Short communication

Immunization of Rock Bream *Oplegnathus fasciatus* at Low Temperature by Immersion with Live Red Sea Bream Iridovirus (RSIV)

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ABSTRACT—Rock bream *Oplegnathus fasciatus* were immersed in cultured RSIV suspension at 17°C for 30 min, and reared for 80 days without controlling temperature. Fish rearing temperature naturally increased at approximately 0.1°C/day from 17°C. Mortalities of fish without and with RSIV at $10^{5.8}$, $10^{3.8}$ and $10^{1.8}$ TCID₅₀/mL were 5.8%, 30.8%, 22.5% and 6.7%, respectively. Surviving fish were reared at 26°C, and intramuscularly injected with RSIV at $10^{1.8}$ or $10^{0.8}$ TCID₅₀/fish. Survivors from RSIV-immersion at $\geq 10^{3.8}$ TCID₅₀/mL were strongly protected against RSIV challenge whereas those at $\leq 10^{1.8}$ TCID₅₀/mL died, suggesting that fish immunization with live RSIV at low temperature could be conducted by immersion route.

Key words: Megalocytivirus, red sea bream iridovirus, immunization, immersion, *Oplegnathus fasciatus*

Red sea bream iridovirus (RSIV), a member of genus *Megalocytivirus* belonging to family of *Iridoviridae*, causes serious economic losses to several marine fishes (Jancovich *et al.*, 2012; Kurita and Nakajima 2012). Commercially available formalin-inactivated RSIV vaccine is efficient in protecting red sea bream *Pagrus major* and yellowtail *Seriola quinqueradiata* (Nakajima *et al.*, 1997, 1999, 2002). However, it offers no protection for fishes belonging to genus *Oplegnathus* (OIE, 2012). Previously, we have demonstrated the potential of immunizing rock bream *O. fasciatus* with live RSIV at low rearing temperature (Oh *et al.*, 2014). After fish received live RSIV at low temperature around 15°C to 17°C, almost no fish died because multiplication rate of

RSIV was too low to develop the disease (Oh *et al.*, 2016c). Inoculated RSIV can infect fish and multiply beyond a threshold level so that fish can mount specific protective immune response. It has been previously shown that fish immunized by live RSIV are highly protected against re-challenges by RSIV (Oh *et al.*, 2014). The same phenomenon has been confirmed by Jung *et al.* (2015) and Matsuyama *et al.* (2016). Previously, fish immunization with live RSIV has been performed by intramuscular injection using virus prepared from spleen homogenate of affected fish. However, intramuscular injection takes a lot of time and effort, thus hindering large scale application. Moreover, preparation of a constant infectious dose of RSIV suspension is difficult using different sources of splenic tissues of affected fish. Recently, we have established primary cell lines such as PI-GF^{RSIV} and RoBE-4. PI-GF^{RSIV} cells are GF cells derived from grunt fin and persistently infected by RSIV (Oh and Nishizawa, 2016b). RoBE-4 cells derived from rock bream embryo exhibit cytopathic effect (CPE) such as cell rounding. RoBE-4 cells also go through completely cytolysis after RSIV infection (Oh and Nishizawa, 2016a). Both cell lines can stably produce high titers of infectious RSIV. The objective of this study was to immunize rock bream by immersion with cultured RSIV at low temperature to determine the minimum dose of RSIV for fish to mount a protective immune response.

Materials and Methods

Virus

RSIV RBHad09 isolate (Oh and Nishizawa, 2016a, 2016b) was cultured in RoBE-4 cells at 25°C. RoBE-4 cells were maintained with Leibovitz's L-15 medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS, Hyclone), 150 IU/mL of penicillin G, and 100 µg/mL of streptomycin (L-15₁₀). Cultured RSIV was centrifuged at 12,000 × g for 15 min at 4°C. The resulting supernatant was harvested and stocked as a standardized RSIV suspension. Infectivity titer of the standardized RSIV suspension was $10^{7.8}$ TCID₅₀/mL (50% tissue culture infectious dose).

