}$ was also under detection limit at 1st dpi at two different temperatures (10 °C and 13 °C). Also, mean VHSV titer ranged from < 1.8 to 5.1 and 3.5–5.3 log $TCID_{50}$/mL at each sampling time point for the fish reared at 10 °C and 13 °C, respectively. At 10 °C, infectious VHSV titer by injection challenge was first detected at 3rd dpi and then rapidly increased at 5th and 7th dpi. Meanwhile at 13 °C, $TCID_{50}$ ranged from 4.8–5.5 log $TCID_{50}$/mL until the end of the experiment and the titer was approximately 100 times lower than at 10 °C. The results for the VHSV infectivity in blood of olive flounder suggests that virus is able to multiply better at lower window of susceptible water temperature. The finding is in line with our previous study, wherein, higher VHSV infectivity was observed in fish organs at 10 °C than at 13 °C (Kim et al., 2016). Additionally, Sano et al., (2009) while studying VHSV infection in Japanese flounder at susceptible/non-susceptible water temperature reported higher cumulative mortality at 20 °C in contrast to 25 °C. However, our study compared $TCID_{50}$ in fish organs and blood of VHSV-infected flounder within susceptible water temperature (Kim et al., 2016) and we found higher viral titers in blood compared with fish organs.

Further, we quantified VHSV copy number by targeting viral nucleoprotein (N) gene as it is most abundant in comparison to other rhabdoviral genes (Chico et al., 2006; Hope et al., 2010; Knüsel et al., 2007; Matejusova et al., 2008; Rose and Schubert, 1987). Fig. 2 reveals VHSV N-gene copy numbers in blood samples from VHSV-infected flounder at 10 °C and 13 °C in the injection challenge group (Fig. 2A) and the immersion challenge group (Fig. 2B). VHSV N-gene copy numbers ranged from 5.7 to 9.9 and 5.9 to 8.9 log copy numbers/mL at 10 °C and 13 °C, respectively. For immersion challenge, VHSV N-gene copy numbers were 5.7–7.9 and 5.8–7.2 log copy numbers/mL at 1st, 3rd, 5th and 10th dpi at 10 °C and 13 °C, respectively. At 10 °C, copy numbers of VHSV-N gene in injection challenged group increased at 5th and 7th dpi, whereas, viral copies slightly increased in blood by immersion challenge. At 13 °C, similar patterns were observed between the injection and immersion challenged groups. However, N-gene copy numbers did not directly correlate with the amount of infectious VHSV. It can be presumed that viral genomic segments present in the fish blood did not assemble further to form infectious virus in the fish body. In addition, we found that VHSV titer and viral copy numbers in the fish blood was similar or higher than tested organs from VHSV-infected flounder (Kim et al., 2016) at the similar sampling period. Iida et al., (2003) studied the fate of VHSV in Japanese flounder and found the viral titer to be highest in heart and blood of infected flounder. Similar findings were also noted by Al-Hussinee et al., (2011a,b), wherein, the authors reported presence of VHSV antigen using immunohistochemistry coupled with extensive gross hemorrhages on serosal surface correlating with presence of viral antigen in blood vessels and tissues. Recently, Qadiri et al., (2019) also reported that VHSV shows a strong tropism for endothelial cells as virus was frequently localized in the areas having direct connection with blood cells.

In conclusion, the present study for the first time investigated the VHSV infection in blood of olive flounder at host susceptible temperatures of 10 °C and 13 °C. Our findings suggest that fish blood may act as a medium for dissemination of VHSV to target organs and subsequently to whole fish body. The results from the current study can pave way for further research to better elucidate the role of blood in VHSV infection in flounder. Also, it can be of value to target future studies by analyzing blood from the same fish at different points without sacrificing the host fish in order to obtain a clear observation about the spread of VHSV in flounder.

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