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Evaluation of blue mussel *Mytilus edulis* as vector for viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT: When viral diseases occur in aquaculture farms, the virus released into the seawater from infected animals can re-infect other susceptible species or accumulate in filter-feeding organisms. We conducted a viral hemorrhagic septicemia virus (VHSV) survivability analysis of blue mussel *Mytilus edulis* digestive enzymes, viral depuration, and infectivity tests via *in vitro* and *in vivo* inoculation to evaluate the infectious state. VHSV particles were not completely digested within 24 h *in vitro* and were maintained for 7 d in the mussel digestive gland. Mussels cohabitating with naturally VHSV-infected olive flounder *Paralichthys olivaceus* could accumulate the viral particles. Although the viral particles in the gill as the entrance of filter-feeding organisms are infectious, the presence of these particles in the digestive gland were not able to induce cytopathic effects *in vitro*. Viral particles detected by RT-PCR from bivalve mollusks (Pacific oyster *Crassostrea gigas* and mussel) from the field did not produce cytopathic effects in cell culture and did not replicate after intraperitoneal injection into olive flounder. Therefore, VHSV particles in blue mussel might be in a non-infectious stage and the possibilities of VHSV transmission to fish under field conditions are scarce.

KEY WORDS: Viral hemorrhagic septicemia · Blue mussel · Digestive gland tissue · Survivability · Depuration

INTRODUCTION

The spread of pathogenic fish viruses depends on several factors, including seawater currents, potential reservoir hosts, and vectors. After the occurrence of a viral outbreak in an open aquaculture farm, release of the virus into the seawater from infected fish should be suspected. It is possible that a pathogenic virus could spread widely and then re-infect other susceptible hosts or accumulate in mollusks by filter-feeding. Bivalve mollusks growing in coastal areas may be contaminated by various pathogens such as human enteric virus, infectious pancreatic necrosis virus, marine birnavirus, and white spot syndrome virus (WSSV) (Mortensen et al. 1992, Atmar et al. 1993, 1995, Suzuki & Nojima 1999, Vazquez-Boucard et al. 2010, Jin et al. 2014, Kim et al. 2016). Due to their filter-feeding nature, bivalves trap many substances, including pathogens. Thus, filter-feeding bivalve mollusks may play a role as a disease transmission vector or carrier.

Viral hemorrhagic septicemia (VHS) is one of the most serious viral fish diseases and is listed as a reportable disease by the World Organization for Animal Health (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2017/). VHS produces annual outbreaks in farmed flounder *Paralichthys olivaceus* in Korea, and high mortality rates (about 50%) have been reported in cultured juvenile and adult flounder (Kim et al. 2009). However, whether VHS transmission occurs via a vector or viral indicator species is unknown.

Pacific oyster *Crassostrea gigas* and blue mussel *Mytilus edulis* are widely distributed in large numbers in the vicinity of aquaculture farms in Korea. These bivalve mollusks may serve as a vector or

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reservoir for fish-pathogenic viruses. Thus, an understanding of how these viruses are maintained in bivalve mollusks and whether they are involved in transmission is crucial for aquaculture.

The aim of this study was to evaluate whether viral hemorrhagic septicemia virus (VHSV)-exposed blue mussels are in an infectious or non-infectious state.

MATERIALS AND METHODS

Viral propagation

Epithelioma papulosum cyprini (EPC; European Collection of Authenticated Cell Cultures [ECACC] 93120820) cells were used to propagate VHSV. The cell line was grown at 20°C in minimum essential medium (MEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic and antimycotic solution (Gibco). The virus used was originally isolated from farmed flounder (10 ± 5 g body weight) infected with VHSV genotype IVa in Jinha in February 2008. The kidneys were homogenized, filtered (0.45 µm pore size), and inoculated (100 mg kidney ml^{-1}; 100 µl) onto EPC cells cultured in T-75 cm^2 culture flasks (Corning). Following development of viral cytopathic effects (CPE) in 5–7 d, virus-infected cells were centrifuged at 1500 × *g* for 10 min and supernatants containing VHSV were stored at –80°C.