Fish immunization

Immunization of rock bream was performed when water temperature was at 17°C. A total of 840 fish (mean body weight of 7 g) were reared in seven aquaria (120 fish per aquarium) with 30 L of seawater turned over 24 times/day. Fish were immersed in RSIV suspension at $10^{5.8}$, $10^{3.8}$ or $10^{1.8}$ TCID₅₀/mL (two aquaria for each dose) at 17°C for 30 min. Fish in the remaining one aquarium were immersed in L-15 medium (diluted 1,000-fold with seawater) in the same manner as mock infection. Fish with RSIV-immersion were then reared for 80 days without controlling seawater temperature. Seawater temperature for rearing fish increased at

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Fig. 1. Changes in rock bream mortalities and genome titers of RSIV after immersion with cultured RSIV suspensions. Fish were reared at 17°C and immersed with RSIV suspension at $10^{5.8}$, $10^{3.8}$, $10^{1.8}$ TCID₅₀/mL or L-15 medium (mock infection). Fish were reared for 80 days without controlling seawater temperature. (A) Mortality of fish immersed with RSIV suspension at different doses and change in water temperature. (B) Changes of RSIV genome titer in splenic tissues of fish immersed with RSIV suspension. Error bars indicate standard deviation.

approximately 0.1°C/day from 17°C to around 25°C (Fig. 1A), along changes of natural water temperatures. On days 0, 1, 3, 5, 7, 10 and weeks 2, 3, 4, 5, 6, 8, 11 after RSIV-immersion, six fish from one of two aquaria for each dose of RSIV-immersion were sacrificed. Fish splenic tissues were individually subjected to nucleic acid extraction and detection of RSIV genomes by quantitative PCR (qPCR) assay as described below. Fish in the remaining aquarium for each dose of RSIV-immersion were monitored for mortality.

Ethics statement

All experiments using rock bream were carried out in strict accordance with recommendations by the Institutional Animal Care and Use Committee of Chonnam National University (Permit Number: CNU IACUC-YS-2015-4).

Re-challenge of fish with RSIV

Fish surviving immersion challenges at different doses of RSIV suspensions were subjected to intramuscular challenges with RSIV at $10^{1.8}$ TCID₅₀/fish or $10^{0.8}$ TCID₅₀/fish. A total of eight aquaria with 20 L

of seawater at 26°C were prepared. A total of 40 fish surviving RSIV-immersion at each RSIV dose ($10^{5.8}$, $10^{3.8}$, $10^{1.8}$ TCID₅₀/mL and mock infection) were divided into two aquaria (20 fish per aquarium). Fish in one aquarium were intramuscularly injected with RSIV at $10^{1.8}$ TCID₅₀/fish. Fish in other aquaria were intramuscularly injected with RSIV at $10^{0.8}$ TCID₅₀/fish. All fish were reared for 21 days to monitor mortality.

Detection of RSIV genomes

qPCR assay targeting RSIV genomes was performed using primers qPst-Had09F (5'-GCA GCC CAG ATT TTT GGT TCG-3') and qPst-Had09R (5'-TGT GCC CAT GTC CTG GTA ATT G-3') under the same condition as described previously (Oh and Nishizawa, 2013). Briefly, total nucleic acids were extracted from fish splenic tissue using standard phenol and chloroform/isomyl alcohol extraction method. Then qPCR assay was carried out in Exicycler 96 real-time quantitative thermal block (Bioneer) using SYBR Green AccuPower Greenstar qPCR mastermix (Bioneer) with the following program: one cycle at 95°C for 2 min followed by 40 cycles of 95°C for 10 s and 58°C for 40 s. Quantitative-detection limit was approximately 10^2 genomes/reaction corresponding to $10^{3.5}$ genomes/mg of fish splenic tissue.

