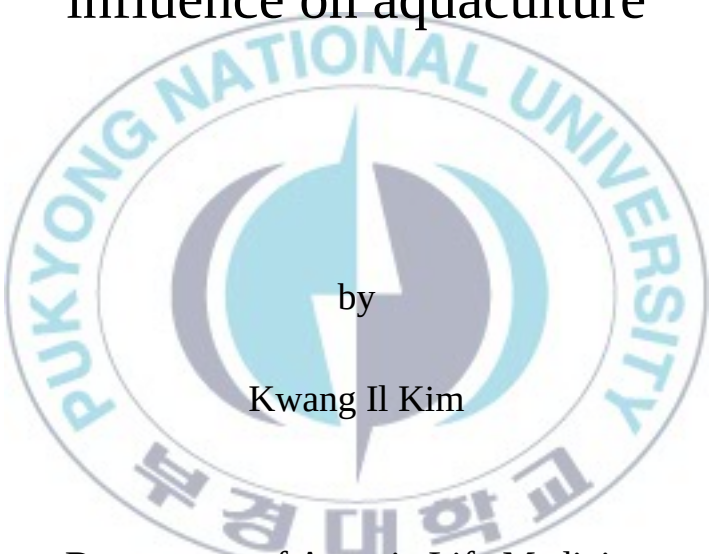
Thesis for Degree of Doctor of Philosophy

Detection of fish disease viruses in shellfish and sea water with assessment of their influence on aquaculture



by

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Pukyong National University

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한국의 패류 및 해수 내 어류 질병 바이러스의 검출과 양식에 미치는 영향

Advisor: Prof. Hyun Do Jeong

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Detection of fish disease viruses in shellfish and sea water with assessment of their influence on aquaculture

A dissertation

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한국의 패류 및 해수 내 어류 질병 바이러스의 검출과 양식에 미치는 영향

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요 약

수생동물 바이러스성 질병의 확산에 가장 큰 영향을 미치는 요인은 감수성 숙주 생물 (susceptible host)를 비롯하여 환경 수와 바이러스 매개체 (vector) 또는 함유생물 (reservoir)이라는 큰 요소가 있다. 바이러스의 축적 및 전파와 관련하여 패류는 여과섭식 (filter-feeding)을 통한 먹이섭취 시 병원체를 비롯한 다양한 물질을 체내로 축적할 수 있으며, 소화과정 동안 불활성화 되지 않거나 중장선 (digestive gland)에 특이적으로 부착하여 존재하는 바이러스 입자를 일정조건에서 배출할 수도 있기 때문에 매개체 또는 함유생물로서의 역할을 할 수 있는 가능성이 있다. 특히, 우리나라의 경우 어류 및 패류 양식장이 밀집된 공간 내 인접하게 존재하고 있으므로, 패류와 해수에 존재하는 수생동물 바이러스성 질병의 검출방법의 개발과 감염성에 대한 평가는 수생동물질병 전파 및 위험성을 최소화하기 위해 필수적이다.

패류와 해수 내의 바이러스는 매우 극 미량으로 존재하기 때문에 이를 검출하기 위해서는 대상 시료로부터 바이러스 농축과정이 필요하다. 패류의 경우 노로바이러스 (norovirus)등 사람 장내 바이러스성 질병 원인체 농축법인 PEG 처리법이 일반적으로 사용된다. 하지만 PEG처리법은 5g이상의 중장선을 사용하기 때문에 처리과정이 복잡하며 소요시간이 긴 단점이 있다. 따라서 바이러스의 농축과정의 개선을 위해서 국내에서 유행하는 바이러스 중 megalocytivirus (DNA 바이러스)와

VHSV (RNA 바이러스)를 표준바이러스로 선정하여 검출에 사용되는 중장선 조직의 양적 비교를 실시하였으며, 그 결과 50mg (PEG 비 처리)의 소량 조직을 사용할 경우에도 정성·정량적 분석이 가능함을 확인하였다. 또한, 소량의 중장선을 이용하는 방법은 기존의 방법 (PEG 처리)에 비해 검출소요시간의 단축 및 개체 별 분석의 장점이 있었다.

해수로부터 바이러스를 농축하기 위해서 음이온 여과 막 농축법을 사용하였으며, glass microfiber (GF/C) 및 니틀로셀룰로즈 (nitrocellulose)를 이용한 이중 필터 방법으로 해수 중 바이러스를 효율적으로 농축할 수 있었다. 그리고 VHSV 감염 넙치 양식장의 유입수 및 사육수에 대한 적용시험을 통해 해수 중 바이러스 농축법으로서의 유효성을 확인하였다.

패류와 해수에는 다양한 수생동물 바이러스성 질병 원인체가 존재하고 있으며, 이를 동시에 효율적으로 검출하기 위한 방법으로 multiplex-nested PCR법을 개발하였다. 국내에서 유행하고 있는 DNA 바이러스들 (megalocytivirus, WSSV)과 RNA 바이러스들 (MABV, VHSV, VNNV)을 표적 바이러스들로 선정 하였다. 본 연구에서 개발된 multiplex-nested PCR법은 대상 바이러스를 특이적으로 검출할 수 있었으며, 검출한계는 중장선 1 mg당 1-10 viral particles으로 나타났다. Multiplex-nested PCR을 이용하여 국내에 서식하고 있는 패류와 주변 해수에 분포하고 있는 바이러스에 대해 모니터링을 실시한 결과, 다양한 바이러스를 검출할 수 있었으며 VNNV를 제외한 다른 바이러스들의 시료 채취 지역별 및 시기적인 양성비율의 유효성 (P<0.05) 있는 차이는 나타나지 않았다. 또한, 패류와 해수에서 검출된 바이러스 중 megalocytivirus와 VHSV는 국내 양식 어류에서 유행하는 유전형과 동일하였으며, VNNV의 경우 국내에서 알려진 RGNNV 타입 이외에 외래

유전형(exotic subtype)인 BFNNV 타입이 검출 됨으로써 외래 바이러스의 국내 유입 및 정착되었음을 보여 주었다.

양식넙치에서 유행하고 있는 VHSV IVa 유전형에 대한 패류의 바이러스 매개체(vector) 또는 함유생물 (reservoir)로서의 평가를 실시하였다. 담치의 소화효소로 인해 VHSV는 24시간 이후에도 완전히 불활성화 되지 않았다. 그리고 인위적으로 VHSV에 오염된 담치의 바이러스 정화능 (depuration)시험 결과, 중장선에 축적된 바이러스는 168시간 이후에도 10 viral particles이상으로 존재하고 있었으며 이는 바이러스가 특이적으로 중장선에 부착하여 장시간 존재할 수 있음 나타낸다. 그러나 담치와 VHSV 감염 넙치간의 cohabitation 모델 실험 결과, VHSV 감염 넙치가 사육수로 배출하는 바이러스의 양은 담치 중장선으로부터 바이러스를 검출할 수 있는 한계 이하였다. 또한, 현장 패류 시료로부터 검출된 VHSV는 CHSE-214 세포주 (*in vitro*) 및 넙치 (*in vivo*)에서 감염을 유도하지 않았다. 이러한 결과는 패류에 존재하는 VHSV는 자연상태에서 넙치에 감염을 유발하기 힘든 양적 수준이며, 또한 중장선에 비 감염 상태로 존재하고 있음을 말해준다.

본 연구에서는 수생동물 바이러스성 질병의 전파와 관련하여 패류와 해수에 존재하는 바이러스의 검출법 개선 및 감염성 평가를 통해 수산양식에 미치는 영향을 분석하고자 하였다. 개선된 바이러스 검출법을 사용하여 국내 해안에 서식하고 있는 다양한 패류와 주변 해수로부터 어류질병바이러스를 검출할 수 있었으며, 어류로부터 유래된 바이러스와의 유전적 동일성을 보여주었다. 넙치에서 유행하는 VHSD을 대상으로 패류의 전파 가능성을 평가한 결과, 현장 패류시료에서 검출된 바이러스는 CHSE-214 세포주와 넙치에서 병원성을 유발하지 않는 비 감염성 상태로 나타났다. VHSV는 패류 내에서 비 감염 상태로 존재하고 있으나 지속적으로 다양한 종류의

어류질병 바이러스들이 패류와 해수에서 검출됨을 고려할 때, 패류의 바이러스성 질병 전파에 대한 잠재적 위험은 배제할 수 없을 것이며 질병 발생 전후로의 패류 및 해수에 대한 모니터링은 필요하다.

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GENERAL INTRODUCTION

Aquaculture has been influenced by aquatic animal diseases along with development of aqua-industries. Of aquatic animal diseases, viral diseases caused by megalocytiviruses including rock bream iridovirus (RBIV), flounder iridovirus (FLIV), and viral hemorrhagic septicemia virus (VHSV), and white spot syndrome virus (WSSV) are present on the aqua farms and lead to serious economic losses in Korea every year. Moreover, those of viral agents are listed as causing remarkable disease by the World Organization for Animal Health (OIE).

After an outbreak of an aquatic animal disease, viral agents released into environmental water from infected hosts could be transmitted to other susceptible hosts or be retained in vector/reservoir species. Additionally, releasing viruses from infected hosts may also accumulate into filter-feeding organisms such as shellfish growing in coastal areas. For viral identification from shellfish and environmental water, viral concentration steps are essential owing to low viral titer or inhibitor substances. To overcome these problems, several methods have been reported such as ethanol precipitation (Kitamura and Suzuki, 2000), filtration method (Katayama et al., 2002), poly polyethylene glycol (PEG) precipitation (Jaykus et al., 1996) and ultracentrifugation (Mehnert et al., 1997). However, most researches have primarily focused on human enteric virus. Although few studies have examined presence of aquatic animal viruses in the

shellfish or environmental water, such as infectious pancreatic necrosis (IPN) (Mortensen et al., 1992), koi herpesvirus (Haramoto et al., 2009), marine birnavirus (MABV) (Kitamura and Suzuki, 2000; Suzuki and Nojima, 1999) and WSSV (Song et al., 2008; Vazquez-Boucard et al., 2010), attempt to investigate viral concentration method for detection of aquatic animal virus in the routine laboratory procedures are lacking. Additionally, various agents are generally present in the environmental water and shellfish. Therefore, to analysis viruses from shellfish and environmental water, effective and sensitive detection method is required.

The spread of aquatic animal pathogenic diseases depends on several factors, such as susceptible hosts, environmental water, and vector or reservoir species. Although surveillance for aquatic animal diseases in susceptible hosts has been implemented for the prevention of outbreak, viral transmission via environmental water or vector/reservoir species remains problematic. Therefore, when considering the spread of aquatic animal viruses, surveillance of viruses from environmental water and shellfish is important for adequately assessing prevention measure.

In addition, a variety of shellfish have been widely distributed in large numbers in the vicinity of aquaculture farms in Korea. These organisms may serve as vector or reservoir for viruses. Thus, an understanding of how fish pathogenic

viruses are maintained in shellfish and whether they are involved in viral transmission is crucial for aquaculture.

The objectives of the present work were to improve viral concentration and detection method for viral identification from shellfish and seawater, and to investigate of several viruses in shellfish and sea water in Korea. In addition, we evaluated the potential of bivalve mollusk as transmitter of VHSV.

Chapter I. Viral concentration method for detection of aquatic animal viruses in shellfish and seawater

I. Introduction

Aquatic animal viral diseases caused by megalocytiviruses, including rock bream iridovirus (RBIV), viral hemorrhagic septicemia virus (VHSV), and white spot syndrome virus (WSSV), are present on aqua-farms and lead to serious economic losses in Korea every year. Although surveillance for aquatic animal diseases has been implemented for the prevention of outbreaks, viral transmission via environmental water or vectors/carriers remains problematic.

Viral agents released from the host into environmental water could be transmitted to other susceptible hosts or retained in vector or reservoir species. Of shellfish mollusks, filter-feeding organisms could potentially accumulate various pathogenic agents from environmental water. Of note, Commission Regulations of the EU (EC, No. 1251/2008) designated the Portuguese oyster (*Crassostrea angulata*), common edible cockle (*Cerastoderma edule*), Pacific oyster (*Crassostrea gigas*), eastern oyster (*Crassostrea virginica*), Donax (*Donax trunculus*), abalone (*Haliotis discus hannai*), rotifers, marine mollusks, and brine shrimp (*Artemia salina*) as vector species of taura syndrome virus and WSSV.

Therefore, when considering the spread of aquatic animal viruses, the detection of viruses from environmental water sources and vector or reservoir species is important for adequately assessing prevention measures.

For viral identification from shellfish or environmental water, viral concentration steps are essential owing to low viral titer or inhibitor substances. To solve these problems, various methods have been reported such as ethanol precipitation (Kitamura and Suzuki, 2000), filtration methods (Katayama et al., 2002), poly polyethylene glycol (PEG) precipitation (Jaykus et al., 1996), and ultracentrifugation (Mehnert et al., 1997). However, most researches have primarily focused on human enteric viruses, including enterovirus (Katayama et al., 2002), norovirus (Atmar et al., 1993; Atmar et al., 1995; Le Guyader et al., 2009), hepatitis A virus (HAV) (Croci et al., 1999; Kim et al., 2008), and poliovirus (Jaykus et al., 1996). Although few studies have examined the presence of aquatic animal viruses in shellfish or environmental water, such as infectious pancreatic necrosis (Mortensen et al., 1992), koi herpesvirus (Haramoto et al., 2009), marine birnavirus (Kitamura and Suzuki, 2000; Suzuki and Nojima, 1999), and WSSV (Song et al., 2008; Vazquez-Boucard et al., 2010), attempts to investigate viral concentration methods for the detection of aquatic animal viruses in routine laboratory procedures are lacking.

Therefore, the objective of the present work was to identify viral concentration methods for shellfish and seawater for the detection of aquatic animal viruses. To

compare concentration steps, we selected megalocytivirus as the DNA virus, and VHSV as the RNA virus (i.e., endemic viruses in Korea). We investigated the compatibility of the PEG treatment method for processing shellfish and the filtration method for processing seawater.

II. Materials and methods

1. Sample

Pacific oysters (*Crassostrea gigas*) were collected from the southeastern seashore (Gwangan, Gijang, Namhae, and Tongyeong) in Korea between November 2007 and March 2010. Influent and cultured seawater from VHSD-positive flounder farms in Gampo and Jeju were collected using a sterilized 1-L bottle in February 2011. All Pacific oyster and seawater samples were directly transported to the laboratory after sampling.

