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Short communication

Juvenile olive flounder immersed in live VHSV at 17 °C and 20 °C shows resistance against VHSV infection at 10 °C

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ABSTRACT

Viral hemorrhagic septicemia (VHS) causes serious economic loss in olive flounder aquaculture industry in Korea. Water temperature is known to play a critical role in VHS disease outbreak. Here, we assessed the potential efficacy of VHSV immersion treatment in relation to resistance conferred at differential water temperatures in olive flounder. VHSV acquired resistance was compared between formalin-killed VHSV immersion treatment and live VHSV immersion treatment at three different water temperatures viz., 10 °C, 17 °C, and 20 °C. At 10 °C, cumulative mortality was around 80% in live VHSV immersed group while 30% cumulative mortality was observed in formalin-killed VHSV treated group. After 4 weeks, surviving olive flounder at 17 °C and 20 °C were challenged with VHSV at 10 °C following which the VHS outbreaks took place at host susceptible water temperature. For the pre-treated flounder at 17 °C, survival rates were 80% and 30% after challenge at 10 °C in live VHSV immersed group and formalin-killed VHSV immersed group, respectively. Whereas, the pre-treated flounder at 20 °C showed survival rate of 75% and 20% after challenge at 10 °C in live VHSV immersed group and formalin-killed VHSV immersed group, respectively. Our results propose the fact that live VHSV immersion using non-susceptible water temperature has the potential to protect olive flounder against VHSV infection. Moreover, the protective efficacy of live immersion treatment in a non-excited immune state without the use of an adjuvant combined with water temperature adjustment was investigated for the first time at 17 °C. Further studies should be targeted to explore the host-associated immune factors responsible for the protective effect and acquired resistance in olive flounder after live VHSV immersion treatment.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV) is the etiological agent of viral hemorrhagic septicemia (VHS), a serious fish disease affecting cultured olive flounder (*Paralichthys olivaceus*) in East Asia (Isshiki et al., 2001; Kim et al., 2009; Skall et al., 2009; Smail, 1999; Wolf, 1988). VHSV is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Trdo et al., 2005). VHS infection predominantly occurs during winter/spring seasons when water temperature is low particularly in the range of 8 °C–15 °C (Isshiki et al., 2001). Cumulative mortality as high as 90% is often recorded (Olesen, 1998; Kim et al., 2009; Kim et al., 2016), however, it gets reduced when rearing water temperature is increased upto 17 °C (Takami et al., 2010; Nishizawa et al., 2011). Interestingly, no mortality of Japanese flounder was observed at 20 °C which highlights the effect of water temperature on VHSV infection (Sano et al., 2009). Previous reports also suggest that water temperature plays a critical role in the spread and transmission of VHSV (Amend, 1970, 1976; Hetrick et al., 1979; Sano et al., 2009; Kim et al., 2016).

Disease prevention and control are essential for sustenance of aquaculture in the long run both economically as well as environmentally. Vaccination plays an important role in high-density commercial fish farming where it is frequently used as a prophylactic measure to control persistent and emerging diseases. Over the last three decades, increased efforts have been made globally to develop an efficient, safe and cost-effective vaccine against VHSV using subunit or killed as well as attenuated virus (de Kinkelin and Béarzotti, 1981; de Kinkelin et al., 1995; Bernard et al., 1983; Leong and Fryer, 1993; Lecocq-Xhonneus et al., 1994; Nishizawa et al., 2011). Although, some of the developed vaccines have shown good protection under the laboratory conditions but none of these are available for commercial application in East Asian Countries which restricts their use at field level. Among the available fish vaccines, formalin-inactivated vaccines are commonly used as the pathogen of interest is killed during vaccine

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preparation which renders it safe to use. On the other hand, live viral vaccines have also proven to be effective in fish and keeping aside their eco safety issues, which is separate topic to debate upon, they have given good results in fish (Ronen et al., 2003; Sommerset et al., 2005). Live vaccines have been reported to induce a strong protective effect against viral diseases by activating specific immune response in fish (Nishizawa et al., 2012; Oh et al., 2014). However, the risk involved in use of a live vaccine under field conditions does remains, but it can be substantially reduced by rearing the fish at 25 °C after immunization (Nishizawa et al., 2011). Moreover, Sano et al. (2009) also reported that VHSV is not present in artificially infected Japanese flounder after 3 weeks of rearing at 25 °C.

