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Potential for a live red seabream iridovirus (RSIV) vaccine in rock bream *Oplegnathus fasciatus* at a low rearing temperature

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ABSTRACT

Serious economic losses have occurred in fingerlings and market-sized rock bream *Oplegnathus fasciatus* in Korea due to red seabream iridovirus (RSIV) infection. We demonstrated previously that viral multiplication in fish is downregulated by maintaining fish at far from optimum temperatures at the onset of disease. We applied this concept to develop a live RSIV vaccine in rock bream. Mortalities in rock bream that were inoculated with RSIV and reared at 21–30 °C were ≥90%, whereas no mortality was observed in fish that received an RSIV inoculation and were reared at ≤18 °C. RSIV kinetics revealed that RSIV multiplied rapidly in fish reared at 24.3 ± 1.3 °C, and achieved the critical level for rock bream (approximately $10^{9.0}$ genomes/mg) within 28 days. In contrast, the RSIV genome was detected on day 10 in fish that received an RSIV inoculation at 15.5 °C, and peaked on day 28 at $10^{5.91 \pm 0.54}$ genomes/mg, then decreasing gradually, and were then maintained under the detection level beginning on day 84 after RSIV inoculation. Furthermore, the fish surviving the RSIV infection at low rearing temperature were strongly protected from re-challenge with homologous RSIV; the threshold level of RSIV for rock bream to mount a protective immune response was $\leq 10^{5.4}$ genomes/mg. Cohabitation experiments revealed that the spread of RSIV from rock bream vaccinated with a live RSIV could be low if it is limited to fish in the late stage (≥84 days of elapse) after vaccination. Thus, it was concluded that when rock bream are reared at ≤18 °C and inoculated with RSIV, the survivors can mount a protective immune response against RSIV, suggesting a positive effect of a live RSIV vaccine for rock bream.

1. Introduction

Red seabream iridovirus (RSIV) infection causes significant mortality in more than 30 species of cultured marine fish throughout many Asian countries [1]. Serious economic losses due to RSIV infection have occurred in fingerlings and market-sized rock bream *Oplegnathus fasciatus* in Korea [2]. Fish infected with RSIV are lethargic and exhibit severe anemia, gill petechiae, enlargement of splenic tissue, and an enlargement of cells and necrosis in renal and splenic hematopoietic tissues [3]. RSIV, a member of the genus *Megalocytivirus* (*Iridoviridae*), is an icosahedral virus of 200–240 nm in diameter and has a single linear ds-DNA molecule of 111 kbp [4–7]. Among megalocytiviruses, the major capsid protein (MCP) gene, the DNA polymerase gene, the *Pst* I fragment gene (containing partial phosphatase and laminintype genes), and the adenosine triphosphatase (ATPase) genes have previously been targeted for viral detection and analysis of their genetic relationships [8–14].

A formalin-inactivated RSIV vaccine has been commercialized, and is efficient for protecting red sea bream *Pagrus major*, yellowtail *Seriola quinqueradiata*, and other fish species [15–17], but is not effective for fishes belonging to the genus *Oplegnathus* [18]. Subunit and DNA vaccines with the RSIV MCP gene have been developed [19,20], but not commercialized. It was recently reported that a polyinosinic-polycytidylic acid [Poly(I:C), a synthetic double-stranded RNA] immunization with a live fish RNA virus, confers protection of fish from the viral infection, such as viral nervous necrosis virus (NNV) infection in the sevenband grouper *Epinephelus septemfasciatus*, viral hemorrhagic septicemia virus (VHSV) infection in Japanese flounder *Paralichthys olivaceus* and infectious hematopoietic necrosis virus infection in rainbow trout *Oncorhynchus mykiss* [21–27]. Although it is conceivable that Poly(I:C) immunization will be applicable to a wide range of fish species and other viruses, it is ineffective against RSIV infection, as RSIV seems to be insensitive to the transient innate immune response induced by Poly(I:C) administration [28].

Nishizawa et al. [24,29] demonstrated that the viral multiplication rate *in vivo* is strongly correlated to virulence and fish mortality, based on data from NNV and VHSV infections; When fish are reared at a temperature within the range of virus multiplication but far from the optimum temperature for disease onset, the virus can multiply beyond the threshold level for fish to mount a protective immune response, but not achieve a critical level [24,29,30].

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These data strongly suggest the potential for live virus immunization in fishes by controlling the rearing temperature. This concept could be promising for fish immunization by taking advantage of the characteristics of viral multiplication in poikilothermic fish species.

In the present study, we applied the same concept of regulating viral pathogenicity by controlling the rearing temperature to RSIV infection in rock bream to demonstrate the potential for a live RSIV vaccine.

2. Materials and methods

2.1. Virus

RSIV RBHad09 isolate [28] was obtained directly from splenic tissues of moribund rock bream by artificially infection. The fish splenic tissues were homogenized with 9 volumes of Leibovitz's L-15 medium (Gibco, USA) and centrifuged (3000 × *g*, 10 min, 4 °C). The supernatants were subdivided and were stored at −80 °C until use.

2.2. Ethics statement

Subsequent experiments using rock bream were carried out in strict accordance with the recommendations in the Guide for the Institutional Animal Care and Use, the Committee of Chonnam National University (Permit Number: CNU IACUC-YS20091).