2. Virus

The megalocytivirus IVS-1 strain and VHSV (as the control) were cultured in grunt fin (GF) and Chinook salmon embryo (CHSE-214) cells, respectively. Cell lines were propagated in Eagle's minimum essential medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and a 1% antibiotic/antimycotic solution (Gibco, Grand Island, NY, USA). The spleen of rock bream (*Oplegnathus fasciatus*) infected with the megalocytivirus IVS-1 strain was used as the inoculum for this virus (Jeong et al., 2003). Additionally, the kidney of VHSV IVa subtype-infected flounder

(*Paralichthys olivaceus*) was used as the inoculum for the VHSV virus. All inocula were filtered (0.45 μm pore size) and inoculated into each susceptible cell line. Following the development of the cytopathic effect, each of the viruses were centrifuged at 3000 × *g* for 10 min, and the supernatant was stored at −80°C prior to the experiment.

3. Sample processing for virus concentration

3-1. Oyster processing using PEG treatment

Oyster samples were aseptically cut open with a knife. The intestinal organs were collected after the separation of the digestive gland by using a scalpel. The digestive glands of individual oysters were pooled as a single specimen. For determining the viral concentration in digestive gland, the method for the detection of norovirus in bivalve mollusks, recommended by the Korea Food and Drug Administration, was modified. Five grams of the digestive gland mixture was homogenized with 0.25 M glycine–0.14 M NaCl buffer (pH 7.5), and the resulting suspension was centrifuged at 10,000 × *g* for 15 min at 4℃. The supernatant was then precipitated with a polyethylene glycol (PEG, Sigma, St. Louis, MO, USA) 8000 solution (final concentration 10% [wt/vol] PEG 8000 with 0.3 M NaCl) for 16 h at 4℃. The resultant polyethylene glycol pellet was

suspended with 0.2% Tween 80–50 mM Tris-HCl and 1× PBS, and then treated with chloroform (Sigma, St. Louis, MO, USA) following precipitation by the PEG 8000 solution (final concentration 20% [wt/vol] PEG 8000 with 0.3 M NaCl) for 4 h at 4℃. The final precipitate was store at −20°C before nucleic acid purification.

3-2. Seawater processing using filtration method

For measuring the viral concentration from seawater, a modified version of the method described by Katayama et al. (2002) was used. One liter of seawater was filtered with a GF/C membrane (1.2 μm pore size; Whatman, Maidenstone, UK) and a nitrocellulose membrane (HA type negatively-charged membrane, 0.45 μm pore size; Millipore, Japan) to eliminate the sediments and absorb the viruses. The filtered membrane was rinsed out the cation using 100 ml of 0.5 mM H_2SO_4. Subsequently, 10 ml of 1 mM NaOH (pH 10) was passed through the membrane in a fresh 50-ml tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100× TE buffer for neutralization. The filtrate was concentrated 3000 × *g* for 10 min at 4℃ by using Amicon Ultra-15 Centrifugal Filter Units (30 kDa; Millipore, Japan), and the concentrate was adjusted to a final volume of 1 ml. The final concentrate was stored at −20℃ before nucleic acid purification.

4. Nucleic acid purification

Nucleic acids were purified from 200 µl of the PEG precipitate, 50 mg of the digestive gland from oyster specimens, and 200 µl of seawater concentrate by using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Dajeon, Korea) for DNA extraction and the RNeasy Plus mini kit (Qiagen, Valencia, CA, USA) for RNA extraction according to the manufacturers' protocols. Total nucleic acids were eluted to 50 µl by TE buffer (pH 8.0).

5. Nested PCR

Specific primer sets were designed from the viral genome based on nucleotide sequences from the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (Table 1). The primer sets for the megalocytivirus was designed from the major capsid protein (MCP) gene. For the VHSV, the primer sets were designed from the glycoprotein (G) gene. cDNA was prepared from RNA (1 µl)-mixed random primers and MMLV reverse transcriptase (Promega, Madison, WI, USA). Nested PCR was performed in a 20-µl reaction mixture with 1 µl of DNA or cDNA, 10 pM of each primer (forward and reverse), 2 µl of 10× PCR buffer, and 200 µM of dNTPs and Taq DNA polymerase (Cosmo Genetech, Seoul, Korea). The cycling conditions for the first-

and nested-step PCR runs were: pre-denaturation at 95℃ for 3 min, 35 cycles of denaturation at 94℃ for 40 s, annealing at 55℃ for 30 s, extension at 72℃ for 30 s, and a final extension step at 72℃ for 7 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

6. Quantitative PCR (Real-time PCR) assay

Viruses were quantified using the LightCycler 480II instrument (Roche, Indianapolis, IN, USA e) according to the manufacturer's instructions. The qPCR reaction mixture contained 1 μl of DNA or cDNA, primers (forward and reverse; Table 1) at a concentration of 500 nM each, and the Light Cycler 480 SYBR Green Master mixture (Roche, Indianapolis, IN, USA). The amplification conditions were as follows: 95℃ for 10 min, followed by 40 cycles of 94℃ for 10 s, 60℃ for 15 s, and 72℃ for 20 s. As a positive control, recombinant plasmids containing 163 bp from the MCP gene (amplified using MC1F/MC1R for megalocytivirus) and 157 bp from the glycoprotein gene (amplified using VqF/VqR for VHSV) were purified from the transformed *Escherichia coli* DH5a strain. A serial 10-fold dilution of the control plasmids was used to establish a standard curve (5.0E + 05 copies/μl to 5.0E + 00 copies/μl). The standard curves were generated using the mean data from experiments performed in triplicate, thus indicating a good linear relationship between the CT values. All samples

used in this study were tested in duplicate, and all PCR reactions were carried out twice.

Table 1. Primers used in this study

Virus	Primers	Sequence (5' to 3')	Amplicon (bp)	Object	Reference
Megalocytivirus	M1F	GCTGCGCATGCCAATCATCT	401	1-step PCR	This study
	M1R	ATGCGATGGAGACCCACTTG			
	M2F	AATGACACCGACACCTCCTC	288	2-step PCR	
	M2R	TGCGATGGAGACCCACTTGT			
	MC1F	GAGGTGCGCATCCACTTC	163	qPCR	Jun *et al*., 2008
	MC1R	CAAGATGATTGGCATGCG			
VHSV	SF1	CACAGATCACTCAACGACC	559	1-step PCR	This study
	SR1	GTGATCATGTGTCCTGGTG			
	SF2	GACTGGGACACTCCACTGTA	467	2-step PCR	
	SR2	CAAACCCCCTCTATGAAGTC			
	VqF	TTTCTTGGTGATTCTGATCATCA	157	qPCR	This study
	VqR	CCGAATCGGAACAAAGGAG			

III. Results

1. Detection of megalcytivirus and VHSV from shellfish

1-1. Comparison of tissue volume for viral detection from oyster

To compare viral concentration methods, nucleic acids purified from 5 g of the digestive gland mixture from 3 individual oysters (PEG treatment, T5g-N) and 50 mg of the digestive gland from individual oysters (no-PEG treatment, sT50mg-N) were utilized. Mean nucleic acid concentrations for T5g-N were 12.87 µg/µl in DNA and 7.80 µg/µl in RNA. Mean nucleic acid concentrations for sT50mg-N were 1 µg/µl in both DNA and RNA, respectively (data not shown). The megalocytiviruses was identified using T5g-N by first-step PCR, whereas VHSV could not be detected using first-step PCR (Fig. 1A, 2A). Furthermore, the megalocytivirus and VHSV were identified by nested-PCR (35-35 cycles) (Fig. 1B, 2B). For viral detection using sT50mg-N, megalocytiviruses were identified in 2 specimens by first-step PCR and 6 specimens by nested-PCR (Fig. 1C, D). Whereas VHSV could not be detected by first-step PCR, the viruses were identified from 6 individual specimens by nested-PCR (Fig. 2C, D).

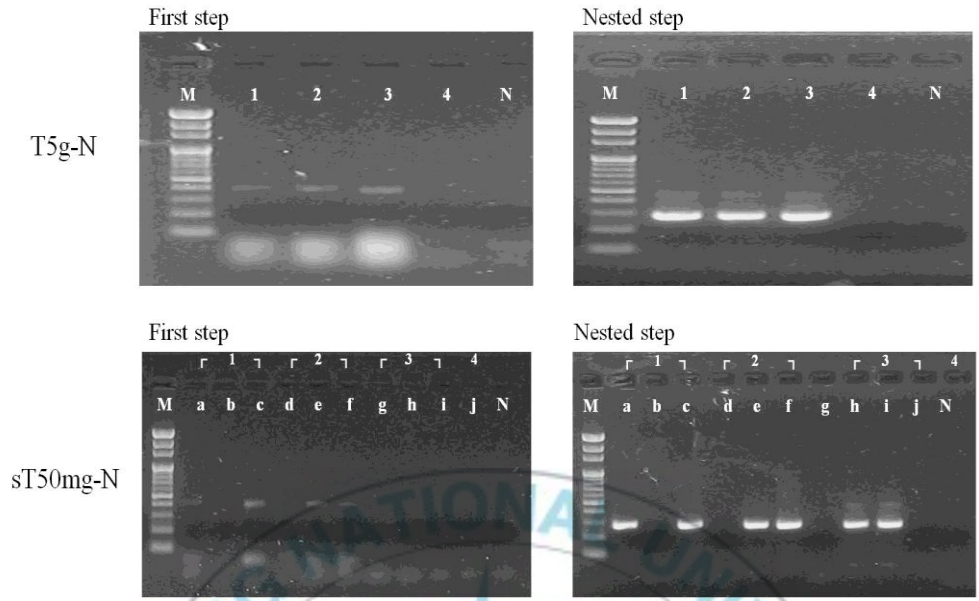

Fig. 1. Comparison of tissue volume for the detection of megalocytivirus in oyster specimens. (A) and (C), first-PCR with T5g-N and sT50mg-N, respectively. (B) and (D), nested-PCR with T5g-N and sT50mg-N, respectively. Template for lanes 1, 2 and 3 in (A) was prepared from individuals of lanes a/b/c, d/e/f, g/h/i, and j in (C), respectively. Lane 4 and j were used as the negative control. Lane N, negative control in PCR without template. Lane M, 100bp DNA ladder.

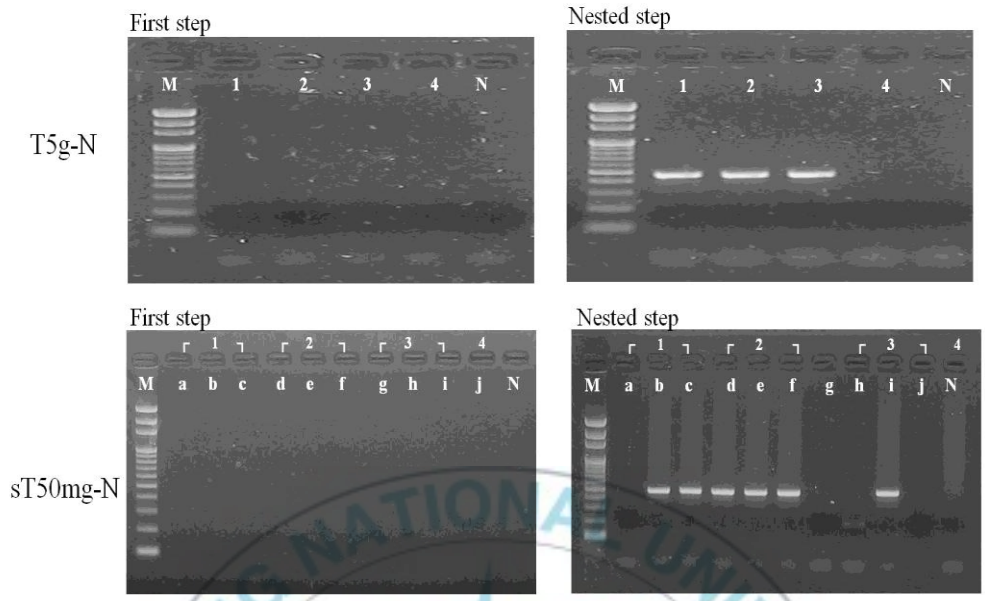

Fig. 2. Comparison of tissue volume for the detection of VHSV from oysters. (A) and (C), first-step PCR with T5g-N and sT50mg-N, respectively. (B) and (D), nested-PCR with T5g-N and sT50mg-N, respectively. Template for lane 1, 2, and 3 in (A) was prepared from individuals of lanes a/b/c, d/e/f, g/h/i, and j in (C), respectively. Lane 4 and j were used as the negative control. Lane N, negative control in PCR without template. Lane M, 100bp DNA ladder.

1-2. PCR inhibitor in digestive gland in oyster

To analyze the PCR inhibitor found in the digestive gland of shellfish, the digestive gland homogenates of oysters and PBS (normal control) were spiked with the cultured megalocytivirus IVS-1 strain for 30 min at room temperature. Spiked concentrations were normalized to 10^3, 10^4, and 10^5 copies/mg of the digestive gland followed by qPCR. The amount of megalocytivirus IVS-1-spiked digestive gland homogenates and PBS were 6.73E + 01, 3.77E + 02, 6.20E + 03 copies/µl and 2.87E + 02, 1.89E + 03, 4.78E + 04 copies/µl, respectively (Table 2). The mean viral recovery yields were 5.57% for the digestive gland homogenates and 32% for PBS. Thus, compared to PBS, the viral recovery yield from the digestive gland tissue decreased by approximately 5-fold by the PCR inhibitor in the digestive gland from oysters.

Table 2. PCR inhibitor test result for the digestive gland from oysters

Spiked copy numbers of IVS-1 on mg tissue	prepared sT50mg-N (50 µl)		PBS (50 µl)	
	C_T[a] value	Copy no./ µl[b]	C_T value	Copy no./ µl
1.00E+05	25.18	6.20E+03 (6.2)	20.57	4.78E+04 (48)
1.00E+03	30.28	3.77E+02 (3.8)	27.35	1.89E+03 (19)
1.00E+02	33.42	6.73E+01 (6.7)	30.78	2.87E+02 (29)

[a] cycle threshold

[b] Viral copy numbers in purified DNA 1 µl from IVS-1 spiked digestive gland tissue or PBS (approximately 1 mg digestive gland = 1 µl sT50mg-N)

Numbers within parentheses indicate percentage to the spiked copy numbers.

1-3. Detection limit of the megalocytivirus in digestive gland of oysters

The detection limit of the megalocytivirus was analyzed using a field-oyster viral-positive specimen [Fig. 1(c)] collected from Tongyeong. Nucleic acids were purified from 10-fold, serial-diluted digestive gland homogenates followed by nested-PCR (35-35 cycles) and qPCR (40 cycles). The megalocytivirus was identified in sT5mg-N (1/10 dilution) by first-step PCR and sT500ug-N (1/1000) by nested-PCR (Fig. 3). The amount of each diluted specimen was 1.20E + 02 to 6.14E + 00 copies, corresponding to 1 µl of sT50mg-N to sT500ug-N (Table 3). Thus, the detection limit of the portion of digestive gland for the detection of the megalocytivirus was 500 µg (6.14E + 00 copies/µl).