Quantitative PCR (qPCR) assay

Total RNA (50 µl) was extracted from blue mussel digestive gland tissue (50 mg) using an RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. The cDNA was prepared from an RNA (1 µl) mixed random primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). To detect VHSV from the bivalve mollusks, primer sets for 2-step reverse transcription-PCR (RT-PCR), real-time PCR, and 6-carboxyfluorescein (FAM)-5′ labeled probes (Integrated DNA Technologies) were designed from the conserved region of the glycoprotein gene of VHSV genotype IVa. Viruses were quantified using LightCycler 480 Probe Master mixture (Roche) according to the manufacturer's instructions. The qPCR reaction mixture contained 1 µl of cDNA, each primer (VqF, 5′-TTT CTT GGT GAT TCT GAT CAT CA-3′ and VqR, 5′-CCG AAT CGG AAC AAA GGA G-3′) at a concentration of 500 nM, and 200 nM of the probe (Vq-probe, FAM-5′-ACT CAA CGA CCT CCG GTC GAG A-3′-IBFQ [Iowa Black Fluorescent Quencher; Integrated DNA Technologies]). Amplification conditions were 95°C for 10 min, followed by 40 cycles of 94°C for 10 s, 60°C for 15 s, and 72°C for 20 s. A recombinant plasmid containing 157 bp from the glycoprotein gene and amplified using the VqF and VqR primer set was purified from the transformed *Escherichia coli* DH5α strain as a positive control. A serial 10-fold dilution of the control plasmid was used to establish a standard curve (5.0×10^5 to 5.0×10^0 copies μl^{-1}). The standard curve, generated using the mean data from experiments performed in triplicate, indicated a good linear relationship between the cycle threshold (C_T) values. All samples used were tested in duplicate, and all PCR reactions were carried out twice.

Survivability of VHSV in mussel digestive enzymes

To determine the survivability of VHSV in mussel, digestive enzymes were extracted from mussel using a method modified from Areekijseree et al. (2004). Digestive gland tissues, pooled from 5 mussels, were homogenized on ice with 0.1 M phosphate-buffered saline (PBS; pH 7.3) at a 1:10 (w/v) dilution. The digestive gland tissue homogenate was centrifuged at 5000 × *g* for 5 min and the upper lipid was discarded, and the supernatant was collected. To minimize inhibitors in the digestive gland tissue, the supernatant was filtered through a membrane (0.45 µm pore size; Millipore). The resulting supernatant was used as the digestive enzyme solution for virus digestion. Digestive enzymes and cultured VHSV (10^8 $TCID_{50}$ ml^{-1}; where $TCID_{50}$ is the tissue culture infectious dose 50% endpoint) were mixed 1:1 (v/v), and the digestive rate was analyzed for 1 and 24 h at 20°C. MEM supplemented with 10% FBS and cultured VHSV mixture were used as the control. The $TCID_{50}$ ml^{-1} was determined in the EPC cell line. The inhibition rate of digestive gland tissue homogenate and the natural decline of the viral titer in MEM were used to calculate the digestive rate. Formulas for calculating the digestive rate were as follows:

$$\text{Inhibition rate by digestive gland homogenate (\%)} = [1 - (\text{Initial virus } TCID_{50}\ ml^{-1} \text{ mixed with digestive gland homogenate / Initial virus } TCID_{50}\ ml^{-1} \text{ on MEM})] \times 100 \quad (1)$$

$$\text{Expected virus } TCID_{50}\ ml^{-1} = \text{Reduced virus } TCID_{50}\ ml^{-1} \text{ during 1 or 24 h incubation period in MEM} \times \text{Inhibition rate by digestive gland homogenate} \quad (2)$$

Digestive rate (%) = [1 − (Virus $TCID_{50}$ ml^{-1} after 1 or 24 h incubation period in digestive gland homogenate / Expected virus $TCID_{50}$ ml^{-1})] × 100 (3)

Estimated shedding rate of virus from VHSV-infected flounder

Olive flounders *Paralichthys olivaceus* (n = 15; 2.75 ± 0.5 g body weight) were acclimatized for 14 d at 15°C in a 10 l tank. Each fish was intraperitoneally injected with 0.1 ml VHSV containing 10^6 viral particles ml^{-1} in MEM. Surviving fish were transferred newly to a 10 l tank for each time point (1, 3, 7, 9, and 11 d post-injection), followed by holding for 6 h, and then 1 l of rearing water was sampled. To determine the concentration of virus particles in the rearing water of the experiments, 1 l of rearing water was concentrated as described in Kim et al. (2016). Briefly, water was filtered with a GF/C membrane (1.2 µm pore size; Whatman) as sediment eliminator and then a negatively charged membrane (HA type, 0.45 µm pore size; Millipore) was used for virus capture. The filter membrane was rinsed with 100 ml of 0.5 mM H_2SO_4 followed by elution with 10 ml of 1 mM NaOH (pH 10). The filtrate was concentrated at 3000 × *g* for 10 min at 4°C using an ultrafiltration system (Amicon Ultra-15 Centrifugal Filter Unit, 30 kDa; Millipore), and then final water concentrate was adjusted to 1 ml with PBS buffer. Concentrated water samples were tested for VHSV by qPCR. The amount of viral particles was divided by the mass of fish in the tank for each time point to determine the quantity of VHSV shed from the fish into 1 l of water. And estimates of shedded viral particles were determined from the quantity of VHSV shed from 1 kg of fish for 1 h (viral particles kg^{-1} h^{-1}) on each sampling day.