2.3. Pathogenicity of the RSIV stock solution at different doses

A total of 100 rock bream (13.9 g MBW) were reared in six aquaria (n = 20) containing 15 l of stagnant seawater at 28 °C, which was exchanged twice per day. Fish in each aquarium were intramuscularly inoculated with 100 μl of the RSIV stock solution diluted 10^3, 10^4, 10^5, 10^6, and 10^7-fold with L-15 medium, and were maintained for 4 weeks to monitor fish mortality.

2.4. Pathogenicity of RSIV at different temperatures

A total of 140 rock bream (13.9 g MBW) were reared in seven aquaria (n = 20) with 15 l of stagnant seawater at 15, 18, 21, 24, 28, 30, or 32 °C, and the seawater was exchanged twice per day. Fish in each aquarium were intramuscularly injected with 100 μl of the virus stock solution diluted 10^3-fold with L-15 medium, and were maintained for 4 weeks to monitor fish mortality.

2.5. Fish immunization with live RSIV and RSIV kinetics in the fish

A total of 600 rock bream (17.7 g MBW) were reared in three aquaria (n = 200) with 1000 l of seawater turned over 24 times/day without control of temperature. When the water temperature reached 15.5 °C, fish in two of the three aquaria were intramuscularly inoculated with 100 μl of the stock solution, which had been diluted 10^3-fold with L-15 medium, whereas fish in the remaining aquarium were intramuscularly injected with 100 μl of L-15 medium alone. On days 1, 3, 5, 7 and 10, and weeks 2, 3, 4, 6, 8, 10, 12, 14, 16 and 18 after RSIV inoculation, six fish each were sacrificed from one of the two aquaria containing RSIV infected fish, so as to obtain splenic tissues for detecting the RSIV genome by qPCR assay as described below. The remaining two aquaria with fish that received RSIV or mock inoculations were used to observe mortality.

Next, a total 600 rock bream (25.5 g MBW) were reared in three aquaria (n = 200) with 1000 l of seawater turned over 24 times/day without control of temperature (24.3 ± 1.3 °C). Fish in two of the aquaria were intramuscularly inoculated with 100 μl of the RSIV stock solution, which was diluted 10^3-fold, whereas fish in the remaining aquarium were injected with L-15 medium alone. Six fish each were sacrificed from one aquarium every 2 or 3 days after RSIV inoculation, to obtain splenic tissues for detecting the RSIV genome by qPCR assay. The remaining two aquaria with fish that received RSIV or mock inoculations were used to observe fish mortality.

2.6. Quantitative detection for RSIV kinetics in infected fish spleen

Nucleic acids were extracted from fish splenic tissue by a standard method with phenol and chloroform/isoamylalcohol solutions, and were subjected to qPCR assay with 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′) targeting the RSIV *Pst* I fragment gene [31]. The qPCR assay was carried out in an Exicycler 96 real-time quantitative thermal block (Bioneer, Korea) using SYBR Green AccuPower Greenstar qPCR premix (Bioneer), with the following program: one cycle of 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, and 58 °C for 40 s. The RSIV genome number in samples was estimated using the regression equation: $y = -0.266x + 10.95$ [y is the number of RSIV genome ($n \log_{10}$), whereas x is the measured Ct value] [31].

2.7. Re-challenge of survivors with homologous RSIV

On day 126 after the primary RSIV inoculation at 15.5 °C (Section 2.5), 60 survivors (42.2 g MBW) were transferred to two new aquaria (n = 30) with 50 l of 24.3 ± 1.3 °C seawater turned over 24 times/day without control of temperature, whereas 60 naïve fish of the same size as the survivors were reared in two additional aquaria (n = 30) in the same manner. The survivors and naïve fish in one of the two aquaria were intramuscularly inoculated with 100 μl of the virus stock solution which had been diluted 10^3-fold with L-15 medium, whereas fish in the remaining two aquaria were intramuscularly injected with 100 μl of L-15 medium. Fish were maintained for an additional 35 days to monitor mortality.

2.8. Evaluation of horizontal infection from fish surviving the RSIV infection to naïve fish

On day 126 after the primary RSIV inoculation at 15.5 °C (Section 2.5), five survivors were transferred to a new aquarium with 50 l of seawater controlled at 28 °C, and were cohabitated with 25 naïve rock bream (42.2 g MBW). After 1 month of cohabitation, all fish were sacrificed to obtain splenic tissue for the detection of RSIV genome by qPCR assay, as described above.

3. Results

3.1. Pathogenicity of the RSIV stock solution

3.1.1. Pathogenicity at different doses

Fish that received RSIV diluted 10^3, 10^4, 10^5, or 10^6-fold began to die 9–12 days after RSIV inoculation, and their cumulative mortality rates were 100, 100, 95, and 20%, respectively, whereas no mortality was observed in fish that were administered RSIV which had been diluted 10^7-fold (Fig. 1A). The calculated 50% lethal dose (LD_{50}) of the RSIV stock solution was approximately a $10^{5.6}$-fold dilution/100 μl/fish (Fig. 1B).

3.1.2. Pathogenicity at different temperatures

Fish inoculated with RSIV at 30 °C and 28 °C began to die 9–10 days after inoculation, and all fish died within 12–13 days. Fish reared at 24 °C and 21 °C began to die 13 and 18 days after RSIV inoculation, respectively. Their cumulative mortalities were 100% and 90%, respectively. No mortality was observed in fish, which