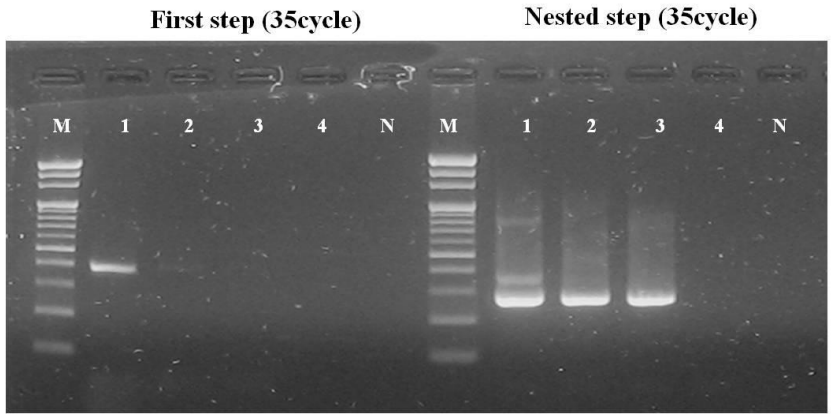

Fig. 3. Detection limit of megalocytivirus by PCR by using serially diluted homogenate of the digestive gland of oysters [lane 1c in Fig. 1 (C) of this study]. Lanes 1–4, DNA templates were prepared with 50 mg, 5 mg, 500 µg, and 50 µg of the digestive gland tissue homogenate, respectively. Lane N, negative control in PCR without template. Lane M, 100-bp DNA ladder.

Table 3. Detection of megalocytivirus in varying amounts of the digestive gland in oysters

Dilution ratio	PCR		qPCR	
	First step	Nested step	C_T [b] Value	Copies no. [c]
10^0 (sT50mg-susN [a])	+	++	30.88	1.20E+02
10^{-1} (sT5mg-susN)	+ [d]	++	34.82	1.21E+01
10^{-2} (sT500ug-susN)	-	++	35.98	6.14E+00
10^{-3} (sT50ug-susN)	-	-	NT	NT

[a] template were prepared using one positive sample (lane 1c in Fig 2 (C))
[b] cycle threshold
[c] copy number/μl prepared template
[d] weak positive; NT, not tested.

1-4. Quantitative analysis of megalocytivirus and VHSV in oysters

From sT50mg-N, viral copies for megalocytivirus and VHSV in the digestive gland of oysters were analyzed by qPCR. Viral amounts of each megalocytivirus-positive oyster specimen from Gijang, Namhae, and Tongyeong were 3.04E + 01, 1.72E + 02 and 4.56E + 02 viral copies in 1 mg of digestive gland tissue, respectively (Table 4). The mean amount of megalocytivirus in the digestive gland tissues was 2.19E + 02 viral copies/mg. For the VHSV-positive oyster specimens from Gijang and Tongyeong, viral amounts in the digestive gland tissues were 1.73E + 02 and 6.85E + 01 copies/mg, respectively. The specimen from Namhae was not identified with VHSV as determined by qPCR. From quantitative analyses of viruses in the digestive gland, we determined that the megalocytivirus presented at a higher rate than did VHSV. These results corresponded with nested-PCR results (Fig. 1 and 2) in that megalocytivirus was detected by first-step PCR.

Table 4. Mean viral copies from the digestive gland in oyster

Virus	Sampling site	Species	Viral copies[a]
Megalocytivirus	Gijang	Oyster (*Crassostrea gigas*)	3.04E+01
	Namhae		4.56E+02
	Tongyeong		1.72E+02
	Mean viral copies/mg		2.19E+02
VHSV	Gijang	Oyster (*Crassostrea gigas*)	1.16E+02
	Namhae		ND[b]
	Tongyeong		6.85E+01
	Mean viral copies/mg		9.22E+01

[a]viral copies/digestive gland tissue mg
(approximately 1 mg digestive gland = 1 µl sT50mg-N)

[a]not detected

2. Detection of megalocytivirus and VHSV from seawater

2-1. Comparison of viral concentration method from seawater

To determine and compare viral concentration methods, glass microfiber (GF/C, 47 mm pore size), cellulose acetate (CA, 0.45 um pore size), and nitrocellulose (HA, 0.45 um pore size) were used as the filtration membranes. The megalocytivirus IVS-1 strain and VHSV were inoculated in 1 L of filtrated seawater (0.22 μm pore size) and the final concentration of each inoculated virus was normalized to 1.00E + 01 viral copies/ml. Nucleic acids extracted from the concentrates were analyzed by nested-PCR by using their corresponding primers. While megalocytivirus was detected by first-step PCR (35 cycles), VHSV was detected by nested-step (35-35 cycles; Fig. 4). For the comparison of filtration methods, although megalocytivirus and VHSV were not detected using either the single GF/C or CA membrane, they were identified using the GF/C+CA or CA with HA membrane.

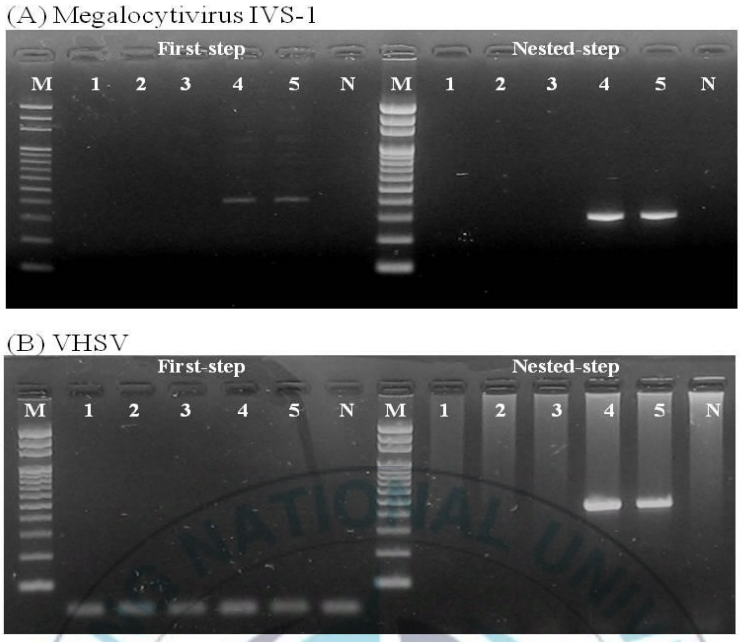

Fig. 4. Detection of viruses in 1 L seawater spiked with megalocytivirus and VHSV. (A) Megalocytivirus IVS strain and (B) VHSV (1.0E + 01/ml seawater, each) following concentration by different membranes. First- and nested-step PCRs were performed for front lanes 1–5 and back lanes 1–5, respectively. Lane 1, general seawater (GF/C+CA); lane 2, GF/C membrane; lane 3, CA membrane; lane 4, GF/C+CA+HA membrane; and lane 5, GF/C+HA membrane. M, 100-bp DNA ladder.

2-2. Sensitivity of nitrocellulose membrane to viral concentrations in seawater

From the megalocytivirus IVS-1 strain and VHSV inoculated seawater (1 L), normalized from 1.20E − 1 to 1.20E + 02 viral copies/ml of seawater, sensitivity and virus recovery yields of the filtration method using GF/C with HA membranes were estimated by nested-PCR. Detection limits of the filtration method were 1.200E + 00 viral copies/ml for the megalocytivirus and 1.22E + 01 viral copies/ml for VHSV (Fig. 5). The means of the recovery yields for megalocytivirus and VHSV in seawater that were obtained using GF/C with HA membranes were 28.11% and 23.00%, respectively (Table 5). For filtration using GF/C with HA membranes in determining viral concentration from seawater, the detection limit of the DNA virus (i.e., megalocytivirus) was approximately 10 times more sensitive than that of the RNA virus (i.e., VHSV). It is possible that the sample incurred a loss of viral RNA during cDNA synthesis for VHSV.

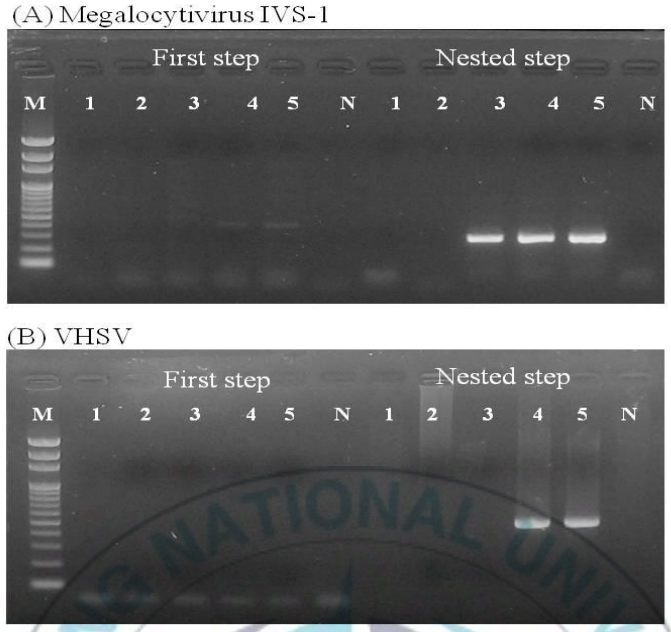

Fig. 5. Sensitivity of the nitrocellulose (HA) membrane used for detecting viral concentrations of (A) megalocytivirus IVS-1 and (B) VHSV in seawater. Lanes 1–5, concentrate of seawater spiked with VHSV or IVS-1 (0, 0.12, 1.2, 12, and 120 viral particles/ml seawater, respectively); N, D.W.; M, 100-bp DNA ladder

Table 5. Viral recovery yield from megalocytivirus and VHSV inoculated seawater

Virus	Inoculated concentration (1ℓ of sea water)	Recovered concentration (1ml of concentrate)	Recovery	PCR	
	Viral particles/ml seawater	Viral particles/ml	(%)[a]	1-step	2-step
Megalocytivirus IVS-1	1.20E+02	3.76E+04	31.33%	+	+
	1.20E+01	3.29E+03	27.42%	+	+
	1.20E+00	3.07E+02	25.58%	ND	+
	1.20E-01	ND[b]		ND	ND
Mean of recovery yield			28.11%		
VHSV	1.22E+02	3.28E+04	26.89%	ND	+
	1.22E+01	2.33E+03	19.10%	ND	+
	1.22E+00	ND		ND	ND
	1.22E-01	ND		ND	ND
Mean of recovery yield			23.00%		

[a]Recovery (%) = (Recovered viral concentration × vol/spiked viral concentration × vol) × 100
[b]ND, not detected

2-3. Detection of VHSV from field specimens

Influent seawater and cultured seawater from 2 VHSD-positive flounder farms in Gampo and Jeju in February 2011 were concentrated using GF/C with CA or HA membranes followed by nested-PCR. Sampling was performed when the cumulative mortality of flounders was approximately 50% in 15℃ ± 0.5℃. Additionally, cultured water from the flounder farms was changed at 16–17 cycles/days. VHSV was not detected in influent seawater by using either the CA or GF/C with HA membranes for filtration (Fig. 6). In cultured seawater, VHSV was detected using GF/C with the HA membrane but not the CA membrane.

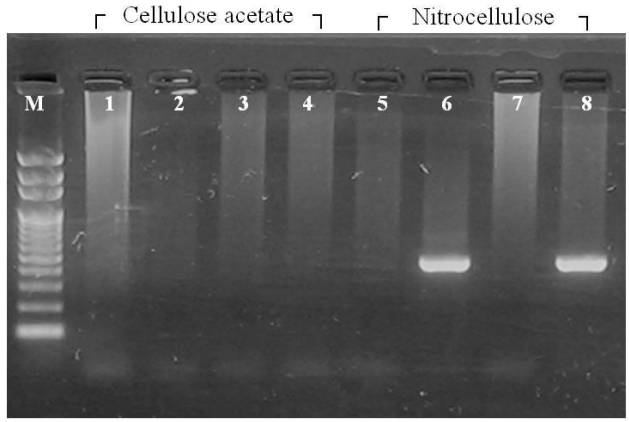

Fig. 6. Comparison of the viral concentration method for seawater from 2 VHSD-positive flounder farms. Influent seawaters from the A farm (Jeju) and B farm (Gampo) were concentrated by CA (lanes 1, 3) or GF/C with HA (lanes 5, 7) membranes followed by nested-PCR. Cultured seawater from the A farm (Jeju) and B farm (Gampo) were concentrated by CA (lanes 2, 4) or GF/C with HA membranes (lanes 6, 8) followed by nested-PCR; M, 100-bp DNA ladder.

IV. Discussion

Since shellfish mollusks and environmental water normally have low viral titers, a series of complicated concentration steps for the detection of viruses is used (Atmar et al., 1995; Jaykus et al., 1996; Katayama et al., 2002). Of the concentration methods, the use of PEG treatment followed by PCR is common because of effective viral precipitation via PEG eluants (Atmar et al., 1993; Atmar et al., 1995; Le Guyader et al., 2009; Suzuki and Nojima, 1999). Although PEG precipitation methods overcome several disadvantages (e.g., low viral titers and carryover of a PCR inhibitor), they are time consuming, involve complicated concentration steps, and require mass portions of sample.

Previous studies reported that the PEG precipitation method for shellfish processing was an effective concentration method (Atmar et al., 1995; Jaykus et al., 1996). However, there are no differences in the results of nested-PCR between PEG (T5g-N) and no-PEG (sT50mg-N) treatments in detecting megalocytivirus and VHSV (Fig. 1 and 2). Although T5g-N, which was pooled from more than 3 individual oysters, was virus-positive as per the nested-PCR analysis, not all individual specimens (sT50mg-N) were virus-positive. In addition, the nested-PCR and qPCR assays of sT50mg-N suggest that the viral concentrations for each individual specimen contributing to the pooled T5g-N sample differed. Interestingly, megalocytivirus could be detected from 500 μg (6.14E + 00 viral

copies/μl) of the digestive gland by nested-PCR despite the presence of PCR inhibitors (Fig. 2, Tables 2 and 3). These findings revealed that sT50mg-N (with nucleic acids purified from 50 mg of digestive gland) was suitable for qualitative and quantitative analyses of aquatic animal viruses from shellfish samples. Additionally, sT50mg-N has several advantages such as an individual analysis and less time-consuming are possible and it has high efficiency.

Among the viral concentration methods from environmental water, Katayama et al. (2002) reported that viral adsorption and an acid rinse followed by elution steps with negatively charged membranes was an efficient method for viral concentration and inhibitor reduction. Therefore, we compared filtration methods using charged membranes (i.e., cellulose acetate [CA] membrane for (-1) negative ions and the nitrocellulose [HA] membrane for (-2) negative ions) to determine aquatic animal viral concentrations.