Adjusting the water temperature coupled with an efficient vaccination strategy can be a useful combination to prevent VHSV infection in flounder. Previous studies have focused on protection efficacy of Poly (I:C) immunized flounder at different rearing temperatures (Nishizawa et al., 2011; Kim et al., 2016), whereas, the effect of water temperature on VHSV infected flounder in a non-excited immune state has not been studied yet. In light of this, the present study was aimed to test the hypothesis that live VHSV treatment at non-VHSV susceptible water temperature might activate fish defense system and confer protection against VHSV infection. Additionally, we tested the protection efficacy between formalin killed and live VHSV treated flounder at differential water temperatures. Fish were reared at a non-susceptible water temperature and afterwards challenged with VHSV at susceptible water temperature. Further, we recorded cumulative mortality and also compared viral load and RNA copy numbers in kidney from vaccinated flounder as well as VHSV challenged-vaccine treated flounder.

2. Materials and methods

2.1. Fish and virus

A total of 540 juvenile olive flounder (8.8 ± 0.9 cm, 6.0 ± 1.2 g) were used in the current study and reared in UV-treated seawater at a separate facility. Prior to the experiments, samples of fish were inspected for VHSV by cell culture and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to confirm the VHSV-free status.

An isolate of VHSV, FYeosu05 (genotype IVa) obtained from a VHSV infected olive flounder farm at Yeosu, Korea, in 2005 (Kim et al., 2009) was used for the present experiment. VHSV was propagated in fathead minnow (FHM) cell line in 75 cm^2 tissue culture flasks at 15 ± 0.5 °C and after the development of complete cytopathic effect (CPE), cell culture supernatant was collected, centrifuged (4000 rpm, 15 min, 4 °C) and stored at -80 °C until use (Kim et al., 2016).

2.2. Immersion of juvenile olive flounder with live VHSV or formalin-killed VHSV at water temperatures of 10 °C, 17 °C, and 20 °C

Infectivity of VHSV stock solution stored at -80 °C was $10^{8.5}$ $TCID_{50}$/mL. In order to inactivate the virus, formalin (2.0% v/v) was added to the stock solution for 24 h at room temperature (20 ± 2 °C). Fish were randomly divided into three groups viz., negative control, formalin-killed VHSV immersion treated and live VHSV treated group with 180 fish per group. A 30 (in duplicate) out of 180 flounder within each negative/VHSV immersed group were reared at 10 °C, 17 °C, and 20 °C, respectively, in 20 L fish rearing tanks containing seawater. Therefore, a total of 540 fish were used at three differential temperatures. After acclimation for 3 days at each temperature, fish were immersed with formalin-killed VHSV or live VHSV at a dose of $10^{5.5}$ $TCID_{50}$/mL at 10 °C, 17 °C, or 20 °C for 1 h. Fish in the negative control group were immersed with $DMEM_0$ medium at 10 °C, 17 °C, or 20 °C for 1 h. During the experiment, two fish in each experimental group were randomly collected for sampling on the 1st, 3rd, 5th, and 7th day after immersion treatment. Kidney samples were aseptically dissected and immediately stored at −80 °C until use and afterwards homogenized with nine volumes of $DMEM_0$ medium for qRT-PCR analysis. In addition, fish mortality was monitored daily for 30 days.

