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Experimental transmission of red sea bream iridovirus (RSIV) between rock bream (*Oplegnathus fasciatus*) and rockfish (*Sebastes schlegelii*)

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Red sea bream iridovirus (RSIV), belonging to the genus *Megalocytivirus*, is the predominant cause of mortality in marine fishes in Korea, including rock bream (*Oplegnathus fasciatus*). Rockfish (*Sebastes schlegelii*) are the host fish for RSIV, exhibiting no clinical signs or mortality. Cohabitation challenges, which mimicked natural transmission conditions, were performed to evaluate viral transmission between rock bream and rockfish, and to determine the pathogenicity and viral loads. In cohabitation challenge, artificially RSIV-infected rock bream were the viral donor, and healthy rockfish were the recipient. The results showed that although the donor rock bream had 95-100 % cumulative mortality (>10^8 viral genome copies/mg of spleen 7-14 days after viral infection), the recipient rockfish did not die, even when the viral genome copies in the spleen were >10^5 copies/mg. These results indicated asymptomatic infections. Notably, in a reverse-cohabitation challenge (artificially RSIV-infected rockfish as the viral donor and healthy rock bream as the recipient), RSIV horizontally infected from subclinical rockfish to rock bream (10^7 viral genome copies/mg of spleen 21 days after cohabitation) with 10-20% cumulative mortality. These results suggest that an asymptomatic, infected rockfish can naturally transmit the RSIV without being sacrificed.

Key words: *Megalocytivirus*, Red sea bream iridoviral disease, Transmission, Rock bream, Rockfish

Introduction

Megalocytiviruses including red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) are pathogens that infect marine and freshwater species (Kurita and Nakajima, 2012). The first outbreak of RSIV infection occurred in red sea bream (*Pagrus major*) in Japan in 1990 (Inouye et al., 1992). Since then, megalocytiviruses have been identified from more than 30 marine and freshwater fish species (OIE, 2019). Understanding viral transmission between hosts is important for disease control. And identifying the natural transmission route and detecting the pathogenic agent are also important criteria for determining susceptible aquatic animal hosts. However, studies reported that some hosts show no clinical signs or mortality after megalocytivirus infection, although viral particles were detected by molecular assays, indicating asymptomatic infections (He et al., 2002; Wang et al., 2007). Therefore, the viral

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transmission between species, pathogenicity, and viral quantitation in viral exposed fish should be evaluated under conditions that mimic the natural pathway.

Rock bream (*Oplegnathus fasciatus*) and rockfish (*Sebastes schlegelii*) and are a valuable marine fish species in Korea, accounting for 1.1% (about 902 tons) and 28.2% (about 22 686 tons) and of cultured marine fish nationwide, respectively (KOSIS, 2018). Notably, in Korea since the 1990s, RSIV-subtype II *Megalocytivirus* has been the predominant genotype in marine fishes, causing high mortality in rock bream (*Oplegnathus fasciatus*) (Jung and Oh, 2000; Jeong et al., 2003; Kim et al., 2019). Cultured rockfish are affected by several pathogens during aquaculture, and a recent study highlighted the parasitic (70.3%) and bacterial (27.3%) infections from diseased rockfish between 2013 and 2016 in aquafarms in Korea (Han et al., 2020). Although parasitic and bacterial infections are the primary cause of mortality in cultured rockfish, RSIVs were still detected from randomly sampled rockfish during the national surveillance for aquatic animals (Kim et al., 2018). Furthermore, RSIVs were detected from seemingly healthy rockfish that showed no clinical signs or mortality, indicating asymptomatic infections (Kwon et al., 2015). Thus, assessing RSIV infections and transmission is important for achieving effective control measures against asymptomatic infections. In this study, the transmission of RSIV (pathogenicity and viral quantitation) between rock bream (the most susceptible host) and rockfish (the asymptomatic host) were experimentally evaluated in a cohabitation experiment.