Comparisons of the concentration methods between membranes with virus-inoculated seawater showed that the nitrocellulose membrane (HA) was efficient for determining viral concentration. Moreover, a double filtration approach, GF/C (1.2 μm) with the HA membranes, could block suspended solids in seawater, as shown in a previous study (Song et al., 2008). The sensitivity of this method shows that the detection limits of megalocytivirus and VHSV in 1 L of seawater were 1.20E + 00 and 1.22E + 01 viral copies/ml, respectively (Table 5). It may be likely that VHSV particles are lost during the cDNA synthesis step. However,

viral recovery yields of this method did not reveal significant differences between viruses; 28.11% for megalocytivirus and 23.00% for VHSV. Previous studies reported that viral recovery yields were virus-specific; >90% for poliovirus, 30~50% for hepatitis virus (HAV) (Katayama et al., 2002), and 3.9% for koi herpesvirus (Haramoto et al., 2009). Although viral recovery yields were low compared to human enteric viruses including poliovirus, VHSV in cultured water of farms with VHSD was identified using this method. Additionally, VHSV was identified in seawater within a 500 meter radius from VHSD outbreak farms with highly cumulative mortality (approximately 50%) of flounder (data not shown). These results revealed that viral concentrations from seawater using GF/C with HA membranes are sufficient to detect aquatic animal viruses in field seawater.

We conclude that aquatic animal viruses (i.e., megalocytivirus and VHSV) in shellfish can be detected using either PEG or no-PEG treatments. Small volumes (e.g., 50 mg) of digestive gland can be enough to qualitatively and quantitatively analyze viruses in shellfish and that of approach has the advantage of being able to conduct efficient, individual analyses compared to PEG treatment. In addition, the filtration method using GF/C with HA membranes allows for the detection of aquatic animal viruses in seawater effectively.

Chapter II. Surveillance of aquatic animal viruses in shellfish and seawater in Korea

I. Introduction

After an outbreak of an aquatic animal disease, pathogens can be easily released from the host into the environment and re-infect susceptible hosts or be introduced into a carrier or vector species that can retain or transfer the pathogen to other species. In addition, the released pathogens may also accumulate in filter-feeding organisms. Shellfish accumulate human enteric viruses such as norovirus and hepatitis A virus at levels sufficient to cause a disease outbreak (Atmar et al., 1993; Atmar et al., 1995). Although shellfish cultured near aquaculture farms can act as carriers or vectors of aquatic animal viruses, studies related to this aspect were not reported fully until recently. Only some studies have examined infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus, marine birnavirus (MABV) and white spot syndrome virus (WSSV) in shellfish and their role as possible vectors or bio-indicators (Gregory et al., 2009; Skar and Mortensen, 2007; Suzuki and Nojima, 1999; Vazquez-Boucard et al., 2010; Vazquez-Boucard et al., 2012).

Various agents are generally present in the ambient seawater. In addition, shellfish as filter-feeding organisms accumulate various substances including pathogenic agents from the water. Of note, the viruses derived from seawater and shellfish were a low copy number. Thus, to analysis the various viruses in seawater and shellfish, more effective and sensitive method than general polymerase chain reaction (PCR) based method is required. The multiplex nested-PCR assay allows various viral pathogens to be detected simultaneously in a single reaction and is very convenient for samples containing mixed infected or contaminated substances such as seawater or shellfish.

Megalocytiviruses including rock bream iridovirus (RBIV) and flounder iridovirus (FLIV) types, viral hemorrhagic septicemia virus (VHSV), and WSSV are annual endemic viral pathogens in Korea and listed as causing remarkable disease by the World Organization for Animal Health (OIE). However, information on the carrier or vector species of these viruses is not available. These viruses could be transmitted through seawater and accumulate in shellfish, particularly if shellfish farms are located near fish farms. For this reason, surveillance of aquatic animal pathogenic viruses in the host, surrounding seawater, and shellfish is important to control and prevent aquatic animal diseases.

In this study, we developed a multiplex nested PCR method for investigation several aquatic animal pathogenic viruses in seawater and shellfish in Korea. We

also investigated a genetic relevance between viruses derived from fish, seawater, and shellfish.

II. Materials and methods

1. Samples

Seawater and shellfish were collected from different sites in the Eastern (Gijang, Jinha), Southern (Geoje, Gosung, Tongyeong, Wando), and Western Sea (Seosan) areas of Korea (Fig. 7). A total of 249 shellfish samples (12 *Chlamys farreri*, 79 *Crassostrea gigas*, 19 *Crassostrea nippona*, two *Haliotis discus hannai*, eight *Meretrix lusoria*, 61 *Mytilus edulis*, two *Panopea japonica*, three *Peronidia venulosa*, 17 *Ruditapes variegates*, 23 *Saxidomus purpurata*, six *Scapharca subcrenata*, two *Sinonovacula constricta*, 10 *Tapes philippinarum*, and three *Tresus keenae*) were collected between January 2010 and November 2011. Sixty seawater samples were collected in sterilized 1 L bottles between February 2011 and November 2011. All seawater and shellfish samples were directly transported to the laboratory after sampling.

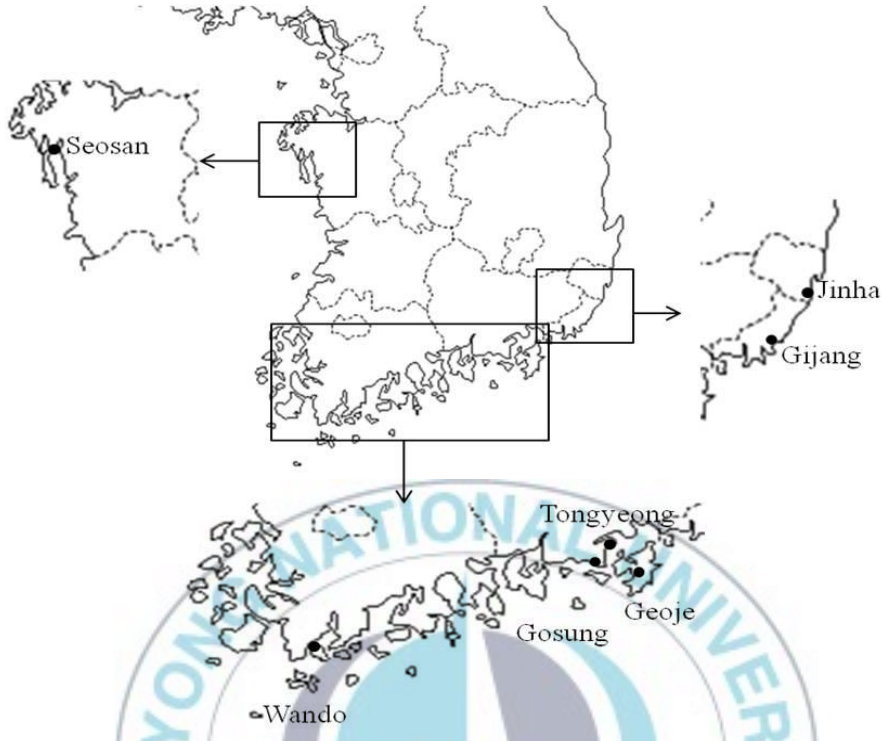

Fig. 7. Sampling site in this study. Each sampling site located near sea shore in Korea.

2. Virus

For viral propagation, Chinook salmon embryo (CHSE-214), epithelial papilloma of carp (EPC), and grunt fin (GF) cell lines were propagated in minimum essential Eagle's medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibiotic and antimycotic solution (Gibco, Grand Island, NY, USA). Spleen from megalocytivirus (IVS-1 strain, Jeong *et al*., 2003)-infected rock bream (90 ± 11.0 g) and brains of viral nervous necrosis virus (VNNV) RGNNV type-infected sea bass were used as viral inoculums for the GF cell line. Kidney from VHSV- and MABV-infected flounder (10 ± 5g) were the viral inoculums for the CHSE-214 and EPC cell lines, respectively. Following the development of cytopathic effects (CPEs), each virus was centrifuged at 3,000 × g for 10 min and the supernatant was stored at -80°C before the experiment. The hepatopancreas of shrimp (*Fenneropenaeus chinensis*) infected with WSSV was used for an experiment.

3. Nucleic acid purification from field samples

Shellfish samples were aseptically opened using a knife, the digestive organs were removed, and the digestive gland was separated using a scalpel. The

digestive gland of three to five individuals were pooled as one specimen and stored at -80℃. The method of Katayama et al. (2002) was modified for the seawater samples. One L of seawater was filtered with a GF/C membrane (1.2 μm pore size, Whatman, Maidenstone, UK) and an HA type negatively charged membrane (0.45 μm pore size, Millipore, Tokyo, Japan) to eliminate sediment and absorb the virus, respectively. The cations were rinsed out of the filtered membrane using 100 ml of 0.5 mM H_2SO_4. Then, 10 ml of 1 mM NaOH (pH 10) was passed through the membrane into a new 50 ml tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100× TE buffer for neutralization. The filtrate was concentrated at 3000 × g for 10 min at 4℃ using an Amicon Ultra-15 Centrifugal Filter Unit (30 kDa, Millipore) and the concentrate was adjusted to a final volume of 1 ml. Nucleic acids were purified from 50 mg of digestive gland tissue and 200 μl of concentrated seawater using an AccuPrep Genomic DNA Extraction kit (Bioneer, Daejeon, Korea) for DNA extraction and an RNeasy Plus mini kit (Qiagen, Valencia, CA, USA) for RNA extraction according to the manufacturer's protocols.

4. Multiplex nested PCR assay

The multiplex nested PCR assay was developed targeting two DNA viruses (meglaocytivirus and WSSV) and three RNA viruses (VHSV, VNNV, and

MABV). Specific primer sets were designed from the viral genome based on the nucleotide sequences from the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Table 6). For the DNA viruses, the primer sets that hybridized to all subtypes of megalcytivirus and WSSV were designed from the conserved region in the major capsid protein gene (MCP) and the VP28 gene, respectively. The primer sets for detecting VHSV and VNNV were designed from the glycoprotein gene and RNA 2 segment gene, respectively. Published primer sets were used to detect MABV (Suzuki and Nojima, 1999). For the RNA viruses (VHSV, VNNV, and MABV), cDNA synthesis was performed using 1 μl of RNA mixed with 1 μl random primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). First-step PCR reactions were performed in a volume of 20 μl with 1 μl of template DNA or 1 μl of cDNA, each of the primer sets (DNA viruses: 0.5 μM primer for megalocytivirus and WSSV; RNA viruses: concentrations of primer were 0.4 μM for VHSV, 0.5 μM for VNNV and 0.6 μM for MABV, respectively), 2 μl 10× PCR buffer, 200 μM of dNTP, and Taq DNA polymerase (Cosmo Genetech, Seoul, Korea). The cycling conditions for the one-step and nested PCRs were a pre-denaturation step at 95℃ for 3 min, 35 cycles of denaturation at 94℃ for 40 s, annealing at 52℃ for 40 s, extension at 72℃ for 40 s, followed by a final extension step at 72℃ for 7 min. The PCR products were identified by electrophoresis on 2% agarose gels and stained with ethidium bromide.

Table 6. Primers for the multiplex nested polymerase chain reaction

	Target virus	Primers	Sequence (5' to 3')	Region	Product size (bp)	Reference
DNA virus	Megalocytivirus	M1F	GCTGCGCATGCCAATCATCT	MCP gene	401	This study
		M1R	ATGCGATGGAGACCCACTTG			
		M2F	AATGACACCGACACCTCCTC		288	
		M2R	TGCGATGGAGACCCACTTGT			
	WSSV	WS1F	GAACATTCAAGGTGTGGAAC	VP28 gene	250	This study
		WS1R	GTCTCAGTGCCAGAGTAGGT			
		WS2F	ACATCAAGAAAGATCAACATCA		125	
		WS2R	CCAACTTCATCCTCATCAAT			
RNA virus	MABV	P1	AGAGATCACTGACTTCACAAGTGAC	Segment A VP2/NS junction part	359	Suzuki *et al.*, (1997)
		P2	TGTGCACCACAGGAAAGATGACTG			
		P3	CAACACTCTTCCCCATG		168	
		P4	AGAACCTCCCAGTGTCT			
	VNNV	NF1	CGTGTCAGTCATGTGTCGCTG	Coat protein gene (RNA2)	440	This study
		NR1	AGTCAACACGGGTGAAGAG			
		NF2	CTTGAGACACCTGAAGAGAC		323	
		NR2	GCTGCTCATCAGAGTAGTAGG			
	VHSV	SF1	CACAGATCACTCAACGACC	Glycoprotein gene	559	This study
		SR1	GTGATCATGTGTCCTGGTG			
		SF2	GACTGGGACACTCCACTGTA		467	
		SR2	CAAACCCCCTCTATGAAGTC			

5. Specificity and sensitivity testing for multiplex nested PCR

Each virus was spiked into digestive gland of oysters (10^2 copies/mg of digestive gland tissue) to determine the specificity of the multiplex nested PCR assay. Sensitivity of the multiplex nested PCR was determined using ten-fold serial dilutions of DNA or RNA extracted from viral-spiked digestive gland of oysters (10^4 copies/mg of digestive gland). Then, the multiplex nested PCR was performed as described above.

6. Sequencing and phylogenetic analysis

The multiplex nested PCR products of megalocytivirus, VHSV, VNNV positive samples were purified using a Gel Extraction kit (GeneAll Biotechnology Co. Ltd, Seoul, Korea**)** and cloned into the pGEM-T vector according to the manufacturer's protocol (Promega). The recombinant plasmids were sequenced at Cosmogenetech (Seoul, Korea) with an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the sequence alignments were compared using the BioEdit program (version 7.0.5). Phylogenetic trees were constructed based on the sequence alignment using the neighbor-joining method with maximum composite likelihood model and 1,000 bootstrap values in the MEGA program (ver. 5.05, http://www.megasoftware.net).

7. Statistical analysis

A one-way analysis of variance was performed for the multiplex nested PCR data using SPSS 12.0.1 (SPSS, Inc., Chicago, IL, USA). A *P*-value < 0.05 was considered significant.

III. Results

1. Specificity and sensitivity of multiplex nested PCR assay

Aquatic animal viruses were simultaneously detected in the nested PCR based on the type of nucleic acids amplified in each corresponding viral nucleic acid specifically and produced amplicons of the expected size of 288 bp for megalocytivirus, 125 bp for WSSV, 467 bp for VHSV, 323 bp for VNNV, and 168 bp for MABV. Primers used for cDNA synthesis using the random oligonucleotide primers or specific primers for each RNA virus did not affect the specificity of the multiplex nested PCR (Fig. 8). The sensitivity of the multiplex nested PCR assay with viral spiked digestive tissue of oysters (one to three different viruses were spiked) appeared to reach one copy for VHSV, VNNV, and megalocytivirus, and 10 copies for WSSV and MABV, respectively (Fig. 9).