2.3. VHSV challenge at 10 °C for surviving flounder after live VHSV immersion and formalin-killed VHSV immersion

At 30 days post-immersion treatment, rearing water temperature was decreased from 17 °C or 20 °C to 10 °C at a rate of 1 °C/hour. After 3 days of acclimation at 10 °C, 40 surviving flounder were chosen for VHSV challenge in each experimental group. Fish were challenged intramuscularly with VHSV at a dose of $10^{4.5}$$TCID_{50}$/100 µL/fish and divided into two tanks. The first tank (n = 20) was used to observe cumulative mortality while as the second tank (n = 20) was used for fish sampling on the 1st, 3rd, 5th, 7th, and 10th day after infection. Three flounder were randomly selected for analyzing VHSV-N gene copy numbers at each sampling time point. Fish kidney samples were aseptically dissected and immediately stored at -80 °C until use. Afterwards, the samples were homogenized with nine volumes of $DMEM_0$ medium for qRT-PCR analysis. Cumulative mortality was monitored daily for 15 days.

2.4. VHSV-N gene copy numbers in kidney of formalin-killed VHSV and live VHSV vaccinated flounder challenged with VHSV

Viral RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Dried RNA pellet was dissolved in RNase-DNase free water (Sigma-Aldrich, St. Louis, MO, USA). Extracted RNA was reverse transcribed to synthesize complementary DNA (cDNA) using ReverTra Ace Kit (TOYOBO, Osaka, Japan) according to manufacturer's instructions. The qRT-PCR primer set (qVN_11 F, GAATCCGTGCAGCTTTTTCAGG; qVN_160R, CAAGTGC ATCCACGATCACCTTC) were designed with Primer3Plus (available online: http://primer3plus.com/cgi-bin/dev/primer3plus.cgi/) based on the N gene of VHSV FYeosu05 genome sequence. The qRT-PCR was performed using the procedure outlined earlier by Kim et al. (2014).

2.5. Ethics statement

All fish experiments were carried out in strict accordance with the recommendations and guideline of the Institutional Animal Care and Use Committee of Chonnam National University (permit number: CNU IACUC-YS-2017-17).

3. Results and discussion

The present study assessed the protective efficiency of VHSV immersion treatment at differential water temperature (10 °C, 17 °C and 20 °C) in olive flounder. As immersion treatment mimics natural course of infection, therefore, it is a promising strategy to evaluate the protective effect in large number of fishes. Cumulative mortality rate at three differential water temperatures are presented in Table 1. In live VHSV immersion group at 10 °C, mortality started on the 7th day after immersion treatment and then rapidly increased from the 8th to 13th day. This suggests that olive flounder were highly susceptible to live

Table 1
Cumulative mortality at three different water temperatures. Olive flounder were immersed with live VHSV or formalin-killed VHSV at a dose of $10^{5.5}$ $TCID_{50}$/mL for 1 h at 10 °C, 17 °C or 20 °C.

Temp./Treated group	Negative	FKC-VHSV	Live VHSV
10 °C	0	30	80
17 °C	0	0	0
20 °C	0	0	0