Materials and methods

Fish

Healthy rock bream (body length 7.28 ± 0.58 cm; body weight 5.72 ± 1.35 g) and rockfish (body length 4.58 ± 0.15 cm; body weight 1.08 ± 0.45 g) were obtained from aquaculture farms in Geoje and Seosan in Korea, respectively. Fish were acclimated at 25.0 ± 0.5°C for 2 weeks in a 500 L circular tank (a flow through system in National Institute of Fisheries Science) and were fed with a commercial diet twice daily. The rearing water was changed continuously (10 L/h). The experimental fish were confirmed to be megalocytivirus infection-free by polymerase chain reaction (PCR) assay, described in the Manual of Diagnostic Tests for Aquatic Animals for RSIVD (OIE, 2019).

Virus culture

Primary cells originating from rock bream fins (Lee et al., 2020) were propagated at 25°C in L-15 medium (Gibco, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic-antimycotic solution (Gibco, USA). The 17Rb24Gs strain (RSIV-subtype II) identified from rock bream was used. Viral infections were induced in a 25-cm^2 tissue culture treated flask (Greiner Bio-one, Germany) containing a monolayer (80-90% confluency) with a multiplicity of infection of 1. Virus inoculated cells were propagated at 25°C in L-15 medium supplemented with 5% FBS and 1% antibiotic-antimycotic solution for 7 days. After the cytopathic effect appeared, the supernatant was collected after centrifuging at 1500 × *g* for 10 min and stored at -80°C until use. A fragmented copy of the major capsid protein (MCP) gene was quantified using peptide nucleic acid (PNA)-based real-time PCR described by Lee et al. (2020) to quantify the cultured virus. Briefly, the quantitative PCR (qPCR) analysis was performed using the CFX 96 Touch Real-time PCR detection system (Bio-Rad, USA) according to the manufacturer's instructions (denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 54°C for 40 s, and 76°C for 50 s). Standards (1.0×10^2 to $\times 10^8$ copies/μL of fragmented MCP gene) were generated using a synthetic oligonucleotide containing the green fluorescent protein, primers, and the PNA-probe sequences.

Cohabitation challenges for mortality

Cohabitation challenges were used to mimic the natural conditions for viral transmission. In the cohabitation challenge, the RSIV-infected rock bream cohabitated with healthy rockfish. In the reverse-cohabitation challenge, RSIV-infected rockfish cohabitated with healthy rock bream. Rock bream (n = 20; cohabitation challenge group) and rockfish (n = 20; reverse-cohabitation challenge group) were intraperitoneally (I.P.) injected with the 17RbGs strain (1.0×10^5 copies/0.1 mL/fish; the donor group) and each placed in a separate tank (50 L), respectively. The megalocytivirus-free rockfish (n = 20; cohabitation challenge group) and rock bream (n = 20; reverse-cohabitation challenge group) as recipient cohabitated with the donor group in a separate tank, respectively. As a negative control, 0.1 mL of phosphate buffered saline (PBS) was I.P. injected into rockfish (n = 20) and rock bream (n = 20) and placed into a separate tank (50 L), respectively. After the viral challenge, the fish were maintained at 25.0 ± 0.5°C using automatic cooling and heating system. The rearing water was continuously changed (5-6 L/h), and effluent was discharged by an automatic ozone treatment system. The experimental fish were observed for mortality for 21 days. The experiment was performed twice. DNA was extracted from the spleen of dead and surviving fish using a Patho Gene-spin™ DNA/RNA Extraction Kit (Intronbio, Korea) according to the manufacturer's protocol and used to determine the viral infection by qPCR as described in section 2.2. The statistical significance of the mortality rate between the recipient and control fish was determined by log-rank test using GraphPad Prism (Ver. 8.4.3). P-values of < 0.05 were considered statistically significant. All experiments were performed with permission from the Animal Ethics Committee of the National Institute of Fisheries Science (Permission No. 2019-NIFS-IACUC-4).

Cohabitation challenges for viral load quantitation

To determine the vial loads from cohabitated fish, 0.1 mL of diluted 17RbGs was I.P. injected into rock bream (n = 30) and rockfish (n = 30) as donor groups. The megalocytivirus-free rockfish (n = 30) and rock bream (n = 30) as recipient groups cohabitated with the injected rock bream and rockfish, respectively. Viral challenged fish were maintained for 3 weeks in 50 L tanks in the same condition as described in section 2.3. As negative controls, PBS-injected megalocytivirus-free rock bream (n = 30) and rockfish (n = 30) were each maintained in a separate tank. Five fish from each group were sampled on post-cohabitation days 5, 7, 9, 11, and 14. Viral genome copy numbers were determined by qPCR using DNA extracted from the spleen as described in section 2.3.

