マダイイリドウイルス病に対する有効なDNAワクチン開発のための研究

Doctoral Dissertation

STUDIES FOR THE DEVELOPMENT OF AN EFFECTIVE DNA VACCINE AGAINST RED SEA BREAM IRIDOVIRUS DISEASE

March 2024

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
Doctoral Course of Applied Marine Biosciences

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to the following individuals and organizations:

1. I am grateful to the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and the Science and Technology Research Partnership for Sustainable Development (SATREPS) program for providing financial support for my research and education.
2. I thank the University for the financial support allowing attending the 21st International Conference on Diseases of Fish and Shellfish, which will be held in Aberdeen, United Kingdom, from September 11–14, 2023.
3. I would like to extend my heartfelt appreciation to the following mentors and colleagues who guided and supported me throughout this journey:

- Thank you to Hidehiro KONDO sensei for being an excellent advisor. He provided me with helpful guidance and a laboratory technique recommendation that may lead to future work. Furthermore, I appreciate your inviting me to participate in the field trip in the Ehime prefecture, which gave me firsthand knowledge of Japanese maritime agriculture.
- Thank you to Ikuo HIRONO sensei for introducing me to the excellent opportunity and experience to study aboard in Japan at the Laboratory of Genome Science.
- I am grateful for Reiko NOZAKI's readiness to impart useful skills and her kind sharing of her life experience and wisdom. Related to genomics including tips on how to live here. Thank you also for the Japanese snacks that provide energy for working every day.
- Motohiko SANO sensei for teaching the cell culture technique.
- Keiichiro KOIWAI sensei

- All the members of the laboratory
- I am grateful to Kaori MIZUNO and Hidemasa KAWAKAMI of the Ehime Prefectural Fisheries Research Center/Agricultural, Forestry and Fisheries Research Institute for providing the red sea bream utilized in the experiment with food and care.
- Finally, I would like to express my gratitude for the use of experimental animal species such as Japanese flounder, rock bream, and red sea bream.

Abstract

The World Organization for Animal Health (WOAH) has classified the red sea bream iridovirus (RSIV) as an infectious agent that affects Japanese aquaculture. The infectious disease caused by RSIV results in significant financial losses. The red sea bream iridovirus is a double-stranded DNA virus with an icosahedral shape and a diameter of 200–240 nm. It is a member of the genus Megalocytivirus, which also contains the infectious spleen and kidney necrosis virus. About 120 possible open reading frames (ORFs) can be found in the roughly 110 kbp long DNA genome (Kawato et al., 2017). Comprehensive analyses of the expression kinetics of viral genes in cultured cells (Lua et al., 2005) and juvenile red sea breams (Dang et al., 2007) have resulted in the classification of these genes as immediate-early, early, and late genes. This data can be utilized to find antigens for the creation of new vaccines as well as to comprehend the mechanism of RSIV infection.

WOAH recommended using vaccines for illness prevention, and these days, commercial vaccines are accessible in Japan. The formalin-killed vaccine against RSIVD have been approved and used in red sea bream, striped jack, and Seriola species in Japan. Nevertheless, it is challenging to monitor immunization effectiveness in the field. Additionally, a different kind of vaccination was being researched. These include a recombinant protein vaccine, which protected against RSIV infection (Hajime et al., 2010), an attenuated lived vaccine, which elicited a protective immune response against RSIV (So-Young et al., 2014), and a DNA vaccine, which produced a foreign protein through immunization with antigen-encoding plasmid DNA and elicited a humoral and cellular immune response (Park et al., 2005).

Since DNA vaccines mimic viral infection in the host organism, they will enable the development of more effective vaccines against viral infections in the future. To develop more

effective vaccines, it is crucial to simultaneously analyze the mechanisms of viral infection and host immunity. Therefore, the objective of this study is to develop DNA vaccines and investigate virus infection mechanisms.

First, DNA and recombinant vaccines encoding two antigen-candidate genes, which have been reported to be antigens in Japanese yellowtail by Matsuyama et al. (2018), were evaluated in Red Sea Bream (*Pagus major*). DNA vaccines were prepared for two candidate antigen genes, ORF 111 and 450. These vaccines were administered intramuscularly to juvenile red sea bream. Four weeks after inoculation, 10^3 and 10^4 copies of the virus were intraperitoneally injected. In the group injected with 10^4 copies, the survival rate of the DNA vaccine group was 89%, compared to 80% in the control group, whereas the survival rates of the DNA vaccine and PBS group were 72% and 80% in the fish injected with 10^3 copies. No increase in antibody titers against each antigen was observed. A recombinant protein for the candidate antigen gene ORF111 was prepared, and the effects of the DNA vaccine and recombinant protein vaccine were compared. Challenge tests were conducted using 10^4 copies of the virus at 4 weeks post-vaccination. Survival rates of 98% and 88% were observed in the DNA vaccine and recombinant protein groups, respectively, with no deaths in the control group. The recombinant protein vaccine increased specific antibody titers against the antigen, but the results of the challenge test did not allow for a conclusive evaluation of the effectiveness of each vaccine.

Second, to investigate how RSIV replicates in the host and how the host responds to the virus, comprehensive gene expression analysis was performed during RSIV infection in rock bream (*Oplegnathus fasciatus*), a species particularly susceptible to the virus. After intraperitoneal injection of the viral solution at 10^4 TCID50/fish, the viral genome copy

number in the spleen was $10^{4.7 \pm 0.2}$ and $10^{5.9 \pm 0.4}$ copies/μg DNA at 3 and 5 days post-injection (dpi), respectively. By transcriptomic analyses using MiSeq, 6 viral genes, including RING finger domain-containing protein and laminin-type epidermal growth factor-like domain genes, were strongly detected at 3 and 5 dpi. The other virus genes were significantly expressed at 5 dpi. By differentially expressed gene analysis, 334 host genes were identified in comparison to those before infection. They were clustered into four groups based on their expression profiles. Among the immune-related genes, interferon-stimulated genes were strongly upregulated. On the other hand, inflammation-related genes, such as granzyme and eosinophil peroxidase genes were downregulated at 3 dpi. Downregulation of certain genes may contribute to the susceptibility of this fish to the virus.

Although DNA vaccines are important for the future development of viral vaccines, the results suggest that their effectiveness may be weak depending on the choice of antigen. In addition, the expression dynamics of viral genes *in vivo* differ from gene to gene, and the results obtained in this study will contribute to future vaccine development. Furthermore, by analyzing the details of the genes whose expression changes with viral infection, it will be possible to develop more effective DNA vaccines through the development of new adjuvants and other means.

Chapter 1

General Introduction

1. Japanese aquaculture

Japan is an island country with > 30 thousand kilometers of coastline. Japan has one of the world's largest exclusive economic zones, covering an area of more than 4 million km^2. Furthermore, the combination of warm and cold currents that flow down the beaches in Japan creates one of the most productive fishing areas in the world. The peak in fish production in Japan since 1988 which high reached 11.2 million tonnes. Japanese marine fisheries are separated into three categories for statistical convenience: offshore, coastal, and distant-water fisheries (FAO, 2024). The marine culture species included Japanese amberjack (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), red seabream (*Pagrus major*), and bluefin tuna (*Thunnus orientalis*) (FAO, 2024).

2. Red sea bream iridovirus

Red sea bream iridovirus (RSIV) is the pathogen causing red sea bream iridovirus disease (RSIVD). The diseased fish displays signs of death, extreme anemia, and depression. The pale gills and misaligned swimming fins are visible through clinical observation. The spleen was enlarged upon necropsy. The histopathology of the lesion shows swelling of the kidney, liver, spleen, and gill cells. Lymphocyte aggregation in cerebral and kidney tissue, along with congestion is further observed. The disease is significant because it can infect over thirty species, including black sea bream, black porgy, amberjack, barramundi, and black rockfish. The high susceptibility of the rock beam (*Oplegnathus sp.*) to this virus is widely recognized (Kawato et al., 2017; OIE, 2019). Moreover, systemic viral proliferation is observed in the organs such as kidney and spleen, as well as the liver, heart, brain, and eyes (Hajime et al., 2009).

The first outbreak of the Red Sea bream iridovirus disease was documented in 1990 on Japan's Shikoku Island (OIE, 2019). Recently, disease incidences from this virus have been reported in many countries, particularly in East and Southeast Asia. Additionally, a disease outbreak resulted in significant financial losses. Thus, the World Organization of Animal Health (WOAH) listed the RSIVD. There is no information on infection by vertical transmission, but horizontal disease transmission occurs when an infected fish sheds virus particles into the environment and infects another fish nearby (OIE, 2019).

RSIV is a member of the Iridoviridae genus Megalocytivirus. The virus causes mass mortality by producing characteristic basophilic inclusion bodies in the enlarged cells of host fish organs in both wild and cultured fish species (Gibson-Kueh et.al., 2003). The primary capsid protein, which forms the icosahedral viral particle 160–180 nm has an envelope-like structure (Kawato et al., 2023). The viral genome is double-stranded DNA, approximately 110 kbp, and has approximately 120 potential open reading frames (ORFs) (Do et.al.,2004; Kawato et al., 2017).

3. Red sea bream iridovirus protection

According to WOAH, red sea bream, striped jack, and Seriola species should be protected against RSIVD using a formalin-killed vaccine against RSIV, which is commercially available in Japan (Dong et.al.,2013; Caipang et al., 2006; Nakajima et al., 1999). However, other types of vaccines against RSIV, such as attenuated lived vaccines (So-Young et al., 2014), recombinant protein vaccines (Hajime et al., 2010), and DNA vaccines (Park et al., 2005, Caipang et al., 2006), have also been reported. Among those, DNA vaccines can elicit cellular as well as humoral immune responses (Park et al., 2005, Caipang et al., 2006).