Cohabitation of mussels with VHSV-infected flounder

In Expt 1, VHSV-free mussels (11 ± 1.2 g body weight) and flounder (12.0 ± 1.0 g) were maintained for 14 d at 15°C in a 40 l tank. Donor flounders (n = 15) were intraperitoneally injected with 0.1 ml VHSV containing 10^6 viral particles ml^{-1} (corresponding to 10^5 $TCID_{50}$ ml^{-1}) in MEM. Blue mussels (n = 20) as recipient were introduced into a 40 l tank to cohabitate with the VHSV-infected flounder on Day 3 post-challenge. The mussels were sampled at 1, 3, 5, 7, and 9 d post-cohabitation. All flounder kidneys and mussel digestive gland tissues were tested for VHSV by qPCR and cell culture.

For Expt 2, to enhance the viral dose in the rearing water in conditions similar to nature, large-sized flounder (550.0 ± 27.5 g) were used in a cohabitation experiment with mussels. VHSV-infected flounder located at a farm on the southern coast of Korea which were exhibiting abnormal behavior were collected and transferred to the laboratory. After 1 d, all dead fish were removed and the remaining flounder were used as donors for VHSV. Recipient mussels (15 ± 1.2 g; n = 40) were cohabitated with the infected flounder (n = 24) for 3 d at 15°C in a 60 l tank. The mussels were sampled at Days 1 and 3 post-cohabitation. After removing gill and digestive glands from 10 mussels, tissues were pooled. Flounder kidneys and mussel tissues (gill and digestive gland) were sampled and tested for VHSV by qPCR and cell culture. To determine the concentration of virus particles in the rearing water of the experiments, 1 l of rearing water was sampled and concentrated as above (see previous subsection). During the cohabitation period, the water in the tank was exchanged daily to mimic the natural flow of seawater in the field.

Viral depuration from mussels after artificial contamination with VHSV

VHSV-inoculated seawater (10^6 viral particles ml^{-1} seawater; 10^5 $TCID_{50}$ ml^{-1}) was used for the depuration experiment. Twenty-five mussels were immersed in a 10 l tank inoculated with VHSV for 6 h at 12 ± 0.5°C to produce VHSV-contaminated mussels (VHSV-Mu). VHSV-Mu were transferred to a new 5 l water bath for 7 d at 12 ± 0.5°C. Five VHSV-Mu were sampled at each time interval of 0, 12, 24, 72, and 128 h. After removing the digestive gland tissue from VHSV-Mu, tissues were tested for VHSV by qPCR. Sampling was conducted before changing the seawater, and the seawater was exchanged completely every day during the depuration period.

Infectivity of VHSV in bivalve mollusks in the field under *in vitro* and *in vivo* conditions

Two field bivalve samples identified as VHS-positive by PCR were used. One was isolated from the digestive gland of a mussel at Gijang in March 2010 (GiJ1003Mu; genotype IVa) and the other from the digestive gland of a Pacific oyster at Tongyeong in January 2010 (TY1001OY; genotype IVa) (Kim et al. 2016). Digestive gland tissue (5 mg) from VHSV-positive field samples were homogenized, centrifuged (8000 × *g*, 10 min), and filtered (0.45 µm pore size) for

the *in vitro* challenge experiment. The filtrate was used as the inoculum. VHSV-free digestive gland tissue spiked with VHSV and cultured VHSV were used as controls. Samples were inoculated in duplicate with 100 µl of a 10-fold dilution series on 6-well tissue culture plates with 70% confluent EPC cells. The plates were incubated at 20°C and observed for 7 d, and then 500 µl of the supernatant of each cell culture was inoculated again on fresh cell layers and incubated for a further 10 d as a blind passage.