Results and Discussion

In the mortality cohabitation challenge, RSIV (17 RbGs strain as RSIV-subtype II)-infected rock bream cohabitated with healthy rockfish. The RSIV-injected rock bream (i.e., the donor) had 95.0 % and 100.0 % cumulative mortality within 14 days post-challenge in experiments 1 and 2, respectively (Fig. 1A and B), and died from the enlargement of the spleen by viral infection. However, the recipient rockfish had neither clinical signs nor mortality after 3 weeks despite RSIV detection in 10 % (experiment 1) and 15 % (experiment 2) of the rockfish (data not shown). In the mortality reverse-cohabitation challenge, RSIV-infected rockfish cohabitated with healthy rock bream. Although the RSIV-injected rockfish (i.e., the donor) possessed the RSIV genomes, there was no mortality. However, the recipient rock bream had 10 % and 20 % cumulative mortality within 21 days post-cohabitation in experiments 1 and 2, respectively (Fig. 2A and 2B). Of the surviving rock bream, 33.3 % (6/18) and 25.0 % (4/16) were RSIV-positive by real-time PCR in experiments 1 and 2, respectively.

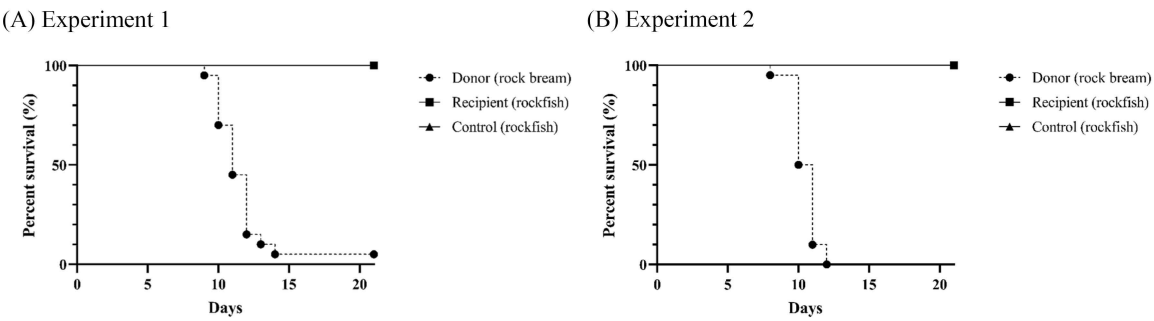

Fig. 1. Cumulative mortality (%) of cohabitated rockfish as the recipient after exposure to artificially RSIV-infected rock bream (10^5 viral genome copies/fish) in the cohabitation challenge. All experiments were repeated twice (A), (B). No significant differences in pathogenicity were observed between the recipient and control rockfish.

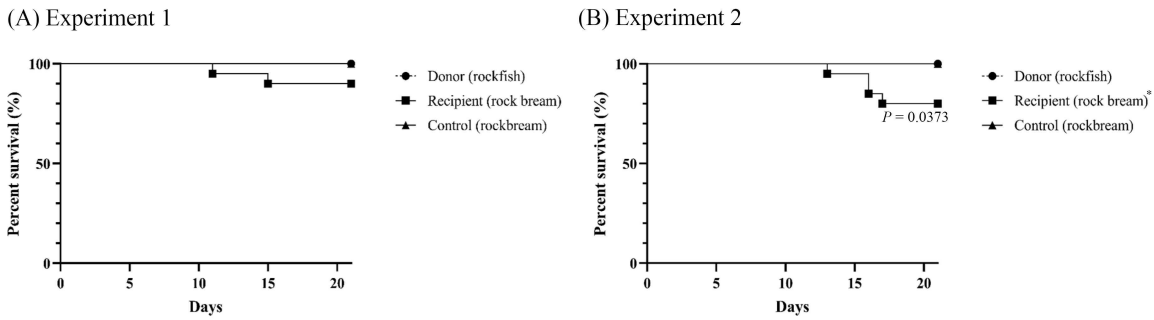

Fig. 2. Cumulative mortality (%) of cohabitated rock bream as the recipient after exposure to artificially RSIV-infected rockfish (10^5 viral genome copies/fish) in the reverse-cohabitation challenge. All experiments were repeated twice (A), (B). A significant difference ($^*P < 0.05$) in pathogenicity was observed between the recipient and control rock bream in experiment 2 (B).