Therefore, this vaccine can increase resistance to infection. Furthermore, DNA vaccines require little preparation time and are stable.

4. Immune response to vaccine

The innate and adaptive immune systems are two interrelated systems that work together to mount a defensive response against pathogen invasion. The innate immune response is non-specific and rapidly targets pathogens through the active involvement of various cells in their elimination. Phagocytic cells such as neutrophils, eosinophils, and macrophages, and other cells such as natural killer cells are commonly involved in the innate immune response. On the other hand, the adaptive immune system uses specially designed receptors to identify foreign invaders based on their unique antigens and responds more slowly, typically over several days. This slower process is orchestrated by the combined actions of lymphocytes, including T cells and B cells (Marshall et.al., 2018).

Vaccination-induced immune responses begins when a host is exposed to a vaccine, as shown in Figure 1. First, phagocytic cells such as dendritic cells and macrophages recognize the vaccine particle through the phagocytosis pathway. Two pathways are then initiated (Pollard and Bijker, 2021):

In the first pathway, antigen-presenting cells (APCs) deliver the protein fragment via MHC class II to $CD4^+$ T cells, also known as helper T cells. Helper T cells then stimulate naive B cells, leading to their differentiation into plasma cells for antibody production and memory B cells for future responses.

Another pathway involves the activation of $CD8^+$ T cells, or killer T cells, either directly by antigen presentation via MHC class I, or indirectly by signals from helper T cells. These activated $CD8^+$ T cells differentiate into effector T cells that play a role in eliminating memory T cells and infected cells.

5. Immune response to DNA vaccine

DNA vaccines generally involve the injection of a plasmid for eukaryotic expression into the host muscle. The immune response to a DNA vaccine is triggered by the uptake of the plasmid DNA by myocytes or keratinocytes. These cells can produce the antigenic protein and present the antigenic peptides via MHC class I. In addition, these antigen-expressing cells undergo apoptosis and are eliminated by phagocytes, and the antigens are then presented via MHC class II. In addition, phagocytes such as macrophages or dendritic cells also take up the plasmid DNA and produce antigens that are presented via MHC class I and class II. The mechanism is shown in Fig. 2. However, these pathways remain uncertain in aquatic animals (Hobernik and Bros, 2018; Kowalczyk and Ertl, 1999). Fish that receive a DNA vaccine exhibit a slightly distinct immune response, with the thymus, gills, spleen, and kidneys being the primary sites of stimulation, particularly in small fish. (Hølvold et.al.,2014)

6. Objectives of this research

DNA vaccines could be more effective against viral infections. This study is to develop a more effective DNA vaccine, To elucidate the mechanisms by which the vaccine can shield

fish from RSIVD and explore the possibility that specific antigen candidates shield red sea bream from the disease. In addition, fundamental information on the mechanism of RSIV infection in fish was evaluated for the future development of effective DNA vaccines.

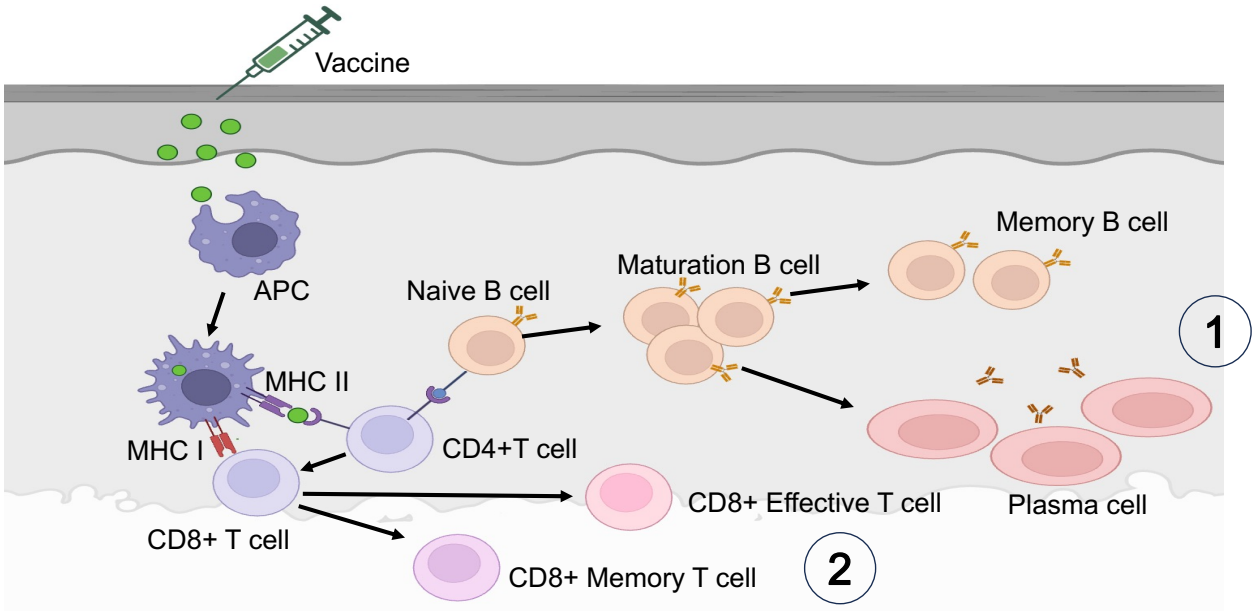

Fig. 1 Schematic illustration of the host's response to the vaccine uses in human and mammalian. Following immunization, B cells are primarily activated to produce antibodies and memory B cells, thereby priming the host for future infection (1) and illuminating the activated T cell pathway (2), which is a minor pathway. The figure was adapted from Pollard and Bijker (2021) with some modifications.

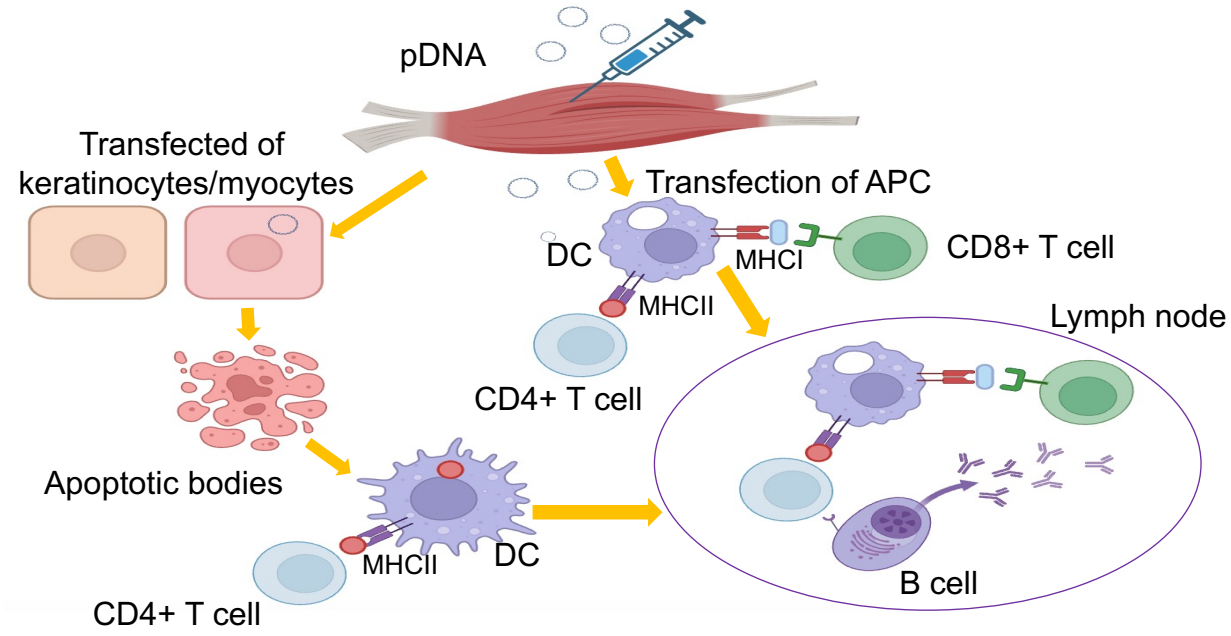

Fig. 2 Schematic illustration of the response to plasmid DNA injection in mammals. The plasmid DNA is taken up by cells like phagocytes and keratinocytes, along with the activation of the host defense pathway against foreign bodies of DNA vaccine. The figure was adapted from Hobernik and Bros (2018) with some modifications.