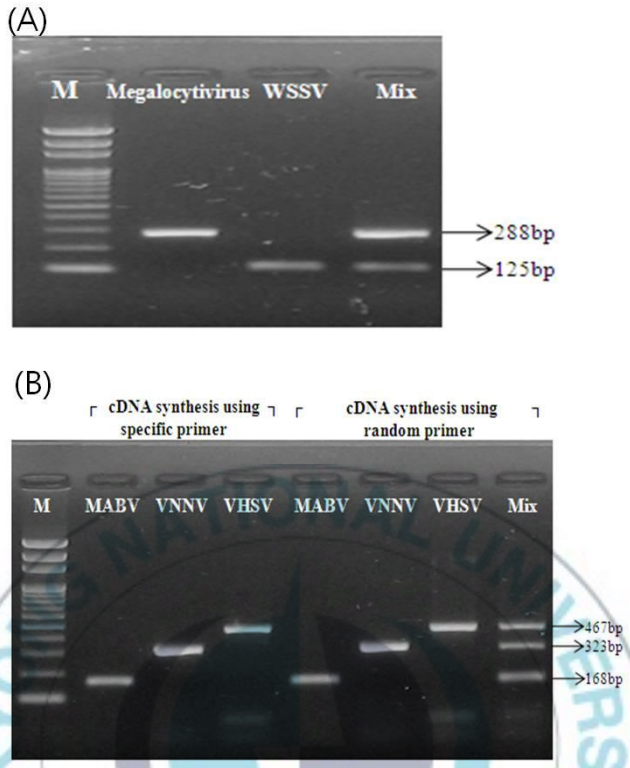

Fig. 8. Specificity of multiplex nested PCR assay. Nucleic acids of individual virus were amplified in PCR reaction containing primer sets. Lane M, 100 bp ladder. (A) detection of DNA viruses (megalocytivirus, WSSV) using multiplex nested PCR; (B), detection of RNA viruses (VHSV, VNNV, MABV) using multiplex nested PCR with cDNA prepared with specific primers or random primers.

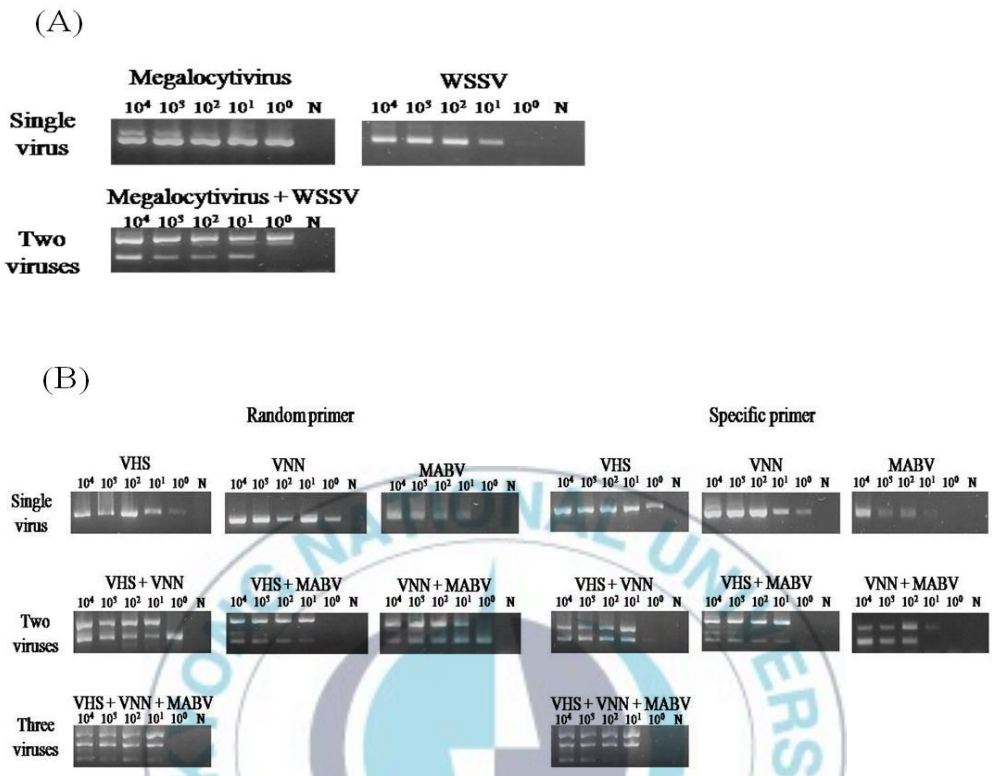

Fig. 9. Comparison of the sensitivity in multiplex nested PCR assay. (A), detection limit of DNA viruses (megalocytivirus, WSSV) using multiplex nested PCR; (B), detection limit of RNA viruses (VHSV, VNNV, MABV) using multiplex RT nested PCR with cDNA prepared with specific primers or random primers.

2. Identification of aquatic animal pathogenic virus in seawater and shellfish

As a shown in Tables 7 and 8, aquatic animal pathogenic viruses were identified from seawater and shellfish samples. Of the 249 shellfish and 60 seawater samples, the most prevalent virus was megalocytivirus in shellfish (n = 95; 38.16%) and seawater (n = 25; 41.7%) samples, followed by VNNV (n = 46; 18.5%), VHSV (n = 36; 14.5%), MABV (n = 15; 6.9%) and WSSV (n = 13, 5.2%) in shellfish and VHSV (n = 4, 6.7%), MABV (n = 2, 3.3%), WSSV (n = 1, 1.7%), and VNNV (n = 1, 1.7%) in seawater. Multiplex nested PCR detection rates of other viruses in shellfish were not significantly different relating to sampling site and time except VNNV which identified from shellfish between eastern and western sea areas (Fig. 10).

Table 7. Detection of aquatic animal viruses in seawater

Sampling site	Positive rate of multiplex nested PCR (%) between February 2011 and November 2011				
	Megalocytivirus	WSSV	VHSV	VNNV	MABV
Eastern sea					
Gampo	1/2 (0.5)	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)	1/2 (0.5)
Gijang	1/2 (0.5)	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)	0/2(0.0)
Jinha	4/10 (40.0)	0/10 (0.0)	1/10 (10.0)	0/10 (0.0)	0/10 (0.0)
Southern sea					
Geoje	1/7 (14.3)	0/7 (0.0)	2/7 (28.6)	0/7 (0.0)	0/7 (0.0)
Gosung	5/13 (38.4)	1/13 (7.7)	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)
Tongyeong	1/4 (25.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
Wando	4/6 (66.7)	0/6 (0.0)	1/6 (16.7)	1/6 (16.7)	0/6 (0.0)
Western sea					
Seosan	8/16 (0.5)	0/16 (0.0)	0/16 (0.0)	0/16 (0.0)	1/16 (6.3)
Total	25/60 (41.7)	1/60 (1.6)	4/60 (6.7)	1/60 (1.6)	2/60 (3.3)

Table 8. Detection of aquatic animal viruses in shellfish

Sampling site	Species	Multiplex nested PCR positive (%) between January 2010 and November 2011				
		Megalocytivirus	WSSV	VHSV	VNNV	MABV
Eastern sea						
Gijang	*Crassostrea gigas*	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)
	Crassostrea nippona	3/8 (37.5)	0/3 (0.0)	3/8 (37.5)	2/8 (25.0)	1/8 (12.5)
	Mytilus edulis	1/10 (10.0)	1/5 (20.0)	4/10 (40.0)	1/10 (10.0)	1/10 (10.0)
	Scapharca subcrenata	1/2 (50.0)	1/2 (50.0)	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)
Hakli	*Mytilus edulis*	2/6 (33.3)	0/6 (0.0)	1/6 (16.7)	0/6 (0.0)	1/6 (16.7)
Jinha	*Crossostrea gigas*	11/18 (61.1)	1/18(5.6)	2/18(11.1)	0/18(0.0)	0/18(0.0)
	Crassostrea nippona	3/5 (60.0)	1/5 (20.0)	2/5 (40.0)	1/5 (20.0)	2/5 (40.0)
	Mytilus edulis	4/18(22.2)	2/18 (11.1)	3/18 (16.7)	3/18 (16.7)	1/18(5.6)
	Meretrix lusoria	2/5 (40.0)	2/5 (40.0)	0/5 (0.0)	2/5 (40.0)	0/5 (0.0)
	Tapes philippinarum	1/1 (100)	0/1 (0.0)	0/1 (0.0)	1/1 (100)	0/1 (0.0)
Southern sea						
Geoje	*Chlamys farreri*	3/6 (50.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)
	Crassostrea gigas	5/9 (55.6)	1/9 (11.1)	1/9 (11.1)	2/9 (22.2)	0/9 (0.0)
	Crassostrea nippona	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	1/6 (16.7)
	Mytilus edulis	2/8 (25.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
	Ruditapes variegatus	5/9 (55.6)	0/9 (0.0)	0/9 (0.0)	2/9 (22.2)	1/9 (11.1)
	Saxidomus purpurata	2/9 (22.2)	1/9 (11.1)	0/9 (0.0)	3/9 (33.3)	0/9 (0.0)
	Tapes philippinarum	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	1/6 (16.7)	0/6 (0.0)

Gosung	*Crassostrea gigas*	1/5 (20.0)	0/5 (0.0)	0/5 (0.0)	2/5 (40.0)	0/5 (0.0)
	Chlamys farreri	1/3 (33.3)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)
	Crassostrea gigas	4/6 (66.7)	0/6 (0.0)	4/6 (66.7)	2/6 (33.3)	0/6 (0.0)
	Meretrix lusoria	1/3 (33.3)	1/3 (33.3)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)
	Mytilus edulis	7/8 (87.5)	0/8 (0.0)	2/8 (25.0)	2/8 (25.0)	0/8 (0.0)
Namhe	*Panopea japonica*	1/2 (50.0)	0/2 (0.0)	1/2 (50.0)	0/2 (0.0)	0/2 (0.0)
	Ruditapes variegatus	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	3/8 (37.5)	0/8 (0.0)
	Scapharca subcrenata	2/6 (33.3)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	1/6 (16.7)
	Sinonovacula constricta	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0/2 (0.0)
	Saxidomus purpurata	3/8 (37.5)	0/8 (0.0)	0/8 (0.0)	3/8 (37.5)	0/8 (0.0)
	Tresus keenae	1/3 (33.3)	0/3 (0.0)	0/3 (0.0)	1/3 (33.3)	0/3 (0.0)
	Chlamys farreri	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	1/3 (33.3)	0/3 (0.0)
	Crassostrea gigas	24/37 (64.9)	0/37 (0.0)	11/37 (29.7)	6/37 (16.2)	3/37 (8.1)
Tongyeong	*Mytilus edulis*	1/10 (10.0)	1/10 (10.0)	0/10 (0.0)	4/10 (40.0)	0/10 (0.0)
	Peronidia venulosa	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	2/3 (66.7)
	Saxidomus purpurata	2/6 (33.3)	0/6 (0.0)	0/6 (0.0)	1/6 (16.7)	1/6 (16.7)
Wando	*Haliotis discus hannai*	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)	0/2 (0.0)
Western sea						
	Crassostrea gigas	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	1/1 (100.0)	0/1 (0.0)
Seosan	*Mytilus edulis*	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)
	Tapes philippinarum	1/3 (33.3)	0/3 (0.0)	1/3 (33.3)	1/3 (33.3)	0/3 (0.0)
Total		95/249(38.16)	13/249 (5.2)	36/249 (14.5)	46/249 (18.5)	15/249 (6.0)

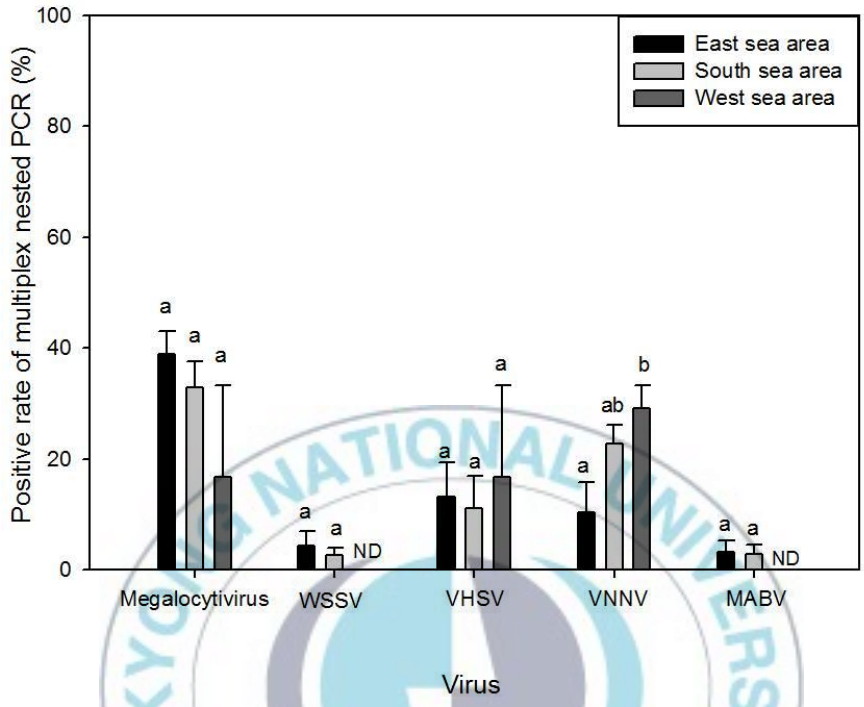

Fig. 10. Detection rate of multiplex nested PCR in shellfish by sampling site, eastern, southern and western sea areas. Significant difference was calculated by a one-way analysis of variance and significant difference (*) was obtained at $P<0.05$.

3. Genetic characterization of aquatic animal pathogenic virus in seawater and shellfish

One aquatic animal pathogenic virus was identified at more than 40% in an individual specimen of shellfish (n = 106; 42.57%) and seawater (n = 28; 46.67%), respectively (Table 9). Two or more viruses were identified in shellfish (n = 44; 17.67%) compared to those in seawater (n = 2; 3.33%) samples.