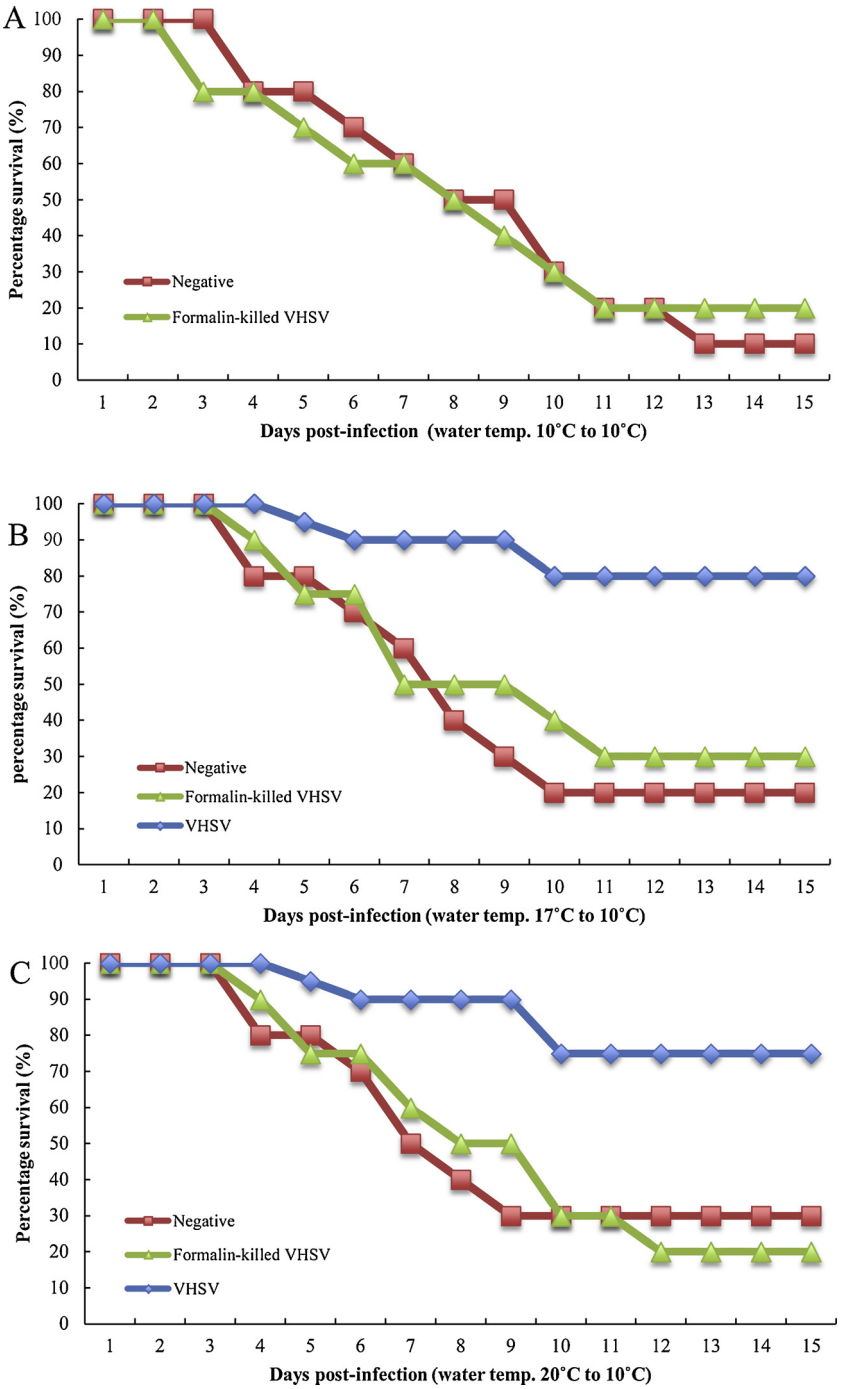

Fig. 1. Water temperature effect of formalin-killed VHSV and live VHSV immersion on survival rate of fish after challenge with VHSV by injection. Surviving flounder were injected with VHSV at $10^{4.5}$ $TCID_{50}$/100 μL/fish at 10 °C. Mortality was monitored daily. (A) Percentage survival of formalin-killed VHSV and negative control at 10 °C; (B) Percentage survival of formalin-killed VHSV, negative control, and live VHSV treated flounder at 17 °C and challenged with VHSV at 10 °C; (C) Percentage survival of formalin-killed VHSV, negative control and live VHSV treated flounder at 20 °C and challenged with VHSV at 10 °C.

VHSV at 10 °C. In other words, it represents that water temperature at 10 °C is pathogenic to flounder as it allows VHSV to replicate and accomplish higher copy numbers. It is expected at 10 °C as the temperature falls within the permissive range for VHSV disease initiation and this observation is in line with our previous finding (Kim et al., 2016). No cumulative mortality was observed at 17 °C and 20 °C in any of the experimental groups. We infer that VHSV infection did not occur in olive flounder at 17 °C and 20 °C. Our results show that cumulative mortality was high in live VHSV immersed flounder at 10 °C. It is also likely that initial bursts of VHSV replication might takes place at 17 °C/20 °C as has been reported previously (Sano et al., 2009; Nishizawa et al., 2011) which can activate the host defense system. This initial immune priming can confer protection against VHSV without actual progression of virus to the infectious stage at non-susceptible water temperature. However, in the present study no increase in N-gene copy number was observed in kidney of olive flounder and we presume that viral copy number in kidney was below the detection limit of the current employed qRT-PCR assay. Also, the possibility of active VHSV replication in other target organs relative to kidney cannot be ruled out. It has been reported that VHSV is rapidly cleared from fish exposed to the virus at temperatures above 20 °C/25 °C (Goodwin and Merry, 2011; Nishizawa et al., 2011).