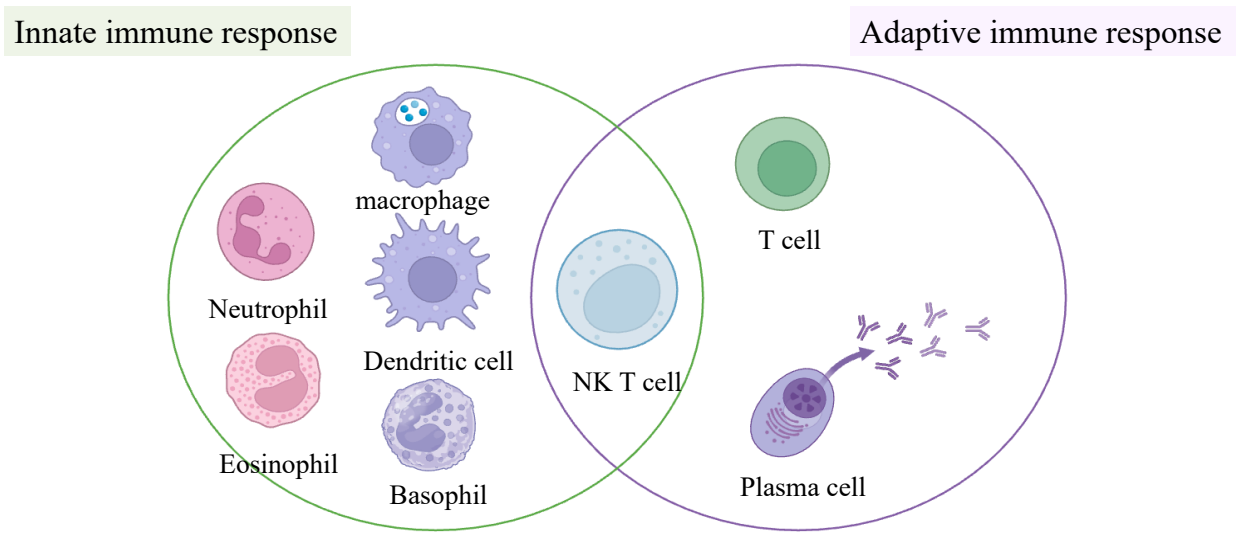

Fig. 3 Schematic illustration of the immune-related cells, including those related to innate and adoptive immune responses. The figure was adapted from Alam (2008) with some modifications.

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Chapter 2

Development of DNA Vaccines Using Novel Antigen Candidate Genes of RSIV in Red Sea Bream (*Pagrus major*)

Abstract

Red seabream iridovirus disease (RSIVD) has caused significant financial losses to Japanese aquaculture. Although formalin-inactivated vaccines against the disease have been approved and used in Japan, there is a need for the development of less expensive and more effective vaccines. DNA vaccines have the potential to be an alternative to formalin-inactivated vaccines. Therefore, the study's objectives were to develop a DNA vaccine by using novel antigen-candidate genes. Based on previous studies, 2 genes, ORF111R and ORF450L of RSIV were selected for the plasmid DNA preparation. The plasmids were intramuscularly injected into rock bream (*Oplegnathus fasciatus*). In the muscle, ORF450L expression was observed but that of ORF111R was marginal. Four weeks after immunization of red seabream (*Pagrus major*) with the DNA vaccine, antibody titers were measured, and a challenge test was performed. There was no difference in antibody titers compared to the control group. The challenge test showed a mortality rate of approximately 20% in the control group, so the differences could not be compared. Furthermore, ORF111R was used to compare the efficacy of DNA and recombinant protein vaccines. The recombinant protein vaccine increased antibody titers compared to the DNA vaccine. In the challenge test, mortality rates were low and differences in efficacy could not be compared.

Keywords: plasmid DNA vaccine, red sea bream (*Pagrus major*) , red sea bream iridovirus, ELISA

1. Introduction

Red seabream iridovirus disease (RSIVD) has caused significant financial losses to Japanese aquaculture. The most efficient strategy for controlling RSIVD is vaccination along with rapid and accurate diagnostic techniques (Puspasari and Widowati, 2022). To prevent RSIVD, formalin-inactivated vaccines have been researched (Nakajima et al. 1990). Recently, the vaccine has been approved and commercially available in Japan.

Although formalin-inactivated vaccines against the disease have been approved and used in Japan, there is a need for the development of less expensive and more effective vaccines. There are reports such as an attenuated vaccine (So-Young et al., 2014) and a recombinant vaccine (Hajime et al., 2010) as alternatives to formalin-inactivated RSIV vaccines. Among the candidates, DNA vaccines have the potential to be an alternative to formalin-inactivated vaccines. A DNA vaccine is a vaccination strategy in which a eukaryotic expression plasmid carrying an antigenic gene is injected into the host muscle to induce the expression of antigenic proteins and confer immunity (Bolhassani and Yazdi, 2009: Kutzler and Weiner, 2008). A DNA vaccine is considered to elicit both humoral and cellular immune responses by engaging with antigens on MHC class I and class II, which are subsequently recognized by $CD4^+$ and $CD8^+$T cells responsively (Pagliari et al., 2023). Furthermore, a DNA vaccine is less costly than traditional vaccinations (Shafaati et al., 2022). The DNA vaccine has proven to be effective in preventing several diseases that affect humans, such as rabies, herpes simplex virus, and malaria (Calarota et al., 1998; Chattergoon et al., 1997, MacGregor et al., 1998).

Development of DNA vaccines has also been reported for RSIVD (Park et al., 2005, Caipang et al., 2006). These vaccines prevent the mortality caused by RSIV, but the antigen genes cannot protect completely against the disease. There have been several reports on the antigen identification for RSIV. Matsuyama et al. (2018) reported that among 72 RSIV ORF genes, some were strongly detected by the serum from convalescent Japanese amberjack (*Seriola quinqueradiata*). Normally, formalin-inactivated vaccines against RSIV have low antibody induction ability. Using some antigen candidates, Antibodies induced by formalin-inactivated vaccines can be detected significantly (Sato, 2022). Sato (2022) also showed that the recombinant ORF111R and 450L can induce strong antibody responses in red sea bream.

From the previous study, ORF111R and 450L might be candidates for protective antigens against RSIV. Therefore, the objectives of this chapter are to produce DNA vaccines using novel candidate genes and evaluate the vaccine efficacy by quantifying the antibody titer and challenge test.

2. Materials and methods

2.1 Preparation of DNA vaccines

2.1.1 Preparation of the Plasmids

DNA was extracted from an infected Japanese amberjack spleen using NucleoSpin® Tissue (MACHEREY-NAGEL GmbH & Co. KG, Germany). The RSIV target genes were then amplified using the DNA and the primers in Table 1. DNA fragments encoding ORF111R and ORF450L were amplified using TaKaRa ExTaq™ DNA Polymerase following the manufacturer's protocol. The mixture containing 2 μl of 10x Ex Taq buffer, 1.6 μl of dNTP, 0.4 μl of forward and reverse primer, 0.1 μl of ExTaq, 13.5 μl of distilled water, and 1 μl of the DNA was applied to 30 cycles of PCR reaction at 1 minute at 95 °C, 30 seconds at 55 °C, and 1 minute of 72 °C, and additional 1-minute reaction at 72 °C. The PCR products were confirmed by electrophoresis.

PCR products were purified using NucleoSpin® Gel accordint to the manufacturer's protocol. The concentration was determined using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, USA). Similarly, PCR was performed using the primers in Table 1 to amplify the linearized the pcDNA™3.1/myc-His B vector (Invitrogen, USA). The PCR products for the virus genes were inserted into the linearized vector by using In-Fusion HD (Takara, Japan) following the manufacturer's protocol. The ligated products were used for the transformation of *E. Coli* JM109. The bacteria were cultured on LB agar plate (1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, 1.5% Agar, and 0.01% Ampicillin) at 37 °C overnight. The inserted DNAs were confirmed by PCR and sequencing using ABI PRISM 3130xl Genetic Analyzer (Life Technologies, USA).

The clones containing the vectors were cultured in 5 mL of LB liquid medium and inoculated into 200 mL of 2xYT/Amp (1.6% Tryptone, 1% Yeast Extract, 0.5% NaCl, and 0.01% Ampicillin) medium for large-scale plasmid preparation. The bacterial cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4˚C. The cells were suspended in solution I (5 ml of 50 mM glucose, 25 mM tris-HCl (pH 8.0) and 10 mM EDTA), lysed by solution II (5 ml of 0.2 N NaOH and 5 ml of 2% SDS), and neutralized using solution III (7.5 ml of 5 M CH_3COOH and 3 M CH_3COOK). After 15 minutes of centrifugation at 9,000 rpm at 4˚C, the supernatant was collected. Following adding isopropanol at a final concentration of 50 %, the precipitate was collected by centrifugation at 7,000 rpm at 4˚C for 15 minutes. After the pellet was resuspended in 3 mL of TE solution (Tris-EDTA buffer) and allowed to sit at 37˚C for an hour, RNA was degraded using RNase. After adding 3.88 g of $CsCl_2$ and 100 μl of EtBr, the solution was added to the OptiSeal tube (Beckman Coultuer, USA). After an ultracentrifugation for 20 hours at 65,000 rpm at 22˚C, the plasmid DNA was collected. The solution was treated with saturated 2-isopropanol to remove EtBr and then dialyzed against TE buffer using a dialysis membrane size 20 (FUJIFILM Wako Pure Chemical Corp., Japan). The DNA concentration was measured using Qubit 2.0 Fluorometer (Life Technologies, Canada) and the solution was stored at -30 °C until use.

2.1.2 Confirmation of gene expression by the Plasmids *in vivo*

Rock bream (*Oplegnathus fasciatus*) was intramuscularly injected with mixed DNA vaccines for 5 μg each of ORF111R and ORF450L. 3 and 7 days after the vaccination, muscles were collected. The mRNA levels were evaluated with the primers shown in Table 2. The muscle was used for total RNA extraction using RNAisoplus according to the

manufacturer’s protocol. cDNA was synthesized using an oligo (dT) primer and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer’s protocol. cDNA fragments encoding ORF111R and ORF450L were amplified using TaKaRa ExTaqTM DNA Polymerase following the manufacturer's protocol. The mixture containing 2 µl of 10x Ex Taq buffer, 1.6 µl of dNTP, 0.4 µl of forward and reverse primer, 0.1 µl of ExTaq, 13.5 µl of distilled water, and 1 µl of the cDNA was applied to 35 cycles of PCR reaction at 1 minute at 95 °C, 30 seconds at 55 °C, and 1 minute at 72 °C, and an additional 1-minute reaction at 72 °C. The PCR products were confirmed by electrophoresis. To ensure that the total RNA sample is free of the plasmid DNA, total RNA samples were also used as a template for the same PCR procedure.