Results and Discussion

Changes in mortality and RSIV-genome titer in rock bream after RSIV-immersion at different doses are shown in Fig. 1. After RSIV-immersion at $10^{5.8}$ TCID₅₀/mL, fish began to die on day 21. Their cumulative mortality reached 30.8% at day 80. RSIV genomes were detected in splenic tissues on day 14 after RSIV-immersion. It reached $10^{6.1 \pm 0.5}$ genome/mg on day 21 after RSIV-immersion. It was then decreased to under the detection limit on day 56 (Fig. 1B). After RSIV-immersion at $10^{3.8}$ TCID₅₀/mL, fish began to die on day 31. Their cumulative mortality reached 22.5% on day 80 (Fig. 1A). RSIV-genomes were detected in splenic tissues on day 21, reaching $10^{4.8 \pm 1.1}$ genome/mg on day 28. It was then decreased to under the detection limit on day 56 (Fig. 1B). After RSIV-immersion at $10^{1.8}$ TCID₅₀/mL and mock infection, no mortality was observed until day 34. However, their cumulative mortalities reached 6.7% (at $10^{1.8}$ TCID₅₀/mL) and 5.8% (mock infection), respectively, at the end of the experiment (Fig. 1A). RSIV-genomes in fish with RSIV-immersion at $10^{1.8}$ TCID₅₀/mL were under the detection limit throughout the experimental period except for samples on the day 35 (Fig. 1B). Although dead fish in the mock infection group were also subjected with RSIV-genome for detection, all were under the detection limit (data not shown).

Fish surviving immersion challenges at different

Fig. 2. Mortality of fish that survived immersion with RSIV suspensions after reinfection by RSIV through intramuscular injection. Survivors from RSIV-immersion were reared at 26°C, and intramuscularly injected with RSIV at $10^{1.8}$ or $10^{0.8}$ TCID₅₀/fish. (A) Mortalities of survivors re-infected by RSIV at $10^{1.8}$ TCID₅₀/fish through intramuscular injection. (B) Mortalities of survivors re-infected by RSIV at $10^{0.8}$ TCID₅₀/fish through intramuscular injection.

doses of RSIV suspensions were subjected to intramuscular challenges with RSIV at $10^{1.8}$ TCID₅₀/fish or $10^{0.8}$ TCID₅₀/fish (Fig. 2). For fish surviving RSIV-immersion at $10^{5.8}$ TCID₅₀/mL, no mortality was observed after RSIV re-infection regardless of RSIV doses injected (Fig. 2). However, fish that survived RSIV-immersion at $10^{3.8}$ TCID₅₀/mL began to die on day 13 or 14 after RSIV re-infection by injection. Mortality in these fish after receiving RSIV by injection at $10^{1.8}$ and $10^{0.8}$ TCID₅₀/fish were 5% and 10%, respectively. Fish that survived RSIV-immersion at $10^{1.8}$ TCID₅₀/mL and naïve fish began to die on day 12 or 13 after RSIV injection. Their mortalities reached to 100% regardless of RSIV doses injected (Fig. 2). These results suggest that fish survived RSIV immersion at $\geq 10^{3.8}$ TCID₅₀/mL might have mounted protective immune response against RSIV re-infection. Therefore, live RSIV immersion at low temperature might have potential to immunize fish against RSIV infection.

It has been reported that a threshold level of RSIV

for rock bream to mount a protective immune response against RSIV infection is less than $10^{5.4}$ genomes/mg (Oh *et al.*, 2014). This was strongly supported by this study, because surviving fish with RSIV multiplication at $10^{4.8 \pm 1.1}$ genome/mg were strongly protected against RSIV infection whereas survivors with almost no multiplication of RSIV were highly susceptible to RSIV infection (Figs. 1B and 2). Nishizawa *et al.* (2011, 2012) have reported that a certain degree of viral multiplication is required for fish to mount a specific protective immune response against injected virus regardless of infection routes, although studies are about different viruses. This finding was validated to be the same in RSIV infection.