Table 9. Characteristics of aquatic animal viruses in shellfish and seawater

No. of virus	Viruses	Shellfish (n*=249)	Seawater (n=60)
1 virus	M[a]	58 (23.29%)	23 (38.33%)
	W[b]	4 (1.69%)	1 (1.67%)
	V[c]	13 (5.49%)	3 (5.00%)
	N[d]	23 (9.24)	0 (0.00%)
	B[e]	7 (2.95%)	1 (1.67%)
Total		106 (42.57%)	28 (46.67%)
2 viruses	M+W	3 (1.27%)	0 (0.00%)
	M+V	15 (6.33%)	0 (0.00%)
	M+N	9 (3.61%)	0 (0.00%)
	M+B	1 (0.42%)	1 (1.67%)
	W+V	0 (0.00%)	0 (0.00%)
	W+N	2 (0.84%)	0 (0.00%)
	W+B	0 (0.00%)	0 (0.00%)
	V+N	1 (0.42%)	0 (0.00%)
	V+B	0 (0.00%)	0 (0.00%)
	N+B	3 (1.26%)	0 (0.00%)
Total		34 (13.65%)	1 (1.67%)
3 viruses	M+W+V	1 (0.42%)	0 (0.00%)
	M+W+N	3 (1.26%)	0 (0.00%)
	M+W+B	0 (0.00%)	0 (0.00%)
	M+V+N	2 (0.84%)	1 (1.67%)
	M+V+B	1 (0.42%)	0 (0.00%)
	M+N+B	0 (0.00%)	0 (0.00%)
	W+V+N	0 (0.00%)	0 (0.00%)
	W+V+B	0 (0.00%)	0 (0.00%)
	W+N+B	0 (0.00%)	0 (0.00%)
	V+N+B	2 (0.84%)	0 (0.00%)
		9 (3.80%)	1 (1.67%)
4 viruses	M+W+V+N	0 (0.00%)	0 (0.00%)
	M+W+V+B	0 (0.00%)	0 (0.00%)
	M+W+N+B	0 (0.00%)	0 (0.00%)
	M+V+N+B	1 (0.42%)	0 (0.00%)
	W+V+N+B	0 (0.00%)	0 (0.00%)
Total		1 (0.42%)	0 (0.00%)
5 viruses	M+W+V+N+B	0 (0.00%)	0 (0.00%)

[a]M,Megalocytivirus; [b]W,WSSV; [c]V,VHSV; [d]N,VNNV; [e]B,MABV; [*]n, total number of samples

4. Genetic relevance between viruses derived from fish, shellfish and seawater

The phylogenetic tree based on the partial nucleotide sequence of the MCP gene revealed that megalocytivirus in seawater samples (Gosung, Jinha, Seosan, Wando) belonged to subgroup II as classified by Imajoh *et al* (2007) (Fig. 11A). Both subgroup II and IV subtypes of megalocyvirus were also identified in shellfish samples and even in a single specimen. From the phylogenetic analysis based on the G gene of VHSV, viruses identified from shellfish belonged to the VHSV IVa genotype (Fig. 11B). From the phylogenetic analysis based on the RNA2 segment gene of VNNV, viruses were divided into two subgroups of RGNNV and BFNNV (Fig. 11C). And viruses identified from the oyster sample from Gosung in March 2013 and seawater samples from Geoje and Wando in April 2011 were in the BFNNV subgroup.

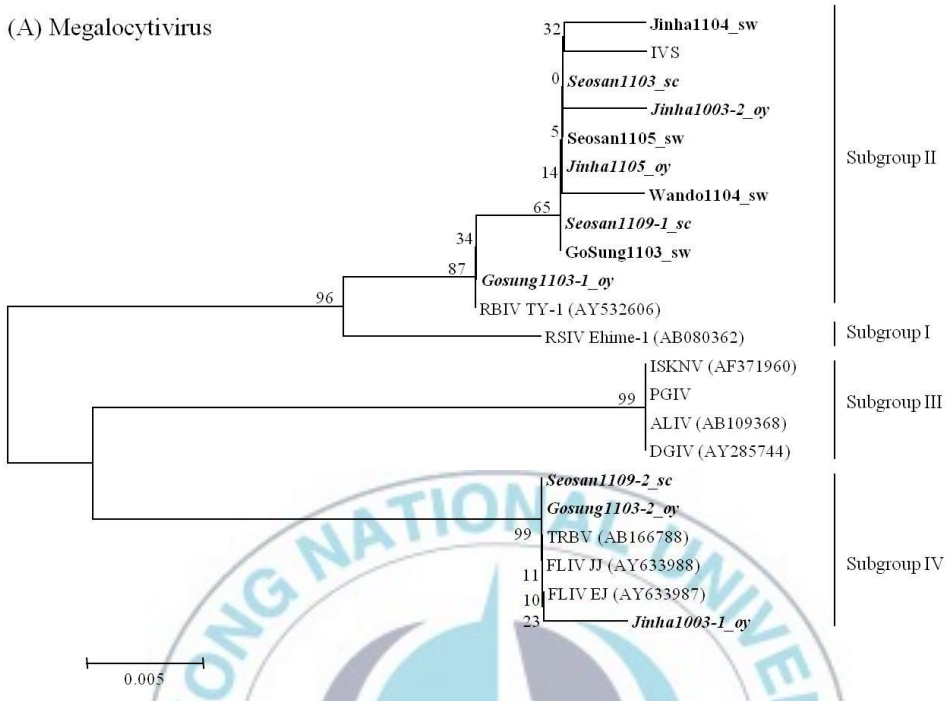

Fig. 11. Phylogenetic analysis of aquatic animal pathogenic viruses identified from seawater and shellfish specimens in the coast of Korea. (A), megaltocytivirus based on major capsid protein (MCP) gene; (B), VHSV based on glycoprotein (G) gene; (C), VNNV based on RNA 2 segment gene. The tree was constructed by neighbor-joining method in the MEGA program (Ver. 5.05). Isolates from seawater and shellfish are highlighted in bold italic and bold Roman fonts, respectively. Abbreviations : mu, mussel (*Mytilus edulis*); oy, oyster (*Crassostrea gigas*); sc, short-necked clam (*Tapes philippinarum*); sw, seawater.

(B) VHSV

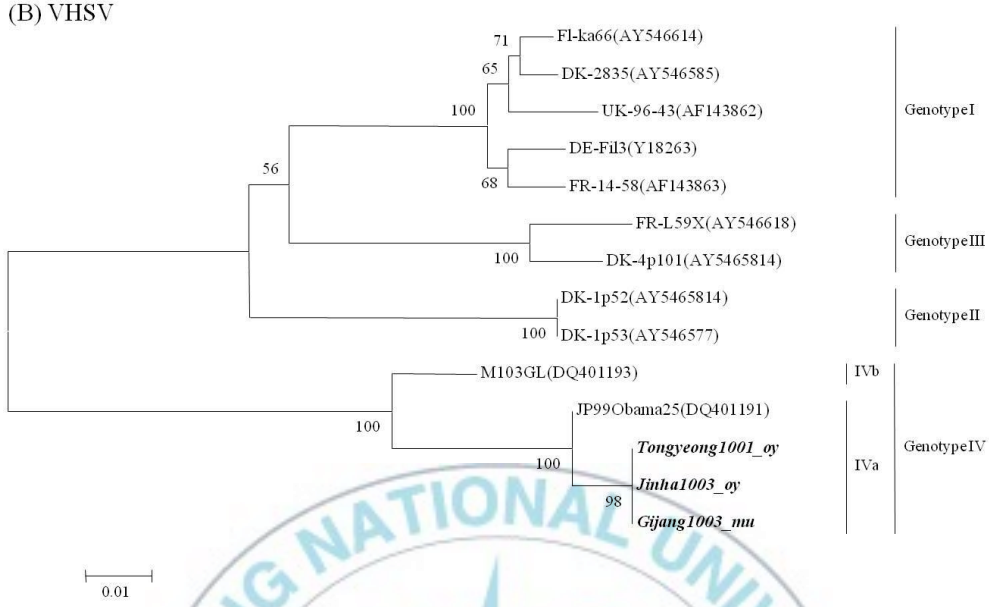

Fig. 11. Continued.

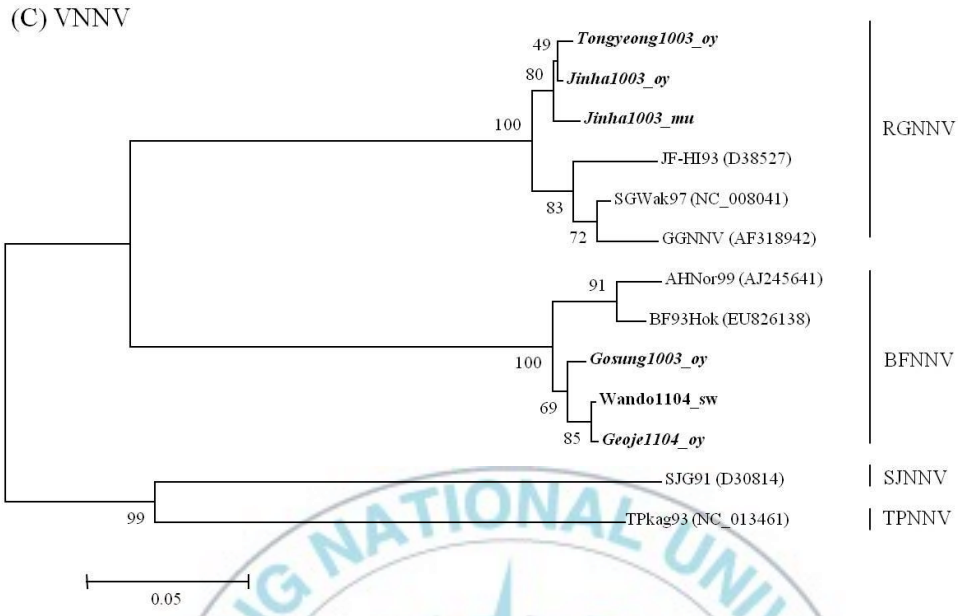

Fig. 11. Continued.

IV. Discussion

Surveillance of aquatic animal pathogenic viruses in hosts, ambient seawater, and shellfish is important to control and prevent aquatic animal disease. A variety of aquatic animal viral diseases are endemic in Korea (Cho et al., 2010; Do et al., 2005b; Gomez et al., 2008; Jeong et al., 2003; Jung et al., 2008; Kim et al., 2009a). But, it is unclear how these viruses are distributed in seawater and shellfish. Thus, we detected megalocytivirus, MABV, VHSV, VNNV, and WSSV from seawater and shellfish using multiplex nested PCR.

Multiplex PCR commonly enables the simultaneous detection of several pathogens in the same reaction tube and is very useful for mixed pathogens or contaminated samples. But, its sensitivity is poorer than that of single PCR or nested PCR (Khawsak et al., 2008). To overcome the drawback of multiplex PCR, multiplex nested PCR is used in common. To identify viruses from seawater and shellfish containing a low copy number, primers for the multiplex nested PCR were designed in the conserved region of each virus. The primers were grouped for DNA pathogens (megalocytivirus and WSSV) and RNA pathogens (VHSV, VNNV, and MABV) due to the cDNA synthesis step from RNA.

Two or three virus spiked samples were slightly less sensitive than one virus spiked sample in the range of 1–10 viral particles. But, that of amount viral particles are considered as error range in nested PCR in common. A previous

study showed that the detection limit of multiplex nested PCR assay is $1–10^2$ copies of respiratory viruses (Lam et al., 2007). In addition, the quantity of megalocytivirus is about 10^2 viral particles in digestive gland tissue of the pacific oyster (*Crassostrea gigas*). These results support multiplex nested PCR assay as an effective detection method for low copy numbers of viruses in seawater and shellfish.

Previous studies reported IPNV (Mortensen et al., 1992), MABV (Inaba et al., 2009), megalocytivirus (Kim et al., 2012), norovirus (Atmar et al., 1995; Le Guyader et al., 2006), and WSSV (Vazquez-Boucard et al., 2010) in shellfish. Moreover, a marine mollusk was suspected to be the wild aquatic animal carrier of WSSV (Manual of Diagnostic Tests for Aquatic Animals in World Organization for Animal Health; OIE). Although the viruses identified in seawater and shellfish were not significantly different due to variations in individual specimens except VNNV, which identified from shellfish between eastern and western sea areas ($P < 0.05$), it is worthwhile to detect various aquatic animal viruses in shellfish regardless of the sampling site and time in Korea. In addition, two or more viruses co-existed in some shellfish samples compared to seawater samples. These results reveal that shellfish might accumulate several pathogens over the long-term. Thus, shellfish may act as vector or reservoir of aquatic animal viral diseases.

The phylogenetic analysis revealed that megalocytiviruses in seawater and shellfish were categorized into two subtypes (II and IV), subtype II including the IVS-1 (Jeong et al., 2003) and RBIV-TY-1 strains (Do et al., 2005b) and subtype IV including FLIV (Do et al., 2005a) known as the main strains found in epizootic aquatic farms in Korea. Of note, two subtypes, II and IV, co-existed in one shellfish specimen. This result suggests that shellfish can co-accumulate several pathogens over the long-term. The VHSV in shellfish belonged to VHSV subtype IVa, which is commonly detected from VHSV-infected flounder in Korea (Kim et al., 2009b). Genetic analysis between viruses derived from fish, seawater, and shellfish indicated that the viruses released into seawater from infected hosts were captured by the shellfish. Interestingly, the barfin flounder nervous necrosis virus (BFNNV), an unknown virus in Korea, was identified in shellfish. This result indicates that a novel subtype of VNNV has been introduced to Korea.

We conclude that multiplex nested PCR was a suitable assay to simultaneously detect various viruses from seawater and shellfish. A variety of aquatic animal viruses were identified in seawater and shellfish regardless of sampling site and time. Shellfish might retain several viral agents for the long-term and play a role as a vector or reservoir of aquatic animal viruses. Additionally, the viral subtypes identified in shellfish and seawater were clustered with endemic subtypes in Korea. Further studies will assess the potential of shellfish as reservoir or vector of aquatic animal viruses.

Chapter III. Evaluation of the potential of bivalve mollusk as transmitter of V*iral hemorrhagic septicemia* Virus (VHSV)

I. Introduction

The spread of fish pathogenic viruses depends on several factors such as susceptible hosts, seawater current and potential reservoir hosts and vectors. After the occurrence of a viral disease on an aquaculture farm, release of virus into the seawater from infected fish should be suspected. Lethal fish pathogenic viruses spread into seawater widely and could re-infect other susceptible species or accumulate in filter-feeding organisms. Bivalve mollusks growing in coastal areas may be contaminated by various pathogens such as human enteric virus, infectious pancreatic necrosis virus (IPNV), marine birnavirus (MABV), and white spot syndrome virus (WSSV) (Atmar et al., 1993; Atmar et al., 1995; Mortensen et al., 1992; Suzuki and Nojima, 1999; Vazquez-Boucard et al., 2010). Due to their filter-feeding nature, bivalves trap pathogenic agents and remain in an infective state if the pathogenic agent is not sufficiently inactivated. 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 diseases and is listed as a remarkable disease by the World Organization for Animal Health (OIE). VHSV produces annual outbreaks in farmed flounder in Korea and high mortality rates (about 60%) have been reported in cultured juvenile and adult flounder. However, VHSV transmission via a vector or viral indicator species is unknown.