2.2 Recombinant protein preparation

ORF111R and ORF450L recombinant proteins were produced using *E.coli* strain BL21DE3Lys carrying the corresponding pColdII vector (Sato, 2022). The bacteria were cultured in 5 mL of LB medium overnight. In the case of ORF111R, the cultured medium was inoculated into 50 mL of LB medium and further cultured for 3 hours at 37 °C. The pre-cultures were subsequently inoculated into 200 mL LB medium containing 0.1 mM IPTG and cultured at 15 °C overnight. The bacterial cells were harvested by centrifugation at 6,000 rpm for 10 minutes at 4 °C. The precipitates were then suspended in 5 mL B-PER™ Complete Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, USA) and incubated for 30 minutes at room temperature. After centrifugation for 10 minutes at 8,000 rpm, the supernatant was collected. The recombinant protein was purified using HisTrap FF (Cytiva) by elutiong

using 0.5 M imidazole in PBS. The purified protein was dialyzed against PBS twice. The samples were kept at -80 °C.

The recombinant proteins were analyzed by SDS-PAGE. The purified protein was mixed with an equal volume of 2X SDS-PAGE sample buffer (1M Tris-HCl pH 6.8, 10% Sucrose, 10% SDS, and 0.01% Bromophenol Blue) and then incubated at 95˚C for 5 minutes. The running gel was prepared by mixing 3.3 mL of H_2O, 4.0 mL of 30% acrylamide mix, 2.5 mL of 1.5M Tris pH 8.8, 0.1 mL of 10% SDS, 0.1 mL of 10% APS, and 4 µL of TEMED, and the stacking gel solution was prepared by mixing 1.4 mL of H2O, 0.33 mL of 30% acrylamide mix, 0.25 mL of 1M Tris pH 6.8, 20 µL of 10% SDS, 20 µL of 10% APS, and 2 µL of TEMED. The electrophoresis was performed with electrophoresis chamber AE-6500 (ATTO, Japan) with the running buffer (25 mM Tris, 192 mM Glycine, and 0.1% SDS). Following electrophoresis, the gel was stained with 10 mL of EzStain Aqua (ATTO, Japan). The photo was taken using the WSE-5300 Printgraph CMOS I (ATTO, Japan).

2.3 Virus preparation

The Kyoritsu Seiyaku Cooperation kindly gifted the RSIV RIE12-1 strain. The TCID50 of the virus solution was calculated by the Behrens-Kärber method using the S-1F cell derived from the caudal fin of rock breams cultured in L-15 medium (Wako, Japan) supplemented with 5% fetal bovine serum.

2.4 Vaccination

2.4.1 The First Vaccine Trial

Experimental groups were prepared for intramuscular injections of phosphate buffer saline (PBS), empty vector, and DNA vaccine, with 50 fish in each group. For the empty vector, 10 μg of pcDNA^{TM}3.1/myc-His B vector was used, and for the DNA vaccine, 5 μg each of ORF111R and ORF450L DNA vaccines were mixed. Each fish was intramuscularly injected with 100 uL of the solution. Four weeks after the vaccination, blood and spleen were collected, and the challenge test was conducted by intraperitoneal injection of 0.1 mL of 1.0 x 10^4 TCID50/ml for the low dosage and 1.0 x 10^5 TCID50/ml for the high dosage of virus copies.

2.4.2 The second vaccine trial

Experimental groups were prepared for intramuscular injections of PBS, empty vector, and DNA vaccine, and intraperitoneal injection of PBS and recombinant protein, with 22 fish in each group. In this experiment, the DNA vaccine and recombinant protein for ORF111R were used. Four weeks after the vaccination, blood was collected, and the challenge test was conducted four weeks after the vaccination by intraperitoneal injection of 0.1 mL of 1.0 x 10^5 TCID50/ml.

2.5 Indirect ELISA

One μg/100 μl well of recombinant protein was added to each well of 96 well plate Immulon 1B (Thermo Fisher Scientific, USA) and incubate at 4˚C overnight. The plate was

then washed 5 times with 150 µL of PBS-T (0.05% Tween-20 in PBS) and blocked with 3% skim milk. After one hour at room temperature, the plate was washed with PBS-T 5 times. Then, 100 µl of red sea bream serum diluted at 1:20 with 1% skim milk in PBS-T was added to the well, followed by incubating for 1 hour. The plate was washed with PBS-T 5 times and incubated with 100 µl of anti-red sea bream IgM-rabbit antiserum diluted with PBS-T containing 1% skim milk at 1:10,000 for 1 hour at room temperature. After washing with PBS-T 5 times, 100 µl of diluted anti-rabbit IgG (Fc) AP Conjugate (Promega Corporation, USA) diluted with PBS-T containing 1% skim milk at 1:5,000 was added and incubated for 30 minutes at room temperature. Then the plate was washed with PBS-T 5 times, and subsequently 100 µl of the substrate (phosphatase buffer: Phosphatase substrate tablet (Sigma-Aldrich, USA), 9.7% Diethanolamine, and 0.104% 1M MgCl in sterile distilled water, pH 9.8) was added. The reaction was stopped with 30 µl of 3M NaOH, and absorbance was measured at 405 nm using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, USA).

2.6 qRT-PCR for MHC class I and II

The mRNA levels for MHC class I and MHC class II were evaluated with the primers shown in Table 2. The spleen was used for total RNA extraction using RNAiso plus according to the manufacture's protocol. cDNA was synthesized using an oligo (dT) primer and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacture's protocol. qPCR was performed using primers shown in Table 2 and using THUNDERBIRD™ Next SYBR® qPCR Mix (TOYOBO, Japan) with a QuantStudio 1 real-time PCR system (Thermo Fisher Scientific, USA) according to the manufacture's protocol. The reaction was performed at 95 °C for 30 seconds followed by 40 cycles of 5 seconds at

95 °C and 30 seconds at 60 °C. The relative mRNA levels were calculated by ΔCtusing the EF-1α and β-actin genes as internal controls (Vandesompele et al., 2002). A one-way analysis of variance (ANOVA) was used for the statistical analysis.

3. Results

3.1 DNA expression

The sequences of the plasmids carrying ORF111R and ORF450L genes were confirmed. Then the plasmids were intramuscularly injected into the fish, and mRNA expressions of the genes were confirmed by RT-PCR (Fig. 1). However, ORF450L mRNA levels seemed to be higher than those of ORF111R. To confirm the contamination of plasmids total RNA was used as a template for PCR, which showed no contaminated plasmid DNA.

3.2 Vaccine trials

In the first vaccine trial, the survival rates of the combined DNA vaccine and PBS were 78.6 % and 69.2 %, respectively, at a low dose challenge (10^4 TCID50/ml). Whereas at high dose challenge (10^5 TCID50/ml.), the survival rates of the combined DNA vaccine and PBS were 78.6 % and 64.3 %, respectively. No statistical significances were observed among the groups (fig.2).

In the second vaccine trial, the survival rate of the DNA vaccine group was 95.2%, and that of the recombinant protein group was 86.4%. The survival rates of intramuscularly and intraperitoneally PBS-injected groups showed 100% and 81.82%, respectively. No statistical significances were observed among the groups (fig.3)

3.3 Antibody level measurement with indirect ELISA

The recombinant proteins for ORF111R and ORF450L were produced (Fig. 4). Using the proteins, specific antibody titers were measured by ELISA. In the first vaccine trial, antibody titers against ORF111R and ORF450L were not significantly different among groups (Fig. 5).

In the second vaccine trial, the recombinant protein group showed the highest antibody titer among the groups (fig 6).

3.4 MHC class I and II expression

MHC class I and class II expressions in the first vaccine trial are shown in Fig. 7. The MHC class I mRNA levels did not change before and after the vaccination (Fig. 7A). On the other hand, the MHC class II mRNA levels increased in the empty vector and DNA vaccine groups after 4 weeks post-vaccination (Fig. 7B).

4. Discussion

RSIV is still an important disease that impacts aquatic cultures. Although formalin-inactivated vaccines have been approved and used in Japan, there is a need for the development of less expensive and more effective vaccines. Based on previous studies, the advantages of the DNA vaccine include inducing both a humoral immune response and a cellular immune response. Moreover, the process of DNA vaccine production is less expensive than the formalin-killed vaccine (Ledesma-Feliciano et.al., 2023). Therefore, DNA vaccine is interested in development in the future, such as efficacy candidate design, improved vaccine delivery, and improved mechanisms of action in the fish body.

The results showed that DNA expression was different while the amount of plasmid injection was the same. According to the plasmid DNA mechanism in the mammal model, those plasmids can be taken in the target cells, such as myocytes or keratinocytes, including groups of antigen-presenting cells. Then the cells produce the antigenic peptides. Whereas in this study shown both candidates could be expressed but the expression of the DNA was different. It indicated that plasmid DNAs in test could entries to host cell. However, it couldn't distingue amount of candidate cooperative process into the cells. Moreover, Wang et al. (2004) reported that the combination of electroporation and intramuscular DNA vaccine injection may lead to a notable improvement in immunization efficacy. This implies that administering vaccines can increase their effectiveness.

