Short Paper

Isolation of Birnavirus from Japanese Pearl Oyster *Pinctada fucata*Satoru Suzuki,^{*1,*2,†} Maki Kamakura,^{*1} and Riichi Kusuda^{*1,††}^{*1}Department of Aquaculture, Kochi University, Nankoku, Kochi 783, Japan^{*2}Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783, Japan

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Since 1994, mass mortality of the Japanese pearl oyster *Pinctada fucata* has widely occurred in the southwestern part of Japan. The cause of mass mortality was investigated in terms of change of current, decrease in feed plankton, increase in seawater temperature, genetic factors, and water pollution. However, the cause of mortality has not been identified. Virus infection is thought to be one of the causes because it has been reported in the past that herpes virus-like particles¹⁾ and birnaviruses²⁾ were found in several bivalves. Pathogenicity of birnavirus against hard clam *Meretrix lusoria* has been reported.³⁾ The authors recently isolated the birnaviruses from Agemaki (jack knife clam) *Sinonovacula consticta* which originated in Ariake Bay, Saga Prefecture, Japan, as well as in an area on the Korean coast.⁴⁾ The strain AGJ-90 that was isolated from the Ariake Bay samples showed pathogenicity against Agemaki when the shells were exposed to physiological stress.⁴⁾ The birnavirus genome was detected in high ratios by PCR, suggesting that the birnavirus is widely distributed in Agemaki.⁴⁾ Therefore, we investigated the existence of viruses in Japanese pearl oyster. In this report, we describe the isolation and detection of birnavirus from Japanese pearl oyster.

Japanese pearl oyster samples were collected in the Uwajima and Uchiumi fishing grounds in Ehime Prefecture, Japan, in October 30, 1996. Three shells taken from the same group were pooled per sample and 6 different samples were employed for analysis. Mainly liver and genital organs were used for the experiment. In order to eliminate virus contamination from seawater or digestive systems, gill, mantle lobe and rectum were not included in a sample. Birnavirus genome was surveyed by using 2-step PCR targeting in the VP2/NS junction region of genome segment A. We previously established this method for detecting birnavirus genome.⁵⁾ The cDNA fragment obtained by nested-PCR was directly sequenced by the dideoxy-terminator method as previously described.⁴⁾ Virus isolation was performed with cultured CHSE-214 and RSBK-2 cells. Liver and genital organs were pooled and weighed; 9-fold volume of Hanks' balanced salt solution was added and homogenated with a stomacker. The homogenates were filtrated through a 220-nm pore filter and 25 and 50 μ l were inoculated to the cells cultured in a 24-well culture

plate. Incubation was performed at 20°C. Supernatant of CPE-appearing cultures was inoculated to newly cultured cells in order to confirm that the CPE was not a cytotoxic effect caused by substances contaminated from the shell organs.

The results of PCR detection and virus isolation are summarized in Table 1. In the 2-step PCR, 168 bp product was obtained in all samples employed for the nested-PCR, indicating that all samples contained birnavirus genome. This fragment was recovered from agarose gel in order to be sequenced. Figure 1, which does not include the primer length, shows a full presentation of the sequence of 134 bp. This was compared with other marine birnavirus (MABV) strains, specifically Y-6,⁶⁾ AGJ-90 and AGK-93.⁴⁾ The sequence of the PCR product shows high homology to the other MABV strains.

In virus isolation, CPE was observed in both cell lines 5 days after inoculation in 4 out of 6 samples (Table 1). In the case of Agemaki, the virus isolation ratio versus PCR positive number was low.⁴⁾ However, the results in this study indicate that the virus isolation ratio was high and suggests that virus replication possibly occurs in the shells. Virus isolation was successful even in non-symptomatic shells. All CPE-positive samples contained more than 10⁴ plaque forming unit/g organ. All isolates were thought to be the same virus, since they indicated the same properties. One of the strains was designated as MABV strain JPO-96. In the neutralization test of JPO-96, in which we used antiserum prepared against MABV strain Y-6,⁶⁾ the isolated virus was neutralized by the antiserum with a neutralizing

Table 1. PCR and virus isolation in Japanese pearl oyster*

	Positive number / tested number
Nested-PCR:	6 / 6
Virus isolation in:	
CHSE-214	4 / 6**
RSBK-2	4 / 6**

* Samples are from: Uwajima (#1, non-symptomatic; #2, symptomatic) and Uchiumi (#3, non-symptomatic; #4, symptomatic; #5, symptomatic; #6, non-symptomatic). Main symptom is a red colouring of mantle cavity and adductor muscle.

** Positives are: #1, 3, 4 and 5.

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strain						
JPO-96	GCGGCACCAC	TCATCGGAGC	CGCCGACCAA	TTCATCGGAG	ACCTGACCAA	50
Y-6						50
AGJ-90				G.		50
AGK-93						50
JPO-96	GACCAACGCA	GCCGGAGGCC	GCTACCTAAC	ACATGCAGCA	GGAGGACGCT	100
Y-6						100
AGJ-90						100
AGK-93					C	100
JPO-96	ACACTGACGT	AATGGACTCC	TGGGCTAGCG	GCAC		134
Y-6			C.....			134
AGJ-90			C.....			134
AGK-93			C.....			134

Fig. 1. Nucleotide sequences of the nested-PCR products obtained from the birnavirus strains. Sequence of JPO-96 is compared with strains Y-6 isolated from yellowtail *Seriola quinqueradiata*, AGJ-90 isolated from Agemaki *Sinonovacula constricta* in Ariake Bay, and AGK-93 isolated from Agemaki in Korea. Dots are the same nucleotides as JPO-96.

titer of 1,771. In the case of the combination of this antiserum with strain Y-6, the neutralizing titer was 40,960. These values suggest that the strains JPO-96 and Y-6 have different antigenicity, although JPO-96 is neutralized. The reason why the neutralizing titer was different although the nucleotide sequence in the VP2/NS junction region was similar might be based on the variation in the VP2 epitope part.

It was concluded that Japanese pearl oysters possess MABV in high ratios, and the isolated strain was very similar to strains derived from other fish and shellfish species in terms of genetic and serological properties. Studies of the pathogenicity of this strain against Japanese pearl oysters are now being undertaken and will be reported in the near future.

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