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Ostreid herpesvirus 1 infection in farmed Pacific oyster larvae *Crassostrea gigas* (Thunberg) in Korea

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Oyster Herpesvirus 1 (OsHV-1) infections cause mortality in the larvae and the juveniles of several bivalve species, including Pacific oyster *Crassostrea gigas*(Thunberg), European flat oyster *Ostrea edulis*(Linnaeus), the grooved carpet shell *Ruditapes decussates*(Linnaeus), Manila clam *Ruditapes philippinarum*(Adams & Reeve) and French scallops *Pecten maximus*(Linnaeus) (Arzul, Renault *et al.* 2001; Da Silva, Renault *et al.* 2008; Segarra, Pépin *et al.* 2010). In particular, a new variant called Ostreid herpesvirus1(OsHV-1 μ Var), characterized mainly by partial sequence data exhibiting a deletion of 12 base pairs in the ORF 4 genome, has caused serious diseases for the oyster aquaculture industry, especially in Europe(Segarra, Pépin *et al.* 2010; Renault, Moreau *et al.* 2012). No association has been found between mortality and host-related factors, except age, where the presence of high viral loads can explain the mortality in spat and in juveniles, but not in adults(Batista, Arzul *et al.* 2007).

The pacific oyster, *C. gigas*, is a very important aquaculture species in Korea. It produces a total output of 647,670 tons worldwide and 240,911 tons in Korea, accounting for 37.2 % of worldwide production (2009, FAO). Pacific oyster farming takes place throughout 8,077 ha within Korean territories, and there are about 40 artificial hatcheries in operation.

Mortality of adult oysters has been reported in South Korea over decades, and some researcher have described this as being due to the alterations in the environment, such as that of water temperature and salinity(Choi, Jung *et al.* 1999). Recently, there a mass mortality incident occurred in an artificial hatchery at the larval stage, and for no identifiable reason. However, monitoring of the herpes virus in pacific oysters has not been conducted before. Therefore, we conducted a monitoring analysis of the herpes virus disease by polymerase chain reaction (PCR) and transmission electron microscopy, for the first time in 2011, and across four oyster hatcheries.

Pacific oyster larvae were collected monthly from January to March 2011 from four hatcheries located in Toongyoung and Gaejae, sample area in Korea (Fig.1). Larvae were collected using a plankton net of mesh size 20 μ m and the larvae were washed three times with sterile-filtered sea water and were resuspended in sterile water for molecular testing and TEM.

Pools of larvae were crushed with a homogenizer (Sigma), and DNA from the oyster larvae was extracted with a high-fidelity PCR template preparation kit (Roche) following the manufacturer's instructions. PCR analyses were performed with OsHV-1-C1/ OsHV-1-C6 primers (5'-TTCCCCTCGAGGTAGCTTTT-3'/5'-GTGCACGGCTTACCATTTTT-3') (Batista, Arzul *et al.* 2007) and Del 36-37F2/Del 36-37R (5'-ATACGATGCGTCGGTAGAGC-3'/5'-CGAGAACCCCATTCCTGTAA-3') (Renault, Moreau *et al.* 2012).

Quantitative real-time PCR was performed using the protocol of Pépin *et al.* 2008. Amplifications were performed using a Mx3000P real-time PCR thermocycler sequence detector (Stratagene), according to the following conditions: initial denaturation for 1min at 95°C, followed by 40 cycles of 15s at 95°C and 30s at 60°C. All samples were analyzed in triplicate.

Specimen preparation for electron microscopy was carried out basically as described in Drury and Wallington (1980). The samples were observed using the transmission electron microscope (JEM-1200EXII, JEOL).

From the pacific oyster larvae collected monthly from January to March 2011 from four hatcheries, there were positive results every month in station 1 and in February in station 2. In January, there was one positive result from station 1. In February, there were two positive

results from station 1 and station 2. In March, there was one positive result from station 1. Herpes virus was not detected in stations 3 and 4 during any month (Fig.1)

For station1, there were 6.18×10^2 Nb of copies/reaction in January, 1.19×10^5 Nb of copies/reaction in February, and 7.96×10^5 Nb of copies/reaction in March. For station 2, there was only one positive result in the February samples $2,33 \times 10^2$ Nb of copies/reaction. Specific PCR products corresponding to the 896 bp fragment were detected by the C1/C6 primer set and 2 sequences were obtained from station 1 in January, which were registered in GenBank (Accession No JQ959597, No JQ959598). Obtained sequences showed 99 % identity with OsHV-1 μ var (Accession number: HQ842610), 97% identity with Chlamys acute necrobiotic virus (Accession number: GQ153938), and 96 % identity with OsHV-1 (Accession number: AY509253).

PCR analyses were performed with Del 36-37F2/Del 36-37R by electrophoresis, and amplified one pattern and sequence showed 99% identity with OsHV-1 from New Zealand(JN800252).