In the first vaccine trial, the survival rates were not different between the DNA vaccine and the control group. Furthermore, in the second vaccine trial, the survival rate did not show a significant difference among the groups. In the challenged test, the virus was inoculated at two different doses: high and low. A low dose (10^4 TCID50/ml) was chosen according to the

challenged dose to evaluate the vaccine efficacy compared to DNA vaccines (Caipang et al., 2006). Furthermore, the high survival rate among the groups in this study was related to the sensitivity of fish to viruses, which reported the transmission of viruses from infected flathead grey mullet to other fish species such as flathead grey mullet, red sea bream, and rock bream (Kim et. al., 2022). For future studies, choosing the sensitivity species was a suggestion.

The post-mixed candidate vaccination was administered to the juvenile fish and compared to the control groups. The fish serum was tested with indirect ELISA to detect the specific antibody level against ORF111R and ORF450L, and both ORFs were shown to be not different among the test groups. Chang et al. (2017) reported the protection and antibody response of two candidates of the DNA vaccine against salmonid alphavirus 3. Different DNA showed different antibody levels. However, designing the DNA vaccine is critical to the vaccine's future development.

The effect of the DNA vaccine was compared with the recombinant protein for ORF111R. The result of indirect ELISA demonstrated that recombinant proteins could generate antibody levels more effectively than DNA vaccination. The mechanism of immune response activation of recombinant protein was occurring past antigen-presenting cells which will signal to $CD4^+$ T cell. DNA vaccine had to enter the cell. This indicated that the amount of plasmid DNA that was taken in the cell and the product of antigen proteins were not enough to elicit the $CD4^+$ T cell-dependent pathway.

The MHC class I expression level was found to be unchanged in all test groups that were evaluated (DNA vaccine, EMP, and PBS groups), including before vaccination. The expression of MHC class I was related to the cell-mediated immune response. In contrast, Caipang et al., (2006) showed that the expression of MHC class I for the DNA vaccine and

formalin-killed vaccine groups was higher than the PBS and empty vector injected groups. Moreover, Yewdell and Del Val (2004) reported the half-life of MHC class I related to cell interaction such as peptide-MHC complexes which more important in cross-priming than direct priming.

The expression of MHC class II showed a slight increase post-vaccination in the empty vector and DNA vaccine group compared to the other groups. Caipang et al., (2006) showed that there was variation in MHC class II expression post-vaccination. Many studies have shown the success of vaccination which the vaccine particle contain with the MHC class II specific binding and pathogen in another site such as DNA vaccines, which could induce antibody protection against influenza A in mice (Grodeland et al., 2013).

In conclusion, based on previous studies, ORF111R and ORF450L were used to produce DNA vaccines against RSIVD and evaluate their efficacy. Although the DNA vaccines seem to be transcribed in vivo, they did not show any significant effects in red sea bream. Therefore For better results, studies should be conducted with more susceptible fish species.

Table 1. List of primers for conventional PCR for DNA vaccine development

Product name	Product size (bp)	Primer name	Nucleotide Sequence （5'->3'）
Primers for plasmid preparation			
ORF111R	945	ORF111R_pcDNA3.1_F	TCCACTAGTCCAGTGATGTCATCGTACCGGTGCCG
		ORF111R_pcDNA3.1_R	CGAGCGGCCGCCACTAATTGGGAAGAGTTCCG
ORF450L	1377	ORF450L_pcDNA3.1_F	TCCACTAGTCCAGTGATGTACAGCCTACTTGAGAT
		ORF450L_pcDNA3.1_R	CGAGCGGCCGCCACTATAATCATCGTCATCAA
Primers for preparation of linearized vector			
		pcDNA31mycHisb-F	CACTGGACTAGTGGATCCGAGCTC
		pcDNA31mycHisb-R	AGTGGCGGCCGCTCGAGTCTAGAG
Primers for insert check and sequencing of the vector			
		T7 promotor (forward)	TAGAAGGCACAGTCGAGG
		BGH (reverse)	TAATACGACTCACTATAGGG
Primers for gene expression analysis			
ORF111R	300	ORF111R_F	TTGGCCGGTACTCTGGTTTC
		ORF111R_R	TGTACAGGTCAGGGGATCGT
ORF450L	300	ORF450L_F	CTCGTCTGGTGGCACGTATT
		ORF450L_R	CCAATGTACACTTGTGCGGC

Table 2. List of primers for RT-qPCR

Product name	Product size (bp)	Primer name	Nucleotide Sequence （5'->3'）
MHC class I	102	MHCI-F	CACCATCGCCATCGTTGCAG
		MHCI-R	CTGGATGAAGCGCAGTTGGC
MHC class II	116	MHCII-F	GGTTCTGGTTCAGGTCCACAGA
		MHCII-R	GAGCGTCAGCTGAGACACAGA
EF-1 α	130	EF-1α-F	CCTTCAAGTACGCCTGGGTG
		EF-1α-R	CTGTGTCCAGGGGCATCAAT
β actin	113	b-actin-F	GGGACAGAAGGACAGCTACG
		b-actin-R	CTTCTCCATGTCGTCCCAGT

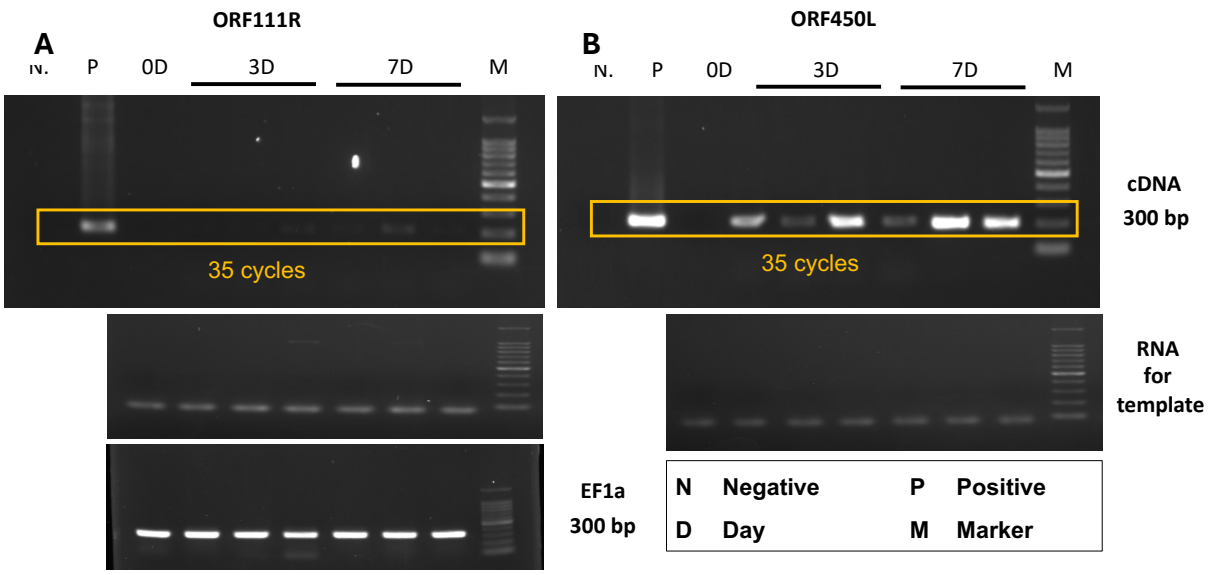

Fig. 1 mRNA expression of the ORF111R (A) and ORF450L (B). cDNA and total RNA were used as templates., EF-1α was used as a housekeeping gene. Samples were collected before and after the vaccination on the indicated days.

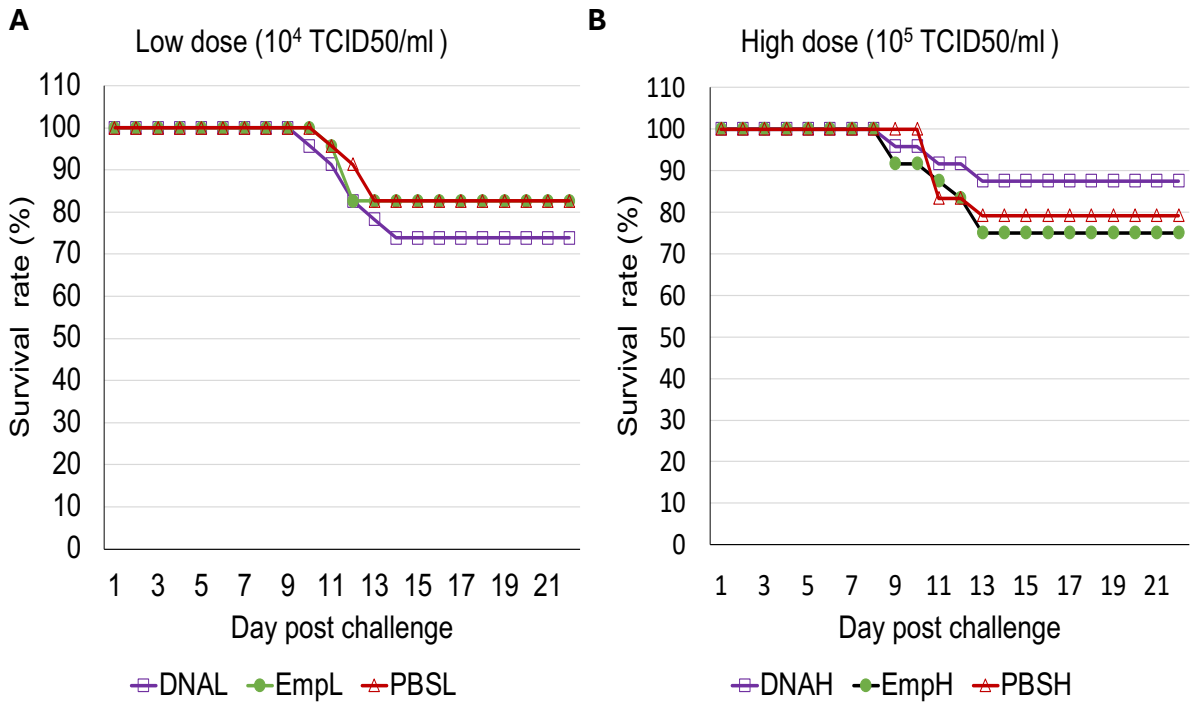

Fig. 2 Survival rate after challenged low dose (10^4TCID50/ml.) (A) and high dose (10^5TCID50/ml.) (B) in the first vaccine trial.