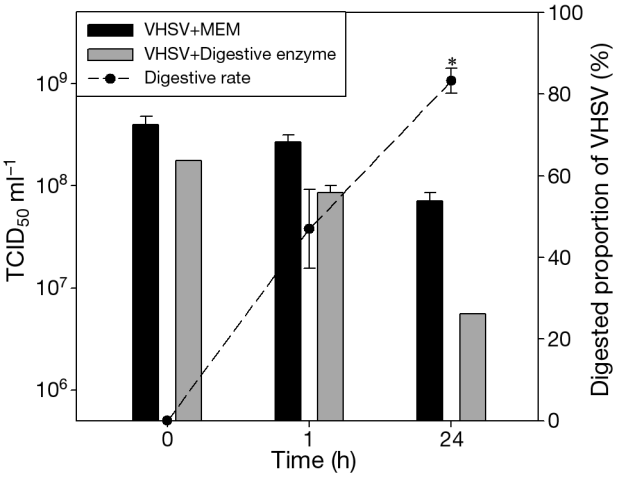

Fig. 1. Digestive rate of viral hemorrhagic septicemia virus (VHSV) by blue mussel *Mytilus edulis* digestive enzymes. Natural declining viral titer ($TCID_{50}$ ml^{-1} in epithelioma papulosum cyprini [EPC] cell line) due to viral stability shown by black bars. Viral titer in digestive enzyme-exposed group shown by grey bars. VHSV digestive rate by blue mussel digestive enzymes shown by circles. Data are means ± SD. *Significant difference (1-way ANOVA, $p < 0.05$). MEM, minimum essential medium

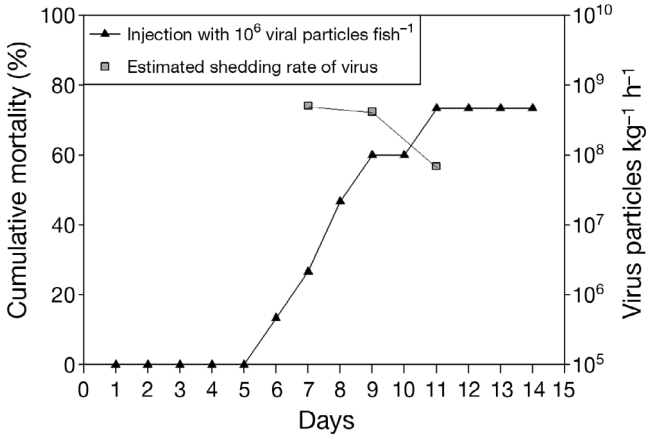

Fig. 2. Relationship between amount of viral hemorrhagic septicemia virus (VHSV) shed into rearing water and cumulative mortality (%) of VHSV-infected flounder *Paralichthys olivaceus*

For the *in vivo* challenge experiment, 2 groups of 5 flounders each (12.0 ± 1.0 g) were injected intraperitoneally with inoculum (0.1 ml) from 1 of the 2 field samples, respectively. Two groups of 5 flounders each were also injected with cultured VHSV (0.1 ml of 10^3 viral particles ml^{-1}) or PBS (0.1 ml) as positive and negative controls, respectively. Injected flounders were observed for clinical signs and mortality for 14 d.

RESULTS

Survivability of VHSV particles in mussel digestive enzymes

The initial mean viral titers of VHSV mixed with MEM and digestive gland homogenate were $10^{8.58}$ $TCID_{50}$ ml^{-1} and $10^{8.25}$ $TCID_{50}$ ml^{-1}, respectively (Fig. 1). Thus, the inhibition rate of digestive gland homogenate on viral infection was on average 52.0% by the inhibition rate formula. The natural decline of VHSV titers in MEM was $10^{8.4}$ $TCID_{50}$ ml^{-1} and $10^{7.83}$ $TCID_{50}$ ml^{-1} after 1 and 24 h at 20°C, respectively. The viral titers of VHSV mixed with the digestive enzymes were significantly reduced compared to MEM at $10^{7.91}$ $TCID_{50}$ ml^{-1} (after 1 h incubation) and $10^{6.83}$ $TCID_{50}$ ml^{-1} (after 24 h incubation) ($p < 0.05$). Therefore, VHSV was digested by 46.99 and 79.45% after 1 and 24 h, respectively. Thus, degradation of VHSV in the digestive enzymes was much faster at an early stage of exposure compared to later stages.