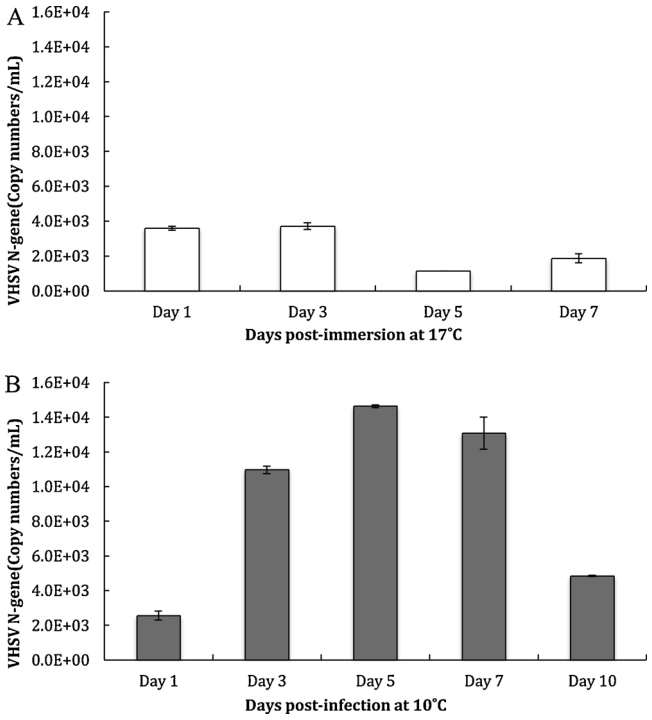

Fig. 2. Quantification analysis of VHSV N-gene in kidney from live VHSV immersion treated flounder at 17 °C and challenged with VHSV at 10 °C. (A) VHSV N-gene quantification analysis in kidney from live VHSV immersion treated flounder at 17 °C. Flounder kidney samples were collected at 1, 3, 5, and 7 days post-immersion at 17 °C. (B) VHSV N-gene quantification analysis in kidney from live VHSV immersion treated flounder challenged with VHSV at 10 °C. The flounder was injected with VHSV at $10^{4.5}$ $TCID_{50}$/100 µL/fish were reared at 10 °C for 15 days. The flounder kidney samples were collected at 1, 3, 5, 7, and 10 days post-infection with VHSV at 10 °C. Y-axis represents VHSV N-gene copy numbers in kidney per mL. Each bar represents the mean of VHSV N-gene copy numbers in triplicate at each sampling date. Error bars are standard deviations.