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References

- Jancovich, J. K., V. G. Chinchar, A. Hyatt, T. Miyazaki, T. William and Q. Y. Zang (2012): Family iridoviridae. In "Virus Taxonomy" (ed. by A. M. Q. King, M. J. Adams, E. B. Carstens and E. J. Lefkowitz). Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, San Diego, CA, pp. 193–210.
- Jung, M. H., S. J. Jung, T. N. Vinay, C. Nikapitiya, J. O. Kim, J. H. Lee, J. Lee and M. J. Oh (2015): Effects of water temperature on mortality in megalocytivirus-infected rock bream *Oplegnathus fasciatus* (Temminck et Schlegel) and development of protective immunity. *J. Fish Dis.*, **38**, 729–737.
- Kurita, J. and K. Nakajima (2012): Megalocytiviruses. *Viruses*, **4**, 521–538.
- Matsuyama, T., T. Minami, Y. Fukuda, N. Sano, T. Sakai, T. Takano and C. Nakayasu (2016): Passive immunization against red sea bream iridoviral disease in five marine fish species. *Fish Pathol.*, **51**, 32–35. (in Japanese with English summary)
- Nakajima, K., Y. Maeno, J. Kurita and Y. Inui (1997): Vaccination against red sea bream iridoviral disease in red sea bream. *Fish Pathol.*, **32**, 205–209.
- Nakajima, K., Y. Maeno, A. Honda, K. Yokoyama, T. Tooriyama and S. Manabe (1999): Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. *Dis. Aquat. Organ.*, **36**, 73–75.
- Nakajima, K., T. Ito, J. Kurita, H. Kawakami, T. Itano, Y. Fukuda, Y. Aoi, T. Tooriyama and S. Manabe (2002): Effectiveness of a vaccine against red sea bream iridoviral disease in various cultured marine fish under laboratory conditions. *Fish Pathol.*, **37**, 90–91.
- Nishizawa, T., I. Takami, M. Yang and M. J. Oh (2011): Live vaccine of viral hemorrhagic septicemia virus (VHSV) for Japanese flounder at a fish rearing temperature of 21°C instead of Poly(I:C) administration. *Vaccine*, **29**, 8397–8404.
- Nishizawa, T., H. J. Gye, I. Takami and M. J. Oh (2012):

- Potentiality of a live vaccine with nervous necrosis virus (NNV) for sevenband grouper *Epinephelus septemfasciatus* at a low rearing temperature. *Vaccine*, **30**, 1056–1063.
- OIE (World Organisation for Animal Health) (2012): Red sea bream iridoviral disease. Chapter 2.3.7, Manual of diagnostic tests for aquatic animals. <http://www.oie.int/international-standard-setting/aquatic-manual/access-online/>.
- Oh, S. Y. and T. Nishizawa (2013): Optimizing the quantitative detection of the red sea bream iridovirus (RSIV) genome from splenic tissues of rock bream *Oplegnathus fasciatus* using a qPCR assay. *Fish Pathol.*, **48**, 21–24.
- Oh, S. Y. and T. Nishizawa (2016a): Multiple passages of grunt fin cells persistently infected with red seabream iridovirus at 15°C or 30°C to yield uninfected cells. *J. Aquat. Anim. Health*, **28**, 214–221.
- Oh, S. Y. and T. Nishizawa (2016b): Establishment of rock bream *Oplegnathus fasciatus* embryo (RoBE-4) cells with cytolytic infection of red sea bream iridovirus (RSIV). *J. Virol. Methods*, **238**, 1–5.
- Oh, S.-Y., W.-S. Kim, M.-J. Oh and T. Nishizawa (2016c): Multiplication rate of red sea bream iridovirus (RSIV) in rock bream *Oplegnathus fasciatus* at different fish rearing temperatures. *Fish Pathol.*, **51**, 194–198.
- Oh, S. Y., M. J. Oh and T. Nishizawa (2013): Potential for a live red seabream iridovirus (RSIV) vaccine in rock bream *Oplegnathus fasciatus* at a low rearing temperature. *Vaccine*, **32**, 363–368.