Estimated shedding rate of virus from VHSV-infected flounder

Infection with VHSV (10^6 viral particles $fish^{-1}$) showed 73.3% (4 of 15 fish survived) cumulative mortality within 11 d in flounder (Fig. 2). Dead flounder generally displayed symptoms of VHSV infection (congested liver and abdominal distension with ascites). The estimated shedding rate of virus peaked at 5.1×10^8 viral particles kg^{-1} h^{-1} at the time of 26.7% cumulative mortality at 7 d post-injection. It decreased to 6.9×10^7 viral particles kg^{-1} h^{-1} at the time of 73.3% cumulative mortality at 11 d post-injection.

Virus accumulation in mussels cohabitated with VHSV-infected flounder

In Expt 1, virus donor flounder in 40 l (n = 15) tanks with mussels (n = 20) were injected intraperi-

toneally with VHSV (10^6 viral particles $fish^{-1}$) and showed 73.3% (4 of 15 fish survived) cumulative mortality within 11 d. Onset of clinical signs such as darkening and abdominal distension appeared at 3 d post-inoculation (dpi) and fish began to die at 5 dpi. Dead flounder carried an average of 4.58×10^6 viral particles mg^{-1} kidney (Table 1). The number of viral particles in rearing water were 5.10×10^2 ml^{-1} at Day 5, 1.02×10^3 ml^{-1} at Day 7, and 6.29×10^1 ml^{-1} at Day 9 post-cohabitation. VHSV were not identified from the digestive gland tissue of any of the cohabitated mussel until VHSV-infected flounder exhibited 73.3% cumulative mortality on Day 11 after inoculation (corresponding to Day 9 post-cohabitation).

In Expt 2, naturally VHSV-infected flounder showed 87.5% (3 of 24 fish survived) cumulative mortality within 3 d of cohabitation, and carried an average of 1.90×10^6 viral particles mg^{-1} kidney (Table 1). The number of viral particles in the rearing water was 1.07×10^5 ml^{-1} and 4.16×10^4 ml^{-1} at 1 and 2 d post-cohabitation, respectively. At 3 d post-cohabitation, viral particles were not detected in rearing water by qPCR. During cohabitation, mussels captured viral particles in both the digestive gland (average 4.53×10^2 mg^{-1} at Day 1 and 7.32×10^1 mg^{-1} at Day 3) and gill (average 3.08×10^2 mg^{-1} at Day 1 and 7.32×10^1 mg^{-1} at Day 3). There was no significant difference in the number of viral particles between tissues. Although VHSV in the digestive gland and gill of mussels were identified by qPCR, virus was barely cultivated and isolated from the gill using blind passage with EPC cells (Table 1, Fig. 3).

Depuration of VHSV from mussels after artificial contamination

During artificial bio-accumulation of VHSV, viral concentration in the digestive gland tissue of VHSV-Mu increased after 6 h (1.61×10^3 viral particles mg^{-1}) (Fig. 4). Viral concentrations in digestive gland tissue of VHSV-Mu then decreased within 12 h during depuration processing (9.7×10^1 viral particles mg^{-1}). However, VHSV was not totally eliminated from the digestive gland tissue even at 168 h (2.42×10^1 viral particles mg^{-1}). No significant difference was found in the concentration of virus in digestive gland between artificial accumulation and depuration processing ($p < 0.05$).

Infectivity of VHSV from field bivalve mollusks

Two VHSV-positive mollusk samples from the field, GiJ1003Mu and TY1001OY, were inoculated onto the EPC cell lines with a 10-fold serial dilution: for TY1001OY it was 3.4×10^1 to 3.4 viral particles $well^{-1}$, and for GiJ1003Mu it was 5.80×10^2 to 5.8 viral particles $well^{-1}$ (Table 2). No CPEs were observed in the cells inoculated with the VHSV-positive field samples after 7 d. In contrast, CPEs of up to 10^2 viral particles and 5×10^1 were observed in the VHSV-free digestive gland tissue spiked with VHSV and cultured VHSV, respectively. No VHSV symptoms were observed in any of the surviving flounder in the field samples and the PBS-injected groups during the 14 d of the *in vivo* experiment. Infection with cultured VHSV (as positive control)