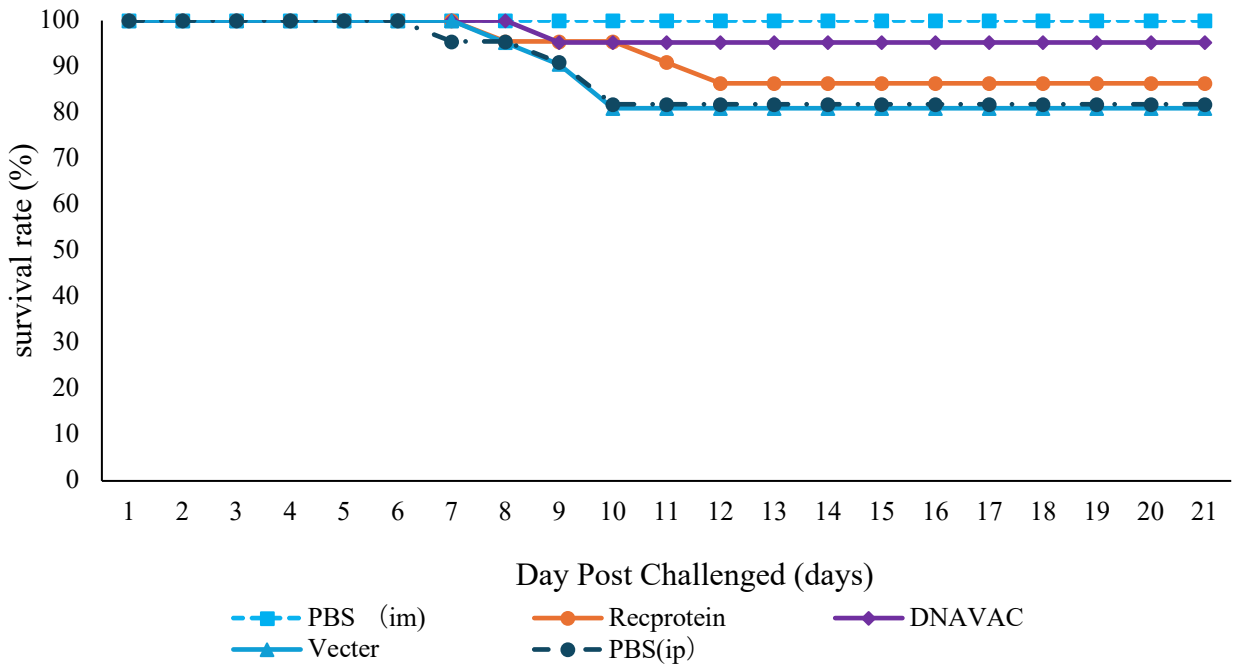

Fig. 3 Survival rate post-challenged test with 10^5 TCID50/ml.in each group in the second vaccination trial.

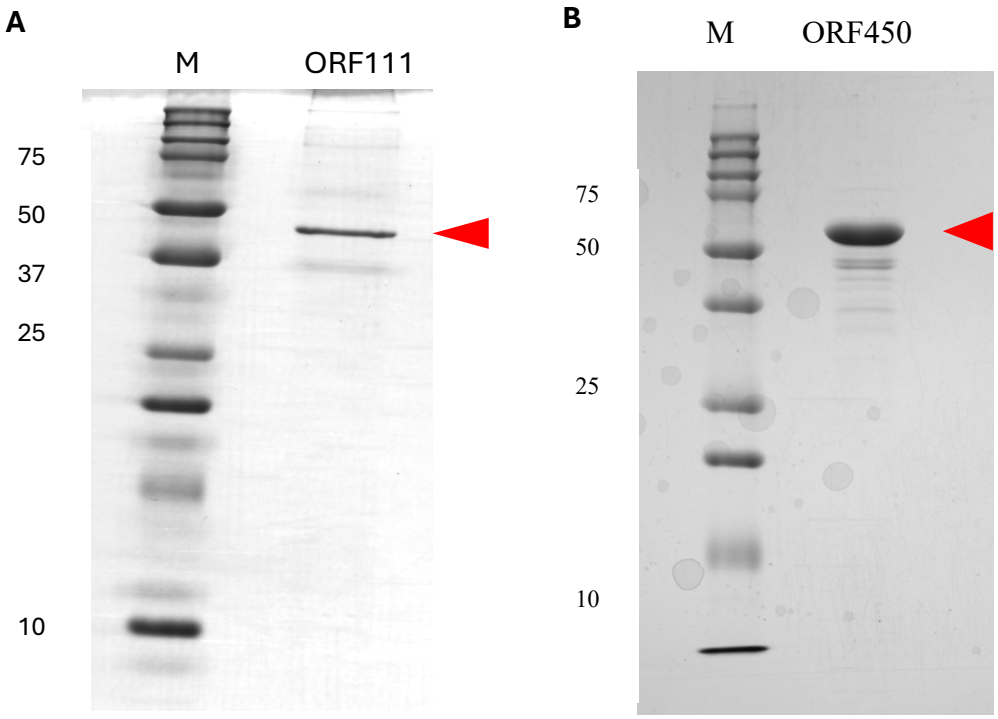

Fig. 4 SDS PAGE result after protein purification.

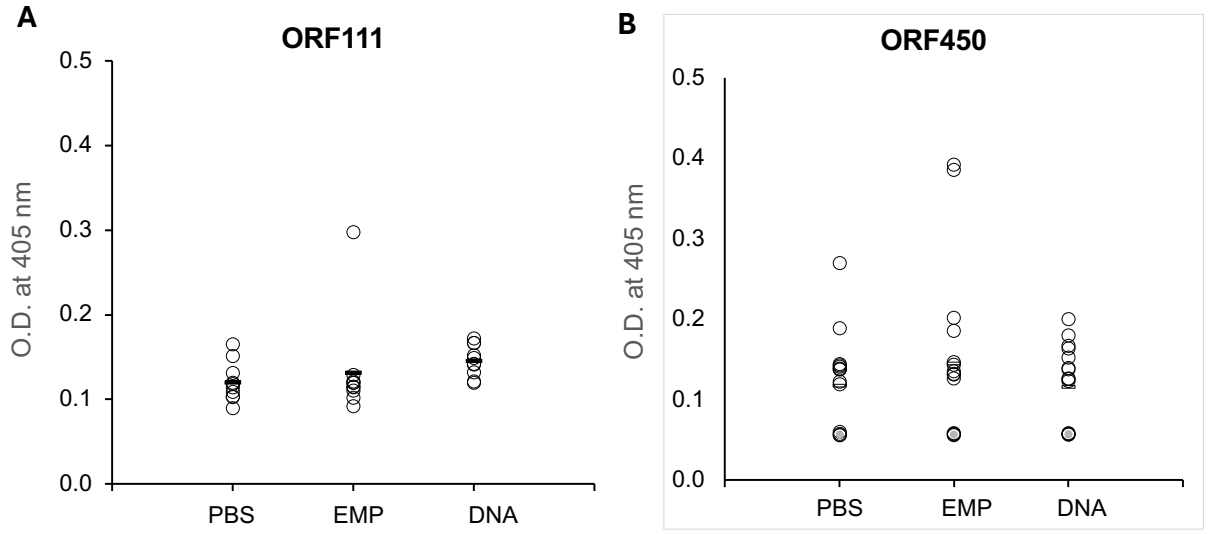

Fig. 5 Indirect ELISA test of ORF111R and ORF450L. Graph shown comparison of antibody level ORF111R (A) and ORF450L (B) in each group in the first vaccine trial.