The 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 vector or reservoir for fish pathogenic viruses. Thus, an understanding of how fish pathogenic 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 VHSV-exposed bivalve mollusks are in an infectious or non-infectious state. We also carried out a viral survivability test with the digestive enzymes of blue mussel, an artificial viral accumulation and depuration test in mussel, and an infectivity test of VHSV in field samples via *in vitro* and *in vivo* inoculation.

II. Materials and methods

1. Bivalve mollusk samples

Twenty-four oyster (*C. gigas*) and 12 blue mussel (*M. edulis)* samples were collected from the Southeastern Sea of Korea (Gijang, Jinha, Tongyeong) between November 2009 and March 2011. The live bivalves were directly transported to the laboratory after sampling, aseptically opened using a knife, and the digestive gland was separated using a scalpel. The digestive gland tissues of individuals were stored at -80℃ before the experiment.

2. Viral propagation

Chinook salmon embryo (CHSE-214) cells were used as propagate VHSV. The cell line was grown at 20℃ in minimum essential medium (MEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% antibiotic and antimycotic solution (Gibco). The virus used was originally isolated from farmed flounder (10 ± 5g) infected with VHSV subtype IVa in Jinha in February 2008. The kidneys were homogenized, filtered (0.45 μm pore size), and inoculated (100 μl) onto CHSE-214 cells cultured in T75 cm^2 culture flasks (Corning, Corning, NY, USA). Following

development of viral cytopathic effects (CPE) in 5–7 days, virus infected cells were centrifuged at 1,500 × g for 10 min and supernatants containing VHSV were stored at -80℃.

3. Detection of VHSV from filed samples

Total RNA (50 µl) was extracted from digestive gland (50 mg) using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. cDNA was prepared from an RNA (1 µl) mixed random primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). To detect VHSV from the bivalve mollusks, primer sets for nested-step reverse transcription-polymerase chain reaction (RT-nested PCR), real-time PCR, and 6-carboxyfluorescein (FAM)-5′ labeled probes (Integrated DNA Technologies, Coralville, IA, USA) were designed from the conserved region of the glycoprotein gene of VHSV subtype IVa.

3-1. RT-Nested PCR

RT-nested PCR assay was performed in 20 µl reaction mixtures with 1 µl of cDNA, 10 pM of each primer (SF1: 5′-CACAGATCACTCAACGACC-3′/ SR1: 5′-GTGATCATGTGTCCTGGTG-3′ for first-step PCR; SF2: 5′-

GACTGGGACACTCCACTGTA-3′/SR2: 5′-CAAACCCCCTCTATGAAGTC-3′ for nested-step PCR), 10× PCR buffer, 2 μl of 200 μM dNTP, and Taq DNA polymerase (Cosmo Genetech, Seoul, Korea). The cycling conditions for the first and nested-step PCR were a pre-denaturation step at 95℃ for 3 min, 35 cycles of denaturation at 94℃ for 40 s, annealing at 52℃ for 40 s, extension at 72℃ for 40 s, followed by a final extension step at 72℃ for 7 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

3-2. qPCR

Viruses were quantified using LightCycler 480 II instrument (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The qPCR reaction mixture contained 1 μl of cDNA, each primer (VqF, TTT CTT GGT GAT TCT GAT CAT CA and VqR, CCG AAT CGG AAC AAA GGA G) at a concentration of 500 nM, 200 nM of probe (Vq-probe, FAM-ACT CAA CGA CCT CCG GTC GAG A-IBFQ), and the Light Cycler 480 Probe Master mixture (Roche). The amplification conditions were 95℃ for 10 min, followed by 40 cycles of 94℃ for 10 s, 60℃ for 15 s, and 72℃ for 20 s. A recombinant plasmid containing 157 bp from the glycoprotein gene and amplified using VqF and VqR was purified from the transformed *Escherichia coli* DH5a strain as a positive control. A serial 10-fold dilution of the control plasmid was used to establish a

standard curve (5.0E + 05 copies/μl to 5.0E +00 copies/ μl). The standard curve, generated using the mean data from experiments performed in triplicate, indicated a good linear relationship between the *C*T values. All samples used were tested in duplicate, and all PCR reactions were carried out twice.

4. Survivability of VHSV in mussel digestive enzyme

To determine the survivability of VHSV in mussel, the digestive enzymes were extracted from mussel using a method modified from Areekijseree et al. (2004). Digestive glands, which were pooled from five mussels, were homogenized on ice with 0.1 M phosphate-buffered saline (pH 7.3) at a 1:10 (w/v) dilution. The digestive gland homogenate was centrifuged at 5000 × *g* for 5 min and the upper lid was discarded, and the supernatant was collected. To minimize the inhibitors in the digestive gland, the supernatant was filtered though a membrane (0.45 μm pore size). The resulting supernatant was used as the digestive enzymes for virus digestion. The digestive enzymes and cultured VHSV (10^8 $TCID_{50}$/ml) were mixed 1:1 (v/v), and the digestive rate was analyzed for 1 and 24 h at 25℃. MEM supplemented with 10% FBS and the cultured VHSV mixture were used as the control. The viral titer was evaluated by $TCID_{50}$. The inhibition rate of digestive enzymes and the natural decline of the viral titer in MEM medium were considered to calculate the digestive rate. The formulas for calculating digestive

rate are as follows:

Inhibition rate of digestive enzymes (%) = [1 – (initial virus $TCID_{50}$ on digestive gland/initial virus $TCID_{50}$ on MEM) × 100

Expected virus $TCID_{50}$ = natural decline viral titer on MEM × inhibition rate of digestive enzymes

Digestive rate (%) = [1 – (virus $TCID_{50}$ after the digestion period/expected virus $TCID_{50}$)] × 100.

5. Cohabitation of mussel with VHSV-infected flounder

5-1. Viral shedding experiment

Total ten flounders (*Paralichthys olivaceus*; 12.0±1.0 g) were acclimatized for 7days before intraperitoneal (I.P.) injection of VHSV. Each fish was injected with 100ul inoculums of VHSV, 10^6 viral particles/fish. Cultured water samples (10ml) were collected 5 days post injection. Cultured water and Kidney of dead or survived flounder were tested for VHSV by qPCR. Viral shedding estimated titers were determined by quantity of VHSV from 1 kg of fish in 1hr.

5-2. Bio-accumulation of mussel via cohabitating with VHSV-infected flounder

Mussels and flounder (*Paralichthys olivaceus*; 12.0 ± 1.0 g) were maintained for 2 weeks at 15℃ in a 40-L tank and confirmed to be virus-free by qPCR. Fifteen flounder were intraperitoneally injected with 0.1 ml VHSV diluted to 10^6 viral particles in MEM. Twenty mussels were co-habitated with the VHSV-infected flounder on day 3 post challenge. The mussels were sampled on days 1, 3, 5, 7, and 9 post cohabitated with VHSV-infected flounder. All flounder kidneys and mussel digestive gland tissues were tested for VHSV by qPCR.

6. Viral depuration from mussels after artificial contamination of VHSV

VHSV-inoculated seawater (10^6 viral particles/ml) was used for the depuration experiment. Mussels were immersed in a 10-L VHSV inoculated tank for 6 h at 12 ± 0.5℃ to produce VHSV contaminated mussels (VHSV-Mu). VHSV-Mus were transferred to a new 5-L water bath for 7 days at 12 ± 0.5℃. The digestive gland of VHSV-Mu was sampled at 12, 24, 72, and 96 h and tested for VHSV by qPCR. Sampling was conducted before changing the seawater, and the seawater was exchanged completely every day during the depuration period.

7. Infectivity of VHSV in field bivalve mollusk via *in vitro* and *in vivo* inoculation

Digestive gland (5 mg) from VHSV-positive field samples was homogenized, centrifuged (8,000 × 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 spiked with VHSV and cultured VHSV were used as controls. The samples were inoculated in duplicate with 100 μl of a 10-fold dilution series on 6-well tissue culture plates with 70% confluent CHSE-214 cells. The plates were incubated at 20℃ and observed for 7 days. Five flounder (12.0 ± 1.0 g) were intraperitoneally injected with each inoculum (0.1 ml) from field samples for the *in vivo* challenge experiment. The injected flounder were observed for clinical signs and mortality for 14 days.

III. Results

1. Identification and quantity analysis of VHSV

Of the 36 bivalve mollusks (24 oysters and 12 mussels), three samples (one mussel and two oysters) were determined to have VHSV by RT-nested PCR. Two samples, a mussel at Gijang in March 2010 (GiJ1003Mu) and an oyster at Tongyeong in January 2010 (TY1001OY), were identified to have the virus by qPCR. The abundance of VHSV in VHSV-positive field samples was 1.16×10^2 and 6.85×10 viral particles/mg digestive gland, respectively. From the phylogenetic analysis of glycoprotein gene showed all VHSV isolated from bivalve mollusk in Korea belonged to VHS IVa genotype (data not shown). The sequence homology of isolates was 91.50% compared with JP99Obama25 (Genbank accession number, DQ401191).

2. Survivability of VHSV particles in mussel digestive enzyme

The initial viral titers of MEM and digestive enzyme of mussel were $5 \times 10^{7.7}$ $TCID_{50}$/ml and $5 \times 10^{7.16}$ $TCID_{50}$/ml, respectively (Fig. 12). Thus, the inhibition rate of digestive enzyme on viral infection was 28.84% by the inhibition rate formula. The natural decline in VHSV titers in MEM was $5 \times 10^{7.7}$ $TCID_{50}$/ml

and $5 \times 10^{6.83}TCID_{50}$/ml after 1 and 24 h at 25℃, respectively. The viral titers of the digestive enzymes mixed with VHSV were reduced from $5 \times 10^{6.83}TCID_{50}$/ml (after a 1 h incubation) to $5 \times 10^{5.83}TCID_{50}$/ml (after a 24 h incubation) at 25℃. Therefore, VHSV was digested in 53.23% and 65.33% after 1 and 24 h, respectively based on the digestive rate formula.

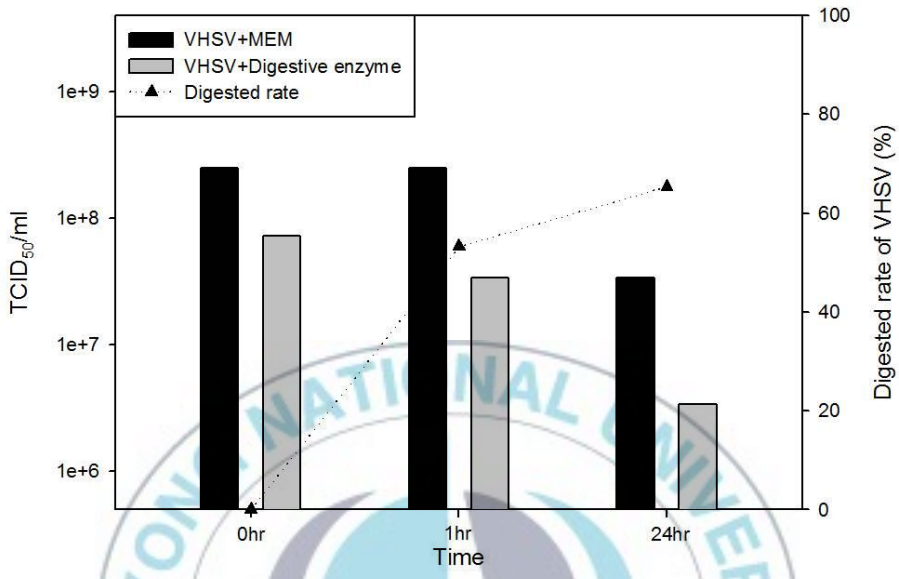

Fig. 12. Digestive rate of VHSV by mussel digestive enzyme. The natural declined viral titer ($TCID_{50}$/ml on CHSE-214 cell line) by viral stability is shown in the black bars. The viral titer in digestive enzyme mixture group is shown in the grey bars. The digestive rate of VHSV by digestive enzyme of mussel is shown in triangles.

3. Cohabitation of mussel with VHSV-infected flounder

3-1. Viral shedding estimated titer from VHSV infected flounder

Infection with VHSV (10^5 viral particles/fish) led to 70% cumulative mortality within 12 days post challenge in flounder. Dead flounders generally displayed symptoms of VHSV infection (congested liver and abdominal distension with ascities). Viral shedding titers peaked at $4.16x10^8$ viral particles/kg/hr in 7 days post injection, in 60% of cumulative mortality (Fig. 13). And titers decreased to $6.93x10^7$ viral particles/kg/hr in 11 days post injection, in 70% of cumulative mortality (not more any fish dead).

3-2. Bio-accumulation of mussel via cohabitating with VHSV-infected flounder

Although infection with VHSV of flounders (10^5 viral particles/fish, led to 70% cumulative mortality within 11 days), VHSV was not identified from digestive gland in cohabitating mussel. Thus, mussel could not accumulate VHSV derived from cohabitate with VHSV-infected flounder. Viral titers in cultured water from VHSV infected flounder (approximately 10^2 viral particles/g/hr/ml) might be low to accumulate into mussel. Or VHSV particles in digestive gland might be below level at the detection limit in qPCR.

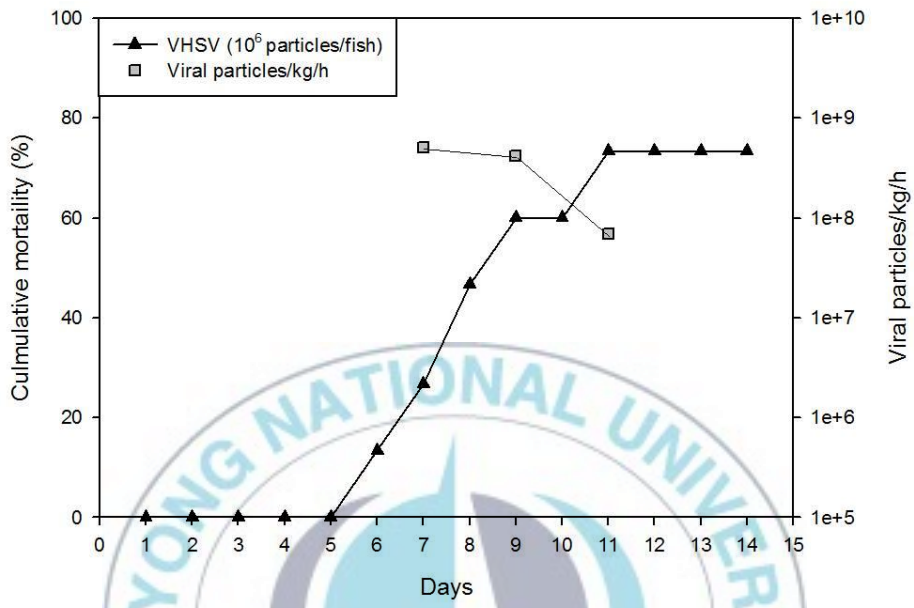

Fig. 13. Viral shedding estimated titer from VHSV infected flounder in relation to cumulative mortality (%). Triangle are shown in cumulative mortality of VHSV infected flounder and Square are shown in viral shedding titer (viral particle/kg/h), respectively.