Table 1. Detection of viral hemorrhagic septicemia virus (VHSV) particles from the mussel *Mytilus edulis* cohabitated with artificially (Expt 1) and naturally (Expt 2) VHSV-infected flounder *Paralichthys olivaceus*. In Expt 1, flounder weight = 12.0 ± 1.0 g, mussel weight = 11.0 ± 1.2 g; in Expt 2, flounder weight = 550.0 ± 27.5 g, mussel weight = 15.0 ± 1.2 g. Cum. mort.: cumulative mortality; nt: not tested; nd: not detected; +ve: positive; –ve: negative

Days post cohabitation	Olive flounder		Blue mussel				Rearing water
	Kidney		Digestive gland		Gill		
	Cum. mort. (%)	Viral particles mg^{-1}	Viral particles mg^{-1}	Culture	Viral particles mg^{-1}	Culture	Viral particles ml^{-1}
Expt 1							
1	0	nt	nd	nt	nt	nt	nd
3	0	nt	nd	nt	nt	nt	nd
5	26.7	$1.49 \pm 0.57 \times 10^6$	nd	nt	nt	nt	5.10×10^2
7	60.0	$8.12 \pm 1.95 \times 10^6$	nd	nt	nt	nt	1.02×10^3
9	73.3	$4.00 \pm 2.56 \times 10^6$	nd	nt	nt	nt	6.29×10^1
Expt 2							
1	33.3	$2.16 \pm 1.95 \times 10^6$	$4.53 \pm 3.7 \times 10^2$	–ve	$3.08 \pm 1.8 \times 10^2$	+ve	1.07×10^5
2	87.5	$2.26 \pm 2.18 \times 10^6$	nt	nt	nt	nt	4.16×10^4
3	87.5	$2.24 \pm 1.37 \times 10^4$	$7.32 \pm 6.5 \times 10^1$	–ve	$7.45 \pm 6.6 \times 10^1$	+ve	nd

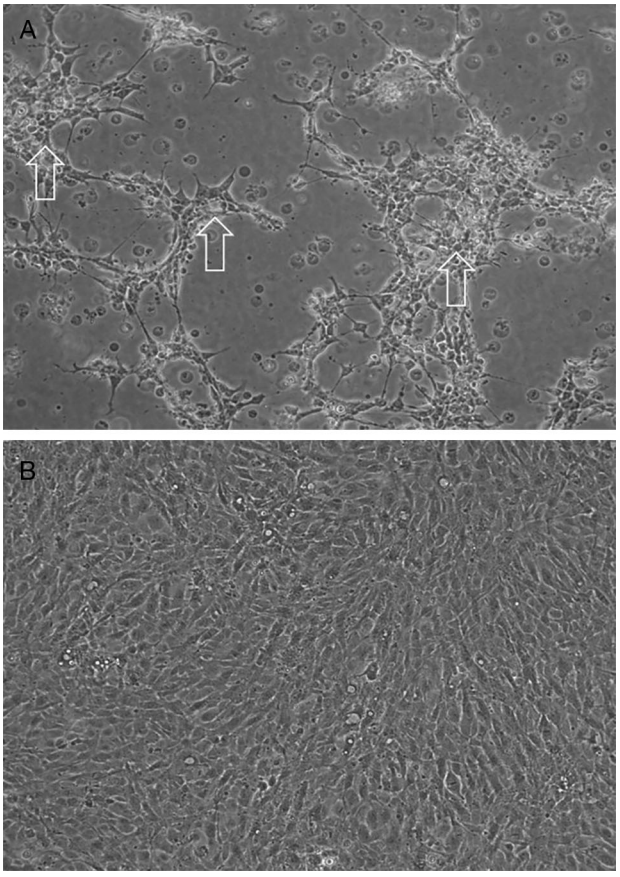

Fig. 3. Cytopathic effects (CPE) of viral hemorrhagic septicemia virus (VHSV) in epithelioma papulosum cyprini (EPC) cells. (A) Lytic CPE of VHSV in EPC at 7 d post-inoculation with gill homogenate of mussel *Mytilus edulis* cohabitated with naturally VHSV-infected flounder *Paralichthys olivaceus* in blind passage (arrows). (B) Negative control inoculated with phosphate-buffered saline

showed 40% (3 of 5 fish survived) cumulative mortality within 14 d post-challenge in flounder. In contrast, VHSV could not be identified by qPCR and the cell culture from fish inoculated with the VHSV-positive field samples.