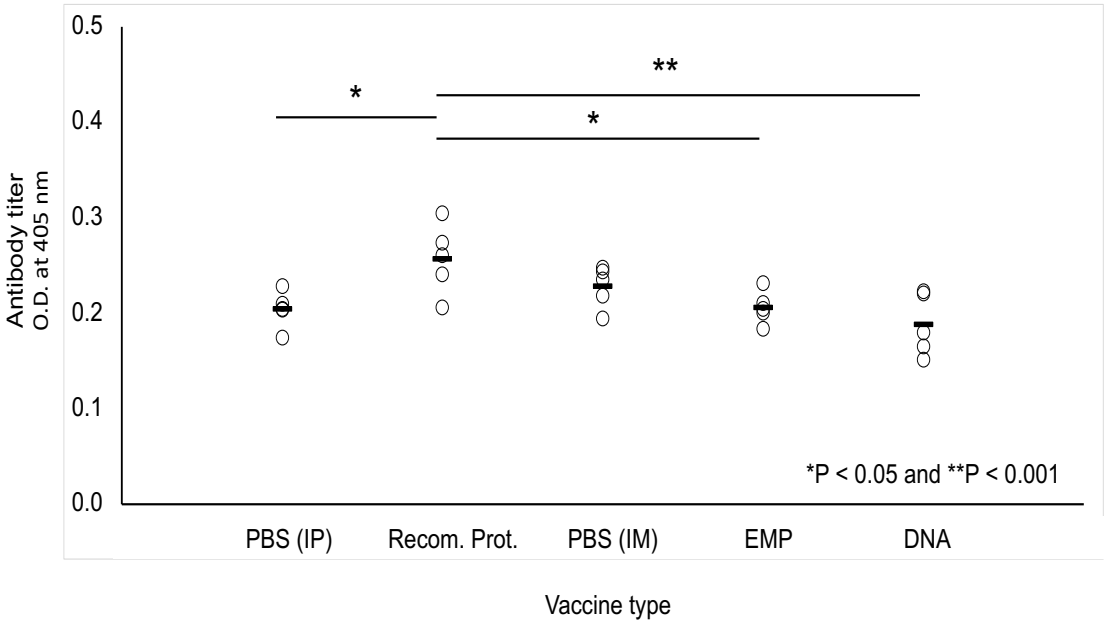

Fig. 6 Indirect ELISA test. Graph shown comparison of antibody level ORF111R in each group in the second vaccine trial.

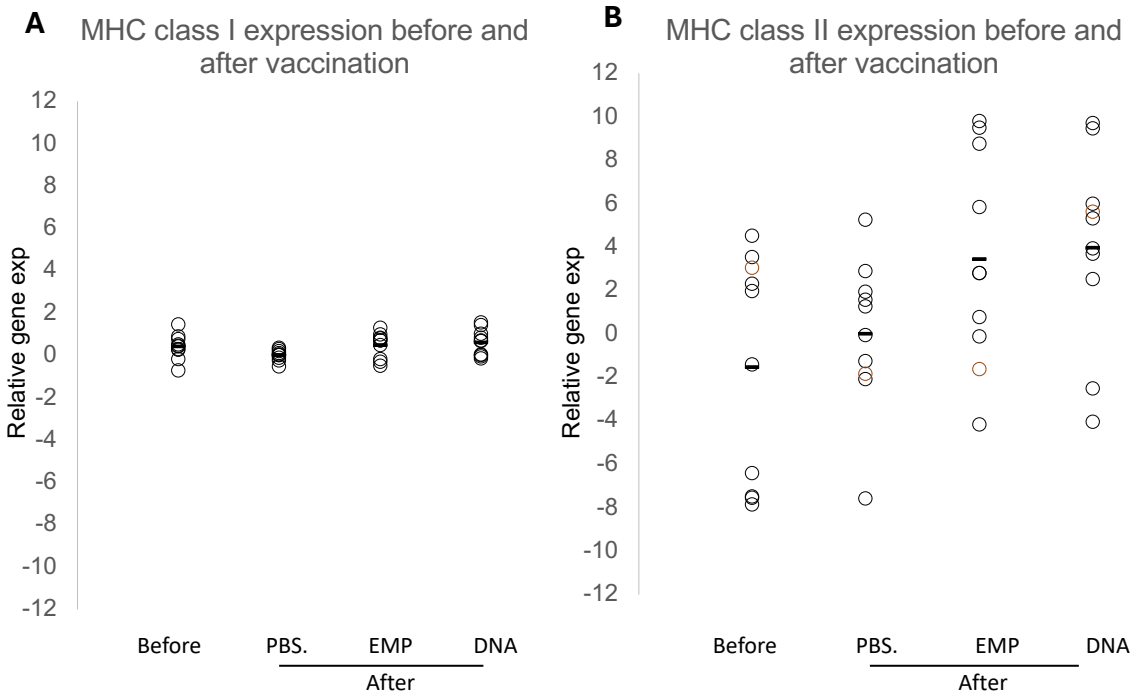

Fig. 7. MHC I (A) and MHC II (B) mRNA levels before and 4 weeks after the vaccination.

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Chapter 3

Transcriptome Analysis in the Spleen of Rock Bream (*Oplegnathus fasciatus*) during RSIV Infection

Abstract

This study investigated the kinetics of red sea bream iridovirus and host gene expression during infection in rock bream (*Oplegnathus fasciatus*), a species highly sensitive to the virus. After intraperitoneal injection of the viral solution at 10^4 TCID50/fish, the viral genome copy number in the spleen was $10^{4.7 \pm 0.2}$ and $10^{5.9 \pm 0.4}$ copies/μg DNA at 3 and 5 days post-injection (dpi), respectively. Using transcriptomic analyses via MiSeq, viral gene transcripts were detected at 3 and 5 dpi. Six genes including RING finger domain-containing protein and laminin-type epidermal growth factor-like domain genes were significantly expressed at 5 dpi. Further, 334 host genes were differentially expressed compared with those before infection. Genes were clustered into four groups based on their expression profiles. Interferon-stimulated genes were more prevalent in groups showing upregulation at 5 dpi and 3 and 5 dpi. In contrast, the group showing downregulation at 3 dpi included inflammation-related genes, such as granzyme and eosinophil peroxidase genes. Downregulation of certain inflammation-related genes may contribute to the susceptibility of this fish to the virus.

Keywords: gene expression, rock bream (*Oplegnathus fasciatus*), red sea bream iridovirus, transcriptome

1. Introduction

Red sea bream iridovirus (RSIV) infection causes mass mortality in farmed marine fish not only in Japan but also in East and Southeast Asian countries (OIE, 2019). Infected fish have shown clinical signs of lethargy, severe anemia, petechiae hemorrhage in the gills, and splenomegaly. Histopathologically, cell swelling and enlargement are observed in multiple organs, including the spleen and head kidney (Inouye et al., 1992). To date, >30 juvenile to adult marine fish species, including red sea bream (*Pagrus major*), have been infected with this virus, and *Oplegnathus* species are known to be highly sensitive to this virus (Kawato et al., 2017; OIE, 2019).

RSIV belongs to the genus Megalocytivirus, which also includes infectious spleen and kidney necrosis virus, dwarf gourami iridovirus, turbot reddish body iridovirus, Taiwan grouper iridovirus, and rock bream iridovirus. RSIV is a double-stranded DNA virus with an icosahedral structure, with a diameter of 200–240 nm. The DNA genome is ~110 kbp long and contains ~120 potential open reading frames (ORFs) (Kawato et al., 2017). The expression kinetics of viral genes have been comprehensively analyzed in red sea bream juveniles (Dang et al., 2007) and cultured cells (Lua et al., 2005) and classified as immediate-early, early, and late genes. This information can be used not only to understand the mechanism of RSIV infection but also to search for antigens to develop new vaccines.

Further, the host gene expression profiles against Megalocytivirus infection have been investigated. For example, transcriptomic analysis in the spleen of rock bream (*Oplegnathus fasciatus*) (Kim et al., 2020), spotted knifejaw (*Oplegnathus punctatus*) (Xu et al., 2022) and Mandarin fish (*Siniperca chuatsi*) (Zhang et al., 2023) following RSIV infection revealed changes in the expression of hundreds of genes and provided information regarding the

interaction between the host and virus. However, these analyses sampled only a time point after the challenge, and the expression kinetics of each gene have not been elucidated.

Herein, we performed transcriptomic analysis of genes in the spleen of RSIV-infected rock bream to elucidate the kinetics of the virus and host gene expression profiles. Based on these analyses, we identified a set of genes that may be related to the viral susceptibility of this fish.

2. Material and methods

2.1 Fish sampling

All animal experiments were conducted according to the guideline issued from the Ministry of the Environment, Japan, and the Regulations on the Handling of Animal Experiments of Tokyo University of Marine Science and Technology. Healthy juvenile rock bream (*O. fasciatus*) (82.6 ± 17.7 g) were kindly provided by Dr. Nobuhiro Hattori (Kinki University, Japan). Before the experiment, fish were reared in a 500-L tank with aerated, recirculating artificial seawater (28 ppt) at 26 °C, and fed twice per day about 1% of body weight per day. The RSIV RIE12-1 strain, which was kindly gifted by Kyoritsu Seiyaku Cooperation, was propagated in the S-1F cell line established from rock bream caudal fin. Cells were cultured in an L-15 medium (Wako, Japan) supplemented with 5% fetal bovine serum. The supernatant of virus propagates was collected, and TCID50 was calculated using the cells by the Behrens-Kärber method. Ten fish were intraperitoneally injected with 0.1 mL of the virus solution at 1.0 x 10^5 TCID50/mL and reared in a 100-L tank with aerated, recirculating artificial seawater (28 ppt) at 26 °C, and fed twice per day about 1% of body weight per day. Spleens were isolated from three fish before (negative control) and at 3 and 5 days post-injection (dpi) and were stored in NAP buffer (Camacho-Sanchez et al., 2013) at −80 °C.

2.2 Evaluation of viral copy numbers via quantitative polymerase chain reaction (qPCR)

DNA was extracted using NucleoSpin® Tissue (Takara, Japan) according to the manufacturer's protocol, and its concentration was measured via NanoDrop™ Lite

Spectrophotometer (Thermo Fisher Scientific, USA). qPCR was performed using THUNDERBIRD™ Next SYBR® qPCR Mix (TOYOBO, Japan) and QuantStudio 1 real-time PCR system (Thermo Fisher Scientific), wherein 10 ng of DNA was mixed with an RSIV major capsid protein (MCP)-specific primer set: RSIV-F (CTGCGTGTTAAGATCCCCTCCA) and RSIV-R (GACACCGACACCTCCTCAACTA). The thermal condition was at 95 °C for 30 sec; 40 cycles of 5 sec at 95 °C and 30 sec at 60 °C. The viral genome copy number was calibrated using a plasmid harboring MCP.