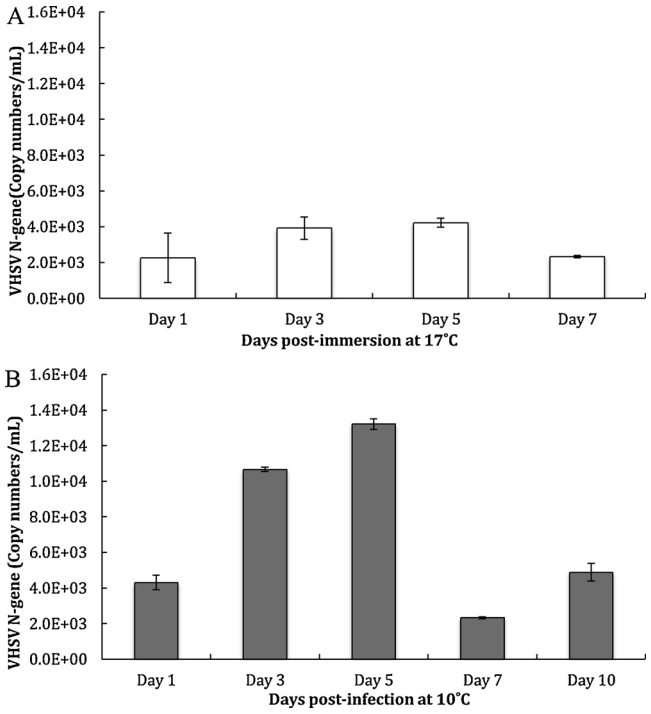

Fig. 3. Quantification analysis of VHSV N-gene in kidney from formalin-killed VHSV immersion treated flounder at 17 °C and challenged with VHSV at 10 °C. (A) VHSV N-gene quantification analysis in kidney from formalin-killed VHSV immersion treated flounder at 17 °C. The flounder kidney samples were collected at 1, 3, 5, and 7 days post-immersion at 17 °C. (B) VHSV N-gene quantification analysis in kidney from formalin-killed VHSV immersion treated flounder challenged with VHSV at 10 °C. The flounder was injected with VHSV at $10^{4.5}$ $TCID_{50}$/100 µL/fish and reared at 10 °C for 15 days. The flounder kidney samples were collected at 1, 3, 5, 7, and 10 days post-infection at 10 °C. Y-axis represents VHSV N-gene copy numbers in kidney per mL. Each bar represents the mean of VHSV N-gene copy numbers in triplicate at each sampling time point. Error bars are standard deviations.

Water temperature is an important factor that affects VHS outbreak in olive flounder (Sano et al., 2009) as well as progression of VHSV infection at 8 °C–15 °C (Basurco et al., 1995; Isshiki et al., 2001; Kim et al., 2009; Snow et al., 1999; Olesen, 1998; Kim et al., 2016). In our study, formalin-killed VHSV immersed flounder was not infected with VHSV at all of the temperatures tested during post-vaccination periods indicating that the virus was successfully inactivated by formalin.

Survival rates at differential water temperatures in flounder after VHSV challenge at 10 °C are shown in Fig. 1. As depicted, survival rate at 10 °C was 10% and 20% in the negative control and formalin-killed VHSV immersed group, respectively. For the VHSV immersed flounder at 17 °C, survival rates were 20%, 30%, and 80% in the negative control, formalin-killed VHSV immersed group and live VHSV immersed group, respectively. Whereas, for the VHSV immersed flounder at 20 °C, survival rates were 30%, 20%, and 75% in the negative control, formalin-killed VHSV immersed group, and live VHSV immersed group, respectively. Less than 30% survival rates were observed in negative control and formalin-killed VHSV immersion vaccine group after challenge with VHSV at 10 °C. This reflects that formalin-killed VHSV vaccinated flounder were not able to mount sufficient immune response against VHSV. We presume that formalin inactivated VHSV partially activates the host defense system in contrast to live virus possibly due to damage to the epitopes or change in the conformational structures of viral proteins (Sanders et al., 2015). However, further studies are warranted to better elucidate and confirm the effect of formalin treatment on VHSV antigenic potential.

In live VHSV immersed group, survival rates were 80% and 75% after challenge with VHSV at 10 °C. These fish were reared at 17 °C and 20 °C for 30 days and afterwards challenged with VHSV by intramuscular injection at 10 °C. High protection efficiency against VHSV challenge was observed indicating efficient protection of olive flounder against VHS by VHSV immersion treatment at 17 °C and 20 °C. Sano et al. (2009) reported that cumulative mortality of VHSV-infected flounder was reduced to 20% when the water temperature was shifted from 14 °C to 20 °C, whereas, the cumulative mortality was 100% when fish were maintained at 14 °C for 14 days. In our study the survival rate was not high in formalin-killed VHSV immersed group at 17 °C, or 20 °C but high survival rate was observed in live VHSV immersed group at 17 °C and 20 °C. The results suggest that live VHSV immersed flounder at 17 °C and 20 °C become refractory to subsequent VHSV infection at host susceptible water temperature.