Previous studies showed that the RSIV-subtype II *Megalocytivirus*-infected rock bream had a high mortality rate (60-100% cumulative mortality; Jung and Oh, 2000; Jeong and Jeong, 2008) and the cumulative mortality of rock bream, which experimentally cohabitated with RSIV- subtype II infected rock bream, reached 100% mortality (Jeong et al., 2008). However, artificially infected-rockfish (body weight 5-7 g) had only a 50% cumulative mortality rate (Jeong and Jeong, 2008). In this study, artificially RSIV-infected rockfish (the recipient in the cohabitation challenge and the donor in the reverse cohabitation challenge) did not die, supporting the conclusions of Kwon et al. (2015), who identified asymptomatic RSIV infections in rockfish. Previous studies also showed that rock bream was the most susceptible species for RSIV subtype II infections. Jin et al. (2011) reported $>10^6$ viral genome copies/mg of tissue at the moribund stage, and Lee et al. (2020) reported 10^8 viral genome copies/mg of tissue. Furthermore, viruses released into the seawater from artificial RSIV-infected juvenile rock bream (10^4 copies/0.1 mL/fish; Kwon et al., 2020) and Japanese amberjack (*Seriola quinqueradiata*; $10^{4.7}$ median tissue culture infectious dose/0.1 mL/fish; Kawato et al., 2016) were approximately 10^4 copies/mL 9 days and $10^{2.3}$ copies/mL 10-14 days after viral injection, respectively. In this study, RSIV-infected donor rock bream also had $>10^8$ viral genome copies/mg of spleen 7 days after the viral injection (Fig. 3A and Table 1). Notably, the rockfish had ap-

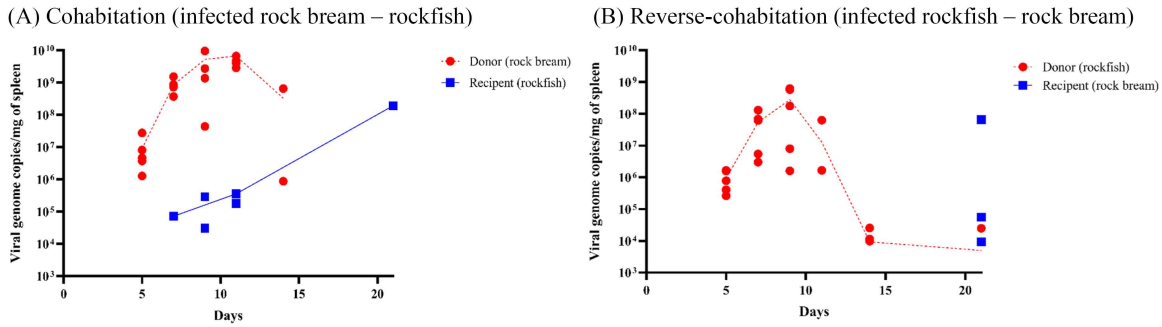

Fig. 3. Viral genome copies in rockfish (A) and cohabitated rock bream (B) as the recipient after cohabitation with artificially RSIV-infected rock bream (A) and rockfish (B) as the donor (10^5 viral genome copies/fish).

proximately 10^5 viral genome copies 9-11 days after cohabitation (Table 1), but the dead RSIV-infected rock bream had this viral load approximately 8-9 days after the viral injection (Fig. 1A and 1B). These results suggest that viruses released from the infected rock bream at the moribund stage could shed enough viral particles into the rearing water, leading to re-infected rockfish.