Infected digestive gland cells in the oyster, *C. gigas* showed numerous autophagosomes, with cell organelles and heterophagosomes with capsids and nucleocapsids. Cytoskeletons of cells indicated necrosis. (Fig. 2A). The fibroblastic cells of the oyster exhibited destroyed mitochondria, rough endoplasmic reticulum. Especially, nucleus was massive condensation with irregularity shape and abnormal marginated chromatin (Fig. 2B). The enveloped virions were circular in shape (approximately 280 nm in diameter) in infected *C. gigas* larvae. The capsids were approximately 100 nm in diameter. Capsids and nucleocapsids were scattered in the cytoplasm of infected cells (Fig. 2C).

For the first time, we report results from a screening conducted for the presence of the ostreid herpesvirus disease, that cause mass larvae mortality in Korean Pacific oyster larvae, which were collected monthly from January to March 2011 four hatcheries. There were positive results every month in station 1 and in February in station 2. Also, herpesvirus was detected in over 90% of mortality that occurred in pacific oysters during the monitoring period, especially in station 1.

Sequence analyses revealed that the virus detected in Korea is closer to OsHV-1 μ var than OsHV-1 or Chlamys virus. So far, OsHV-1 μ var has been detected in Europe, in Australia and in New Zeland (OIE 2011).

Also, PCR analyses was performed with Del 36-37F2/Del 36-37R, which amplified 3 different patterns as 989 bp, 384 bp, or no amplification (Renault, Moreau *et al.* 2012). The virus detected in Korea has a large deletion pattern (384bp), the same as the two strains detected in Japan and China. This means that the virus in Asias strains are similar, but not identical to OsHV-1 μ var detected in *C. gigas*.

Herpes-like virus infections in oyster are caused by viruses presenting morphological features, cellular locations and size rages characteristic of virions belonging to the Herpesviridae family. Renault *et al.* (2000) reported that the diameter of the enveloped virus particles was approximately 117 nm in *C. gigas* larvae. However, in this study, a diameter of the enveloped virions was bigger than that of *C. gigas*. In infected cells of *C. gigas*, abnormal endoplasmic reticulum was observed. Herpes-like virus infected Pacific oyster larvae often observed virus particles in hemocyte, fibroblastic-like cells and connective tissue, but in this study, they were only observed in the common epithelial cells. This difference may be due to the infection degree of the virus.

The original source of OsHV-1 μ var remains unknown. To investigate the dynamics of this virus in Korea, monitoring of the artificial hatchery, the wild oyster infection, food organisms, and the influent and effluent sea waters is required. Although adult bivalves are apparently less sensitive to such infections compared to the young stage, oysters require monitoring at all stages. Also, further molecular characterization is necessary in order to investigate the original source by sequencing the other part of the viral genome.

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Figures

Figure 1. Sampling area of the oyster, *Crassostrea gigas* in Toongyeong and Gaejae Korea

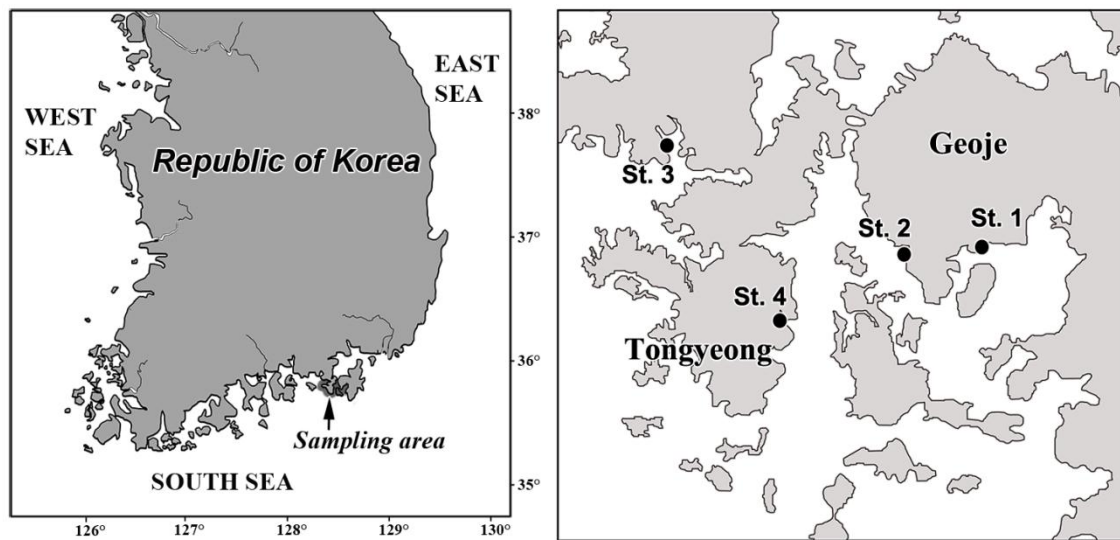


Figure 2. Transmission electron microscopes of the oyster, *Crassostrea gigas* larvae. (a) virus particles in the cytoplasm, note the containing empty capsids (white arrowhead) and enveloped virus particles (black arrowhead). (b) showing the degeneration of the mitochondria (black arrowhead), rough endoplasmic reticulum (white arrowhead) and nucleus (asterisk). (c) enveloped virus particles showing the viral envelope and brick-shaped capsid with high electron dense (arrowhead) and scattered capsids and nucleocapsids (asterisk).