Table 2. Infectivity of viral hemorrhagic septicemia virus (VHSV) from digestive gland of bivalves in the *in vitro* experiment. nd: not done; ++/+/–: strong/weak/zero cytopathic effect, respectively

VHSV source	Dosage (viral particles well^{-1})			
	5×10^2	10^2	5×10^1	5×10^0
Oyster *Crassostrea gigas* sample TY1001OY[a]	nd	nd	–	–
Mussel *Mytilus edulis* sample GiJ1003Mu[b]	–	nd	–	–
Mussel digestive gland spiked with cultured VHSV	nd	+	–	–
Cultured VHSV	nd	++	++	+

[a]Viral concentration in digestive gland well^{-1}: 3.43×10^0 to 3.43×10^1 particles
[b]Viral concentration in digestive gland well^{-1}: 5.80×10^0 to 5.80×10^2 particles

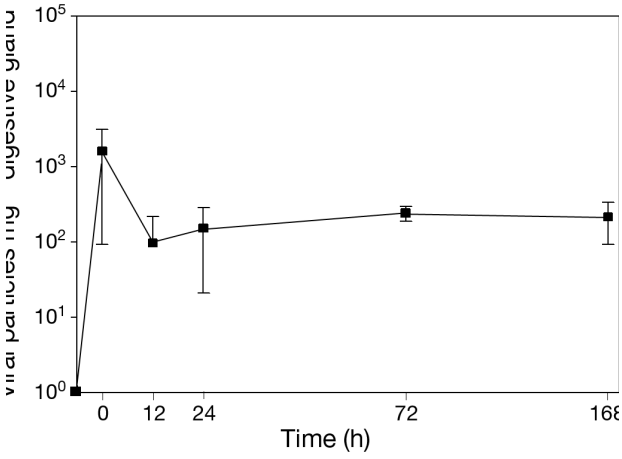

Fig. 4. Quantitative pattern of viral hemorrhagic septicemia virus (VHSV) in *Mytilus edulis* digestive gland tissue during mussel depuration process. Data are means ± SD

DISCUSSION

The interaction of aquatic animal viruses with bivalve mollusks is important in evaluating whether the mollusks serve as vectors or reservoirs for the viruses. Previous studies have shown the presence and persistence of viruses including infectious salmon anemia virus (ISAV), megalocytivirus, and WSSV investigated in bivalve mollusks (Skar & Mortensen 2007, Vazquez-Boucard et al. 2010, Molloy et al. 2014, Kim et al. 2016). Furthermore, a recent study showed that redspotted grouper nervous necrosis virus (RGNNV)-contaminated Manila clam *Ruditapes philippinarum* could experimentally release viable viral particles into the surrounding environment (Volpe et al. 2017).

The Pacific oyster and blue mussel are widely distributed in the vicinity of aquaculture farms in Korea. In a previous study, we first identified VHSV from digestive gland tissue of oysters and mussels by PCR assay (Kim et al. 2016). A number of studies have shown that a variety of virus particles can be identified in the digestive gland, gill, and mantle tissue of bivalve mollusks (Mortensen et al. 1992, Atmar et al. 1995, Suzuki & Nojima 1999, Vazquez-Boucard et al. 2010, Jin et al. 2014, Kim et al. 2016). In particular, norovirus particles bind specifically to the oyster digestive tract via a carbohydrate structure (Le Guyader et al. 2006). Although there are no data on aquatic animal viruses that specifically bind to the oyster digestive tract, other viruses could bind to the digestive

tract of bivalve mollusks. Phylogenetic analysis revealed that the VHSV from bivalve mollusks belonged to the VHSV genotype IVa (Kim et al. 2016), which is commonly detected in VHSV-infected flounder in Korea. This likely indicates that VHSV released into seawater from infected flounder was captured by the bivalve mollusks.

Our general PCR results could not distinguish between infectious and non-infectious states of the virus. Previous studies have shown that VHSV particles are infectious at 4°C in freshwater (Hawley & Garver 2008) and that organic materials such as ovarian fluids or blood products enhance the stability of the virus (Kocan et al. 2001). Viral stability (natural reduction of titer in MEM media) and activity of bivalve mollusk digestive enzymes have been considered as VHSV survivability tests in the mussel digestive gland. Although we found that VHSV particles had a digested rate of 79.45% by 24 h at 20°C, some of the viral particles maintained an infectious state under *in vitro* conditions (Fig. 1). Therefore, viral particles in the digestive tract of mussels are not completely inactivated by digestive enzymes within 24 h.