The minimal infectious dose for inducing asymptomatic infection in rockfish is still unclear. In the field, asymptomatic RSIV infections in rockfish (the viruses were detected by nested-PCR) were identified in 23.1-70.0 % of fish in 15 aquaculture farms between July and December in Tongyeong in 2013, but RSIV

Table 1. Identification and viral loads of RSIV in rock bream and rockfish after cohabitation or reverse-cohabitation challenge

Experimental group	Days	Donor[a]		Recipient[b]		Control[c]	
		real-time PCR	Viral gene copies/mg of spleen	real-time PCR	Viral gene copies/mg of spleen	real-time PCR	Viral gene copies/mg of spleen
Cohabitation[d]	5	5/5	$9.10\times10^6\pm1.06\times10^7$	0/5	N.D.[f]	0/5	N.D.
	7	5/5	$8.54\times10^8\pm4.26\times10^8$	1/5	7.2×10^4	0/5	N.D.
	9	5/5	$5.31\times10^9\pm5.57\times10^9$	2/5	$1.61\times10^5\pm1.84\times10^5$	0/5	N.D.
	11	5/5	$6.71\times10^9\pm4.86\times10^9$	2/5	$2.68\times10^5\pm1.24\times10^5$	0/5	N.D.
	14	2/2	$3.31\times10^8\pm4.67\times10^8$	0/5	N.D.	0/5	N.D.
	21	-	-	1/5	1.98×10^8	0/5	N.D.
Reverse-cohabitation[e]	5	5/5	$9.36\times10^5\pm6.48\times10^5$	0/5	N.D.	0/5	N.D.
	7	5/5	$5.40\times10^7\pm5.27\times10^7$	0/5	N.D.	0/5	N.D.
	9	5/5	$2.78\times10^8\pm3.04\times10^8$	0/5	N.D.	0/5	N.D.
	11	2/5	$3.24\times10^7\pm4.35\times10^7$	0/5	N.D.	0/5	N.D.
	14	3/5	$1.55\times10^4\pm8.56\times10^3$	0/5	N.D.	0/5	N.D.
	21	1/5	2.50×10^4	2/5	$2.19\times10^7\pm3.79\times10^7$	0/5	N.D.

[a]Donor, RSIV-infected rock bream in cohabitation challenge group or rockfish in reverse-cohabitation challenge group;
[b]Recipient, healthy rockfish in cohabitation challenge group or rock bream in reverse-cohabitation challenge group;
[c]Control, phosphate buffered saline injected rockfish in cohabitation challenge group or rock bream in reverse-cohabitation group;
[d]Cohabitation, RSIV-infected rock bream cohabitated with healthy rockfish;
[e]Reverse-cohabitation, RSIV-infected rockfish cohabitated with healthy rock bream;
[f]N.D., Not detected

identified from field-derived rockfish did not lead to mortality in rock bream by I.P. injection (Kwon et al., 2015). In the reverse-cohabitation challenge, RSIV replicated in rockfish after I.P. injection (>10^7-10^8 viral genome copies 7-11 days after viral injection; Fig. 3B and Table 1). However, viral particles released from the infected rockfish could not induce acute re-infection and death in the rock bream for a shorter period than in the cohabitation challenge. Nevertheless, in reverse-cohabitation challenge, rock bream cohabitated with RSIV-infected rockfish showed 10 % and 20% mortality within 21 days (Fig. 2A and 2B) and had a viral load of 2.19 × 10^7 viral copies/mg of spleen on day 21 (Table 1). These results suggest that asymptomatic infected rockfish can transmit the RSIV without their natural sacrifice. Future studies are needed to determine the minimal infectious dose and develop a risk assessment model for asymptomatic transmission between fish species at the field level. Especially, active surveillance to asymptomatic infected fish species, such as rockfish, needs to be conducted in aquaculture regions with various fish species, such as Tongyeong and Namhae.

Pathogenicity and viral loads of RSIV-subtype II *Megalocytivirus* were determined by cohabitation challenges between rock bream and rockfish. RSIV infected and replicated in asymptomatic rockfish without mortality from artificially infected rock bream. RSIV infections were also induced in rock bream by asymptomatic-infected rockfish through silent viral transmission.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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