Quantification of VHSV N-gene was done from kidney samples of live VHSV immersed group and formalin-killed VHSV immersed group at 17 °C for which sampling was performed at 1, 3, 5, and 7 days after immersion treatment. We also quantified VHSV-N gene copy number from the live VHSV and formalin killed VHSV pre-treated flounder at 17 °C after challenge with VHSV at 10 °C and the sampling for the same was done at 1, 3, 5, 7, and 10 days after immersion treatment. VHSV N-gene quantification results at 17 °C are presented in Fig. 2. In the live VHSV immersed group at 17 °C, the VHSV N-gene copy number was estimated to be 3.6×10^3, 3.71×10^3, 1.14×10^4 and 1.87×10^3 copy numbers/mL at 1, 3, 5, and 7 days post immersion treatment, respectively (Fig. 2A). After challenge with VHSV at 10 °C, VHSV N-gene quantification in live VHSV immersed group was estimated to be 2.55×10^3, 1.10×10^4, 1.46×10^4, 1.31×10^4, and 4.85×10^3 copy

numbers/mL at 1, 3, 5, 7, and 10 days post immersion treatment, respectively (Fig. 2B). VHSV N-gene copy number in live VHSV immersed group was not increased when fishes were maintained at 17 °C, whereas, VHSV-N gene copy number got increased at 3 to 7 days post infection and afterwards rapidly decreased on 10^{th} day. When we compare the survival rate and viral copy numbers in live VHSV immersed flounder, the copy numbers was increased after challenge with VHSV at 10 °C but the survival rate was 80% in olive flounder challenged with VHSV at 10 °C. It is likely that live VHSV immersion treatment at 17 °C and 20 °C allows the fish to acquire immune response against VHSV infection in olive flounder.

In the formalin-killed VHSV immersed group at 17 °C, the VHSV N-gene quantification at 1, 3, 5, and 7 days post immersion treatment was estimated to be 2.27×10^3, 3.93×10^3, 4.23×10^3, and 2.33×10^3 copy numbers/mL, respectively (Fig. 3A). After challenge with VHSV, the VHSV N-gene was estimated to be 4.31×10^3, 1.07×10^4, 1.32×10^4, 2.33×10^3, and 4.88×10^3 copy numbers/mL at 1, 3, 5, 7 and 10 days post immersion treatment, respectively (Fig. 3B). The copy number got increased at 3–5 days post-infection and then rapidly decreased at 7 days post-infection but the survival rate in formalin-killed VHSV vaccinated flounder was 30%, indicating that VHS outbreak occurred in formalin-killed VHSV vaccinated flounder at 17 °C after challenge with VHSV at 10 °C. It could be interpreted that flounder immersed in formalin killed VHSV were not able to mount sufficient immune response against VHSV infection and the fish succumbed to death. Avunje et al. (2012) reported temperature dependent viral replication and immune response against VHSV infection in olive flounder, wherein, the authors concluded that immune response in VHSV-infected olive flounder plays a crucial role in the survival of the host at higher temperature. Matsuyama et al. (2012) reported that antiviral responses and protein expression in Japanese flounder are dependent on size and life stages of the fish. Interestingly, the VHSV successfully gained entry in the fish body by immersion treatment at 17 °C and 20 °C. VHSV N-gene copy number was detected but not increased in kidney of live VHSV immersed and formalin-killed VHSV vaccinated flounder. Taken together, live VHSV vaccinated flounder at 17 °C and 20 °C acquired resistance against VHSV infection at 10 °C. Our results indicate that VHSV immersion vaccine at water temperature over 17 °C might confer resistance to the fish against VHS outbreaks. Moreover, the protective efficacy of live VHSV immersion treatment in a non-excited immune state was investigated for the first time at 17 °C. The current findings unveil the protective effect of live and formalin killed vaccine in combination with water temperature adjustment in flounder fishes. However, future studies should be targeted to better explore the host associated immune factors responsible for immune protection at differential water temperatures in relation to vaccine treatment in fishes.

Declaration of Competing Interest

None.

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