Cohabitation and depuration experiments were conducted to assess experimental viral accumulation and release from the bivalves. In cohabitation Expt 1, mussels cohabitating with VHSV-infected flounder did not have detectable VHSV particles. A previous study showed that VHSV shedding occurred prior to the death of infected flounder and is secreted from moribund or dead fish into rearing water with an average titer of $10^{3.5}$ $TCID_{50}$ ml^{-1} (Muroga et al. 2004). In the present study, the highest shedding rate of virus was observed at 7 d post-injection as 5.1×10^8 viral particles kg^{-1} h^{-1} when VHSV-infected flounder showed 26.7% cumulative mortality (Fig. 2). The results of Expt 1 suggest that a high dose of virus in the water or a longer exposure time was needed for VHSV particles to accumulate in the mussels. Alternatively, mussels may have digested the VHSV particles below the detection limit of molecular-based methods (qPCR). In cohabitation Expt 2, naturally VHSV-infected flounder (550.0 ± 27.5 g body weight), which were almost 50 times larger than those (11 ± 1.2 g body weight) of Expt 1, might enhance the viral dose in rearing water (above 10^4 viral particles ml^{-1}) to mussels as shown in Table 1. Although sufficient viral particles in rearing water led to viral accumulation in the mussel (average 2.16×10^2 viral particles mg^{-1} of digestive gland and 1.67×10^2 viral particles mg^{-1} in gill), VHSV from digestive gland tissue of mussels cohabitated with naturally infected flounder could not be replicated in EPC cells. In contrast, VHSV particles from gill were recovered, suggesting that the gill as the entrance to these filter-feeding organisms contained more replicable virus particles than the digestive gland. From cohabitation experiments, it is thought that VHSV particles carried by mussel might be converted to a non-infectious stage. In the depuration experiment, VHSV-Mu had 1.61×10^3 viral particles mg^{-1} digestive gland even though viral exposure time was only 6 h with a high dose of virus (10^6 viral particles ml^{-1} seawater). VHSV abundance was maintained at more than 2.42×10^2 viral particles mg^{-1} in the digestive gland during the depuration process. However, no significant difference was observed in the number of VHSV particles between the accumulation and depuration processes due to variations in individual mussel samples. Skar & Mortensen (2007) reported that ISAV from ISAV-challenged mussels is not detectable 4 d after depuration, due to digestion or inactivation of the virus. In contrast, norovirus in oyster digestive gland tissue was not totally eliminated and was detectable even at 10 d of depuration processing post-artificial contamination by norovirus-contaminated feces for 72 h (Ueki et al. 2007). These results indicate that VHSV may have been maintained in blue mussel for a longer time (>7 d), similar to norovirus.

Viral isolation from bivalve mollusk tissue based on a cell culture system was difficult due to the cellular cytotoxicity of the digestive gland tissue. Therefore, we conducted viral isolation using an interval-diluted digestive gland homogenate from VHSV-positive field samples and VHSV-inoculated samples. Although artificially VHSV-inoculated digestive gland homogenate developed CPEs to 10^2 viral particles, VHSV in field bivalves did not. A previous study showed that ISAV particles in mussel digestive gland tissue do not replicate in CHSE-214 cells (Molloy et al. 2014). In infectivity tests, VHSV in field bivalves may have insufficient cell infective concentrations. Alternatively, those samples may have had inactivated VHSV particles that could only be detected by molecular assays. In addition, VHSV particles in field bivalves did not replicate after intraperitoneal injection into olive flounder. Interestingly, the minimum infectious dose of VHSV in flounder was 10^2 viral particles $fish^{-1}$ by intraperitoneal injection and 10^4 viral particles ml^{-1} by the immersion method, respectively (authors' unpubl. data), suggesting that the quantity of VHSV in field bivalves was insufficient to infect or that those samples might be in a non-infectious state.

In summary, VHSV particles were not totally digested within 24 h *in vitro* and were maintained for more than 7 d in the blue mussel. Although mussels cohabiting with naturally VHSV-infected flounder carried the viral particles, viral particles in digestive gland did not maintain infectivity. Moreover, field mollusks (Pacific oyster and mussel) which showed VHSV-positive results by PCR did not possess infectivity *in vitro* or *in vivo* experiments either, suggesting that the viral particles in field bivalves might be non-infectious. Hence, it might be difficult to conclude that mussels play a role as a VHSV transmitter, and also the possibilities of virus transmission to fish under field conditions are scarce.

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