4. Viral depuration of mussel after artificial contamination

During artificial bio-accumulation of VHSV, viral concentration in the digestive gland of VHSV-Mu increased after 6 h (1.61×10^3 viral particles/mg) (Fig. 14). The viral concentration in digestive gland of the VHSV-Mu decreased within 12 h (9.7 × 10 viral particles/mg) during depuration processing. However, VHSV particles were not totally eliminated from the digestive gland tissue within 168 h (2.42 × 10 viral particles/mg). And the amount of virus in digestive gland was not significant difference ($P<0.05$) between bio-accumulation and depuration processing due to variation of individual specimens.

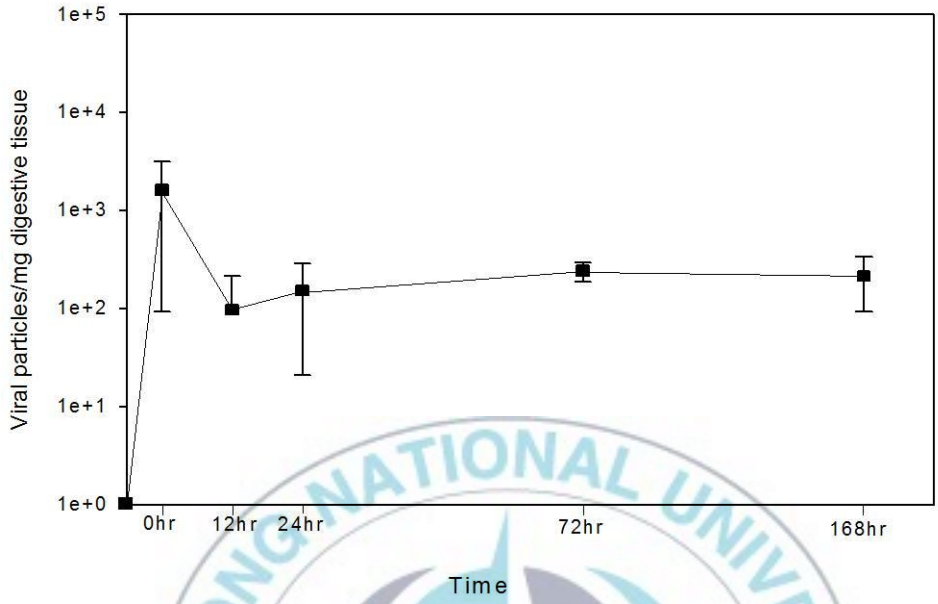

Fig. 14. Quantitative pattern of VHSV in mussel during depuration. Black square are shown in viral particles/ mg of digestive gland in mussel.

5. Infectivity of VHSV in field bivalve mollusk via *in vitro* and *in vivo* inoculation

Two VHSV-positive field samples, GiJ1003Mu and TY1001Y, were inoculated into the CHSE-214 cell line with a 10-fold serial dilution: TY1001OY (3.4×10^1 viral particles to 3.4 viral particles/well), GiJ1003Mu (5.80×10^2 viral particles to 5.8 viral particles/well) (Table 10). 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 were observed in the VHSV-inoculated digestive gland homogenate. No VHSV symptoms were observed in any of the surviving flounder during the 14 days of the *in vivo* experiment (Fig. 15). Moreover, VHSV could not be identified from two field samples injected flounders by qPCR and not be isolated on the CHSE-214 cell.

Table 10. Infectivity of VHSV derived from digestive gland of bivalve via *in vitro* inoculation

Samples[a]	$5x10^2$	10^2	$5x10^1$	10^1	$5x10^0$	10^0
TY1001OY[b]	nd[d]	nd	-	nd	-	nd
GiJ1003Mu[c]	-	nd	-	nd	-	nd
Digestive gland spiked with cultured VHSV	nd	+	-	nd	-	-
Cultured VHSV	nd	++	++	nd	+	+

[a]added viral particles/well;

[b]viral concentration in digestive gland/well: 3.43E+01 to 3.43E-01 particles;

[c]viral concnetration in digestive gland/well: 5.80E+02 to 5.8E+00 particles;

[d]nd, not done

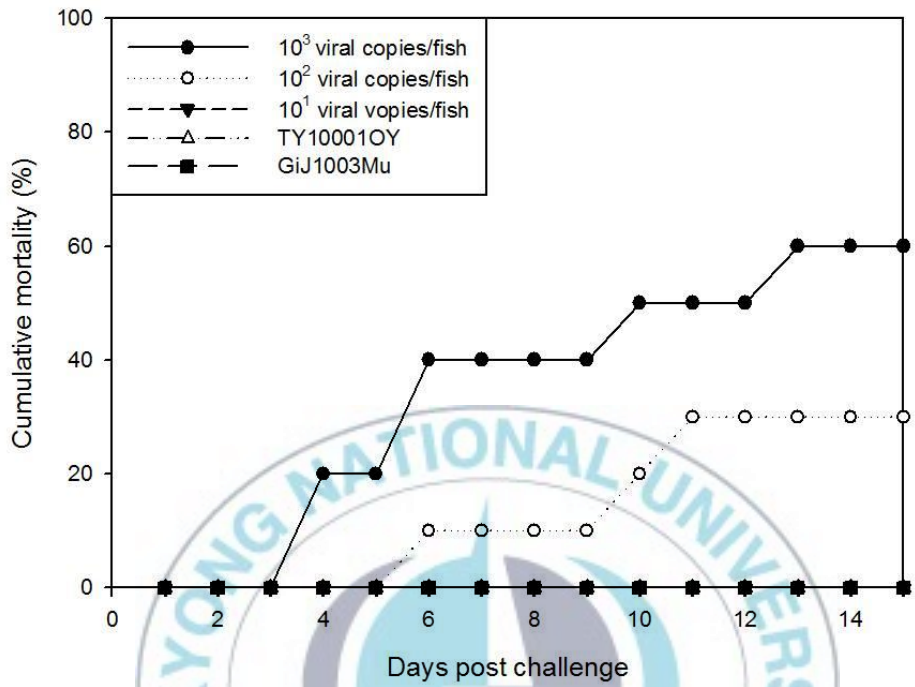

Fig. 15. Cumulative mortality of VHSV derived from field bivalve mollusk. Injection with cultured VHSV used as positive control group. Injection with cultured VHSV used as positive control group. Injection amount of TY100OY was 3.40E+01 viral copies/fish and of GiJ1003Mu was 5.80E+02/fish.

IV. Discussion

Little is known about the role of bivalve mollusks as a vector or reservoir for aquatic animal viruses, although they are a well known reservoir for human enteric viruses due to their filter-feeding activity (Atmar et al., 1993; Atmar et al., 1995). Similar to human enteric viruses, bivalve mollusk might be either vector or reservoir of aquatic animal viruses by their filter-feeding activity.

The Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus edulis*) are widely distributed in the vicinity of aquaculture farms in Korea. In the present study, we identified VHSV from digestive gland of the Pacific oysters and blue mussels. Previous studies have shown that a variety of virus particles can be identified in the digestive gland, gill, and mantle of bivalve mollusks (Atmar et al., 1995; Mortensen et al., 1992; Suzuki and Nojima, 1999; Vazquez-Boucard et al., 2010). 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, several viruses may bind the digestive tract of bivalve mollusks. The phylogenetic analysis revealed that the VHSV from bivalve mollusks belonged to VHSV subtype IVa, which is commonly detected in VHSV-infected flounder in Korea indicating that VHSV released into seawater from infected flounder was captured

by the bivalve mollusks.

General PCR results could not distinguish between infectious or non-infectious state of the virus. Previous studies have shown that VHSV particles are infectious at 4℃ in freshwater for 1 year (Hawley and Garver, 2008), and organic materials such as ovarian fluids or blood products enhance stability of the virus (Kocan et al., 2001). Viral stability (natural reduction titer in MEM media) and activity of bivalve mollusk digestive enzymes have been considered as VHSV survivability in the mussel digestive gland (Koehn and Siebenaller, 1981; Supannapong et al., 2008). Although we found that VHSV particles were digested in 65.33% for 24 h, the viral particles maintained an infectious state at 25℃ (Fig. 12). This result indicates that viral particles in the bivalve mollusk digestive tract are not totally inactivated by digestive enzymes in within 24 h.

Two experiments were conducted to assess experimental viral accumulation and release from the bivalves. In the experiment 1, mussel cohabitating with VHSV-infected flounder have not detectible VHSV particles. Previous study showed that ISA shedding was identified before inoculated-fish dead and rose to a peak during highest mortalities (Gregory et al., 2009). Although viral titers releasing from VHSV-infected flounder was $4.1x10^{8}$ viral particles/kg/h in 60% of mortality (Fig. 13), viral titers in cultured water (approximately 10^{2} viral particles/g/hr/ml) might be low to accumulate into mussel. The experiment 1 results suggested that a high dose of virus in the water or a longer exposure time was needed for VHSV

particles to accumulate in mussel. Alternatively, the mussel may have digested the VHSV particles below the detection limit of molecular based methods. In experiment 2, VHSV-Mus had 1.61×10^3 viral particles/mg digestive gland even though viral exposure time was only 6 h with a high dose of virus (10^6 viral particles/ml seawater). VHSV abundance was maintained at more than 10 viral particles 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 at 4 d after depuration, due to digestion or inactivation of the virus. In contrast, norovirus in oyster digestive gland was not totally eliminated and was detectable even after 7 d of artificial immersion in norovirus contaminated feces (Ueki et al., 2007). These results indicate that VHSV may have accumulated in bivalve mollusks for a longer time similar to the norovirus.

Viral isolation from bivalve mollusk tissue based on a cell culture system was difficult due to the severe cell cytotoxicity of the digestive gland. Therefore, we conducted viral isolation using serial diluted digestive gland homogenates from VHSV-positive field samples and VHSV-inoculated samples. Although artificially VHSV-inoculated digestive gland homogenate developed CPEs to 10^2 viral particles, VHSV-positive field samples did not. A previous study showed that ISAV particles in mussel digestive gland tissue do not replicate in CHSE-214

cells (Molly et al., 2012). Similar to that study, VHSV-positive samples may have similar or a below the sensitivity of cell infective concentration. Alternatively, those samples may have had inactivated VHSV particles that could only be detected by PCR based method. In addition, VHSV-positive field sample inoculated flounder did not show any VHSV clinical signs or mortality. Interestingly, the minimum infectious dose of VHSV in flounder was 10^2 viral particles/fish by intraperitoneal injection and 10^4 viral particles/ml by the immersion method, respectively (unpublished data), suggesting that the quantity of VHSV in field samples was insufficient to infect or that those samples might be in a non-infectious state.

In summary, we identified VHSV in bivalve mollusks. The VHSV particles were not totally digested within a short time (24 h) and were maintained for more than 7 d in the blue mussel. Viral particles might be in a non-infectious state in the VHSV-field positive samples. However, we could not completely rule out the possibility of a risk for VHSV transmission through bivalve mollusks. Therefore, investigations of virus in bivalve mollusks before or during an ongoing VHSV outbreak in aqua-farms should be conducted.

SURMARRY

After viral disease occur on aquaculture farms, viruses released into the seawater from viral-infected aquatic animals can re-infected other susceptible hosts or retained in vector or reservoir species. Filter-feeding organisms can accumulate various substances including viral agents such as human enteric virus and aquatic animal viruses. Additionally, the viruses are generally present in the shellfish and the ambient water. Therefore, surveillance of aquatic animal viruses in susceptible hosts, shellfish, and seawater is important for adequately assessing prevention measure of aquatic animal disease.

Since shellfish mollusks and environmental water normally have low viral titers, a series of complicated concentration steps for the detection of viruses is used. Comparison of tissue volumes for viral detection revealed that small volumes (50 mg) of digestive gland can be enough to use in qualitative and quantitative analysis. In addition, that of approach has the advantage of being able to conduct individual analysis compared to PEG treatment. And the filtration method using GF/C with HA (nitrocellulose, negatively charged membrane) membranes allows for the detection of aquatic animal viruses in seawater effectively.

Primer sets for multiplex nested PCR were developed to simultaneously detect aquatic animal viruses (megalocytivirus and WSSV as DNA viruses, and VHSV, VNNV and MABV as RNA viruses) from shellfish and seawater. The multiplex

nested PCR amplified each virus specifically with a detection limit of about 10 viral copies, and facilitated detection of several fish pathogenic viruses from shellfish and seawater. The megalocytivirus was the most prevalent virus in shellfish (n=95; 38.16%) and seawater (n=25; 41.7%) specimens. The detection rate of viruses were not significantly different regardless of sampling site and time except VNNV, which identified in shellfish between eastern and western sea areas (P<0.05). And several viruses were co-existed in shellfish regard less of sampling site and time. These results revealed that shellfish might be accumulated several viruses for long-term time and could play roles as reservoir of viruses derived from aquatic animals. Additionally, the phylogenetic clusters of viruses derived from fish, shellfish, and seawater revealed that viruses released into seawater from infected host were captured by the shellfish.

From surveillance of aquatic animal virus in shellfish and seawater, viral haemorrhagic septicaemia virus (VHSV) subtype IVa was identified in digestive gland of bivalve mollusk and seawater. To evaluate the potential of bivalve mollusk as transmitter of VHSV, viral survivability in mussel digestive enzyme, viral depuration, and infectivity test via *in vitro* and *in vivo* inoculation were carried out. The viral particles were not completely digested within 24h and were maintained 7d in digestive gland of mussel. But, virus derived from field samples could not be isolated in CHSE-214 cells and did not replicate in flounder. These findings revealed that VHSV identified in bivalve mollusks might be a non-

infectious state.

Although VHSV derived from shellfish was present in a non-infectious state, the possibility of the viral transmission through bivalve mollusks should not be ruled out owing to the presence of various viruses. Furthermore, the investigations of viruses in bivalve mollusks before or during an ongoing disease outbreak in the aqua-farms should be carried out.

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