2.3 Transcriptomic analysis of next-generation sequencing data

Total RNA was isolated from each sample using RNAiso Plus (Takara) according to the manufacturer's protocol. RNA was separately used for mRNA library construction using TruSeq Standard mRNA Library Prep (Illumina, USA). Sequencing was performed using MiSeq reagent kit (version 2) 300 cycles (Illumina) and Illumina MiSeq (Illumina). Reads were assembled and analyzed for differentially expressed genes (DEGs) using Trinity version 2.8.4 (Grabherr et al., 2011). Host DEGs were selected with a *p*-value of <1e−3, fold change of >22, and length of >500 bp. Host DEGs were annotated and applied to Gene Ontology (GO) enrichment analysis using the BLAST2GO program (Götz et al., 2008).

For RSIV gene expression profiling, the reads were mapped onto the genes of RIE12-1 (accession no. AP017456). For normalization among the samples, the top 2,000 genes highly expressed in the host were used. The Trimmed mean of M-values (TMM) for the expression of the RSIV gene was calculated using Trinity version 2.8.4 (Grabherr et al., 2011).

2.4 qRT-PCR

Total RNA was extracted from the same samples used in the transcriptomic analysis. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with oligo (dT) primer. All procedures were performed following the manufacturer's instructions. We designed primer sets based on the sequences determined for the tissue expression analysis (Table 1). For qPCR, THUNDERBIRD™ Next SYBR® qPCR Mix (TOYOBO, Japan) was used on a QuantStudio 1 real-time PCR system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The thermal condition was at 95 °C for 30 sec; 40 cycles of 5 sec at 95 °C and 30 sec at 60 °C. EF-1α and β-actin genes were used as internal controls and the relative expression levels of the target gene were calculated (Vandesompele et al., 2002). The expression level of each sample was expressed as a percentage with the average value of the negative control as 100. The statistical significance was evaluated using a one-way analysis of variance (ANOVA) and Dunnett's post hoc test.

3. Results and discussion

Fish at 3 and 5 dpi showed no apparent symptoms, but their spleen was enlarged. In addition, all remaining fish died by day 7. The viral genome copy at 3 and 5 dpi was $10^{4.7 \pm 0.2}$ and $10^{5.9 \pm 0.4}$ copies/μg DNA, respectively. Kim et al. (2022) reported that rock bream infected with viral genome copies of 10^8, 10^6, and 10^4 died at 7, 11, and 14 dpi (at 25 °C), respectively, and their viral copy numbers in the spleen were almost saturated at 5, 7, and 10 dpi, respectively. This study detected viral genome copies at 3 and 5 dpi. Thus, it is speculated that the virus was sufficiently replicated at 3 dpi.

Paired-end sequencing of approximately 3 million fragments per sample was performed (accession no. DRA016434). The reads were mapped onto the RSIV genes, and their expression profiles were evaluated. Among the 108 predicted ORFs in the genome of the RSIV RIE12-1 strain, transcription of 104 genes was detected (Supplemental Table 1). Based on hierarchical clustering, genes were clustered into two groups (Fig. 1). The expression of genes in group 1, which were identical to ORF016L, ORF037R, ORF226R, ORF291L, ORF539R, and ORF543R (Kurita et al., 2002), significantly increased at 3 and 5 dpi (Table 1). Except for ORF539R, the other five genes were also detected in the RSIV-infected red sea bream P. major at 5 and 7 dpi (Dang et al., 2007). In contrast, the mRNA level of MCP, an important component of the virus capsid, was approximately 0.1% of the maximum value (see Supplemental Table 1). Along with MCP, the expression of 29 genes was strongly detected in red sea bream at 5 and 7 dpi (Dang et al., 2007). Although it is difficult to compare the different experiments directly, it is speculated that the transcriptional regulation of viral genes is controlled by different mechanisms in different species.

Among the six genes, ORF291L, a homolog of the laminin-type epidermal growth factor-like domain, is an antigen of the monoclonal antibody M10, which is commonly used for disease diagnosis in Japan (Takano et al., 2019). Although this molecule is expected to be highly antigenic and predicted to be a membrane protein, a DNA vaccine using this gene did not show any protective activity in a challenge test (Caipang et al., 2006). This molecule does not form a viral particle but is present on the cell surface of infected cells (Takano et al., 2019). Thus, genes with high expression levels may not necessarily be candidates for protective antigens.

The reads were assembled into 103,264 contigs, which included 70,654 contigs considered single gene sequences. By DEG analysis, 334 genes showed differential expression (Supplemental Table 2 and Supplemental material). They were clustered into four groups: upregulated at 5 dpi (group 1), upregulated at 3 and 5 dpi (group 2), downregulated at 5 dpi (group 3), and downregulated at 3 dpi (group 4) (Fig. 2).

By annotation and GO enrichment analysis, GO was enriched in groups 1, 2, and 4 (Fig. 3). GOs related to immunity were enriched in groups 2 and 4. Genes related to immunity are listed in Table 2. Group 1 also contained genes involved in immunity, such as cytokines. Among the genes, a gene homologous to interferon (IFN)-β-like and IFN response genes were found in groups 1 and 2. Type I IFNs (IFN-I), including IFN-β-like, induce gene expression, such as the Mx protein gene involved in innate immunity, especially those showing antiviral activity. IFN-I is induced by a viral infection and increases during RSIV infection (Zhang et al., 2016). However, IFN-I induced by polyIC does not protect the fish against RSIV infection (Kim et al., 2012), although it can reduce the viral load (Jung and Jung, 2017).

In contrast, group 4 contained genes related to inflammatory responses, including genes encoding cytokine receptors, eosinophil peroxidase-like, and granzyme-like protein 1. As the expression of genes was downregulated at 3 dpi, it is speculated that the number of cells associated with these genes decreased or their activity was inhibited. In particular, granzyme, a member of serine proteases, is released by cytotoxic T cells and natural killer cells (Barber, 2001; Barry and Bleackley, 2002). These cells are important for eliminating virus-infected cells (Cook et al., 2014; Ma et al., 2021). The viral copy number increased at 3 dpi, revealing that the suppression of these immune cell functions may have allowed the virus to multiply efficiently.

The expression profiles of certain genes involved in leukocytes function were confirmed by qPCR after RSIV infection (Fig. 4). The mRNA levels of CXC chemokine receptor1 (CXCR1), eosinophil peroxidase (EPO), and matrix metalloproteinase 9 (MMP9) in the spleen markedly decreased on 3 and 5 dpi. CXCR1, also known as the interleukin-8 receptor, is an important molecule in the chemotactic activities of neutrophils in mammals (Baggiolini and Clark-Lewis, 1992). Furthermore, EPO functions in eosinophils and involves the killing of pathogens (Dunn et al., 1968; Arnhold and Malle, 2022), and MMP9 functions in neutrophils and involves migration across the basement membrane., respectively (Delclaux et al., 1996; Owen and Campbell, 1999). It should be noted that the CXCR1 mRNA levels in the rock bream spleen increased 6 hours after RSIV infection and returned to the normal level 24 hours after the infection (Umasuthan et al., 2014). In mammals, leukocytes including neutrophils decrease in response to viral infection (Newburger and Dale, 2013; Guo et al., 2021). The typical histopathological changes in RSIV infection are the presence of enlarged cells, which are infected with the virus, in the lymphoid tissues including the spleen (Kawato et al., 2017). Therefore, our results suggest that leukocytes such as neutrophils and eosinophils

were the target of the virus and the susceptibility of these cells to the virus might relate to a high viral load and high susceptibility of the fish.

Comprehensive gene expression analysis in the spleen associated with RSIV or RSIV-like Megalocytivirus infection has been conducted. After the virus infection, a series of inflammatory signaling molecules were upregulated in the spleen of spotted knifejaw and Mandarin fish (Xu et al., 2022; Zhang et al., 2023). The findings are consistent with the gene expression patterns in groups 1 and 2 (see Table 3). On the other hand, Kim et al. (2023) showed that the genes involved in the immune system, including the B cell receptor signaling pathway and platelet activation, were downregulated. On the other hand, our results showed that the number of leukocytes, including neutrophils and eosinophils, might reduce or the function of such cells might be downregulated.

In conclusion, we performed transcriptomic analyses of genes in the spleen of RSIV-infected rock bream, a species highly sensitive to the virus. Based on clustering analyses, we revealed that certain viral and host genes were differentially expressed at 3 and 5 dpi. The expression of host genes involved in inflammatory responses was downregulated at 3 dpi. As these genes might be involved in immunity against RSIV, their downregulation may contribute to the viral susceptibility of the fis

Table 1. List of primers for RT-qPCR

Gene ID	Product name	Product size (bp)	Primer name	Nucleotide Sequence （5’-3’）
Target genes				
TRINITY_DN815_c0_g1	C-X-C chemokine receptor type 1-like	182	CXCR1_F	TACCCGGGAACCTGTTAGTG
			CXCR1_R	GCTGAGGAACTTGCACATGA
TRINITY_DN1568_c0_g1	Eosinophil peroxidase-like	177	EPO_F	CTCATCGCAAGACAGTTCCA
			EPO_R	GTTTGAGAGGACGCTGAAGG
TRINITY_DN1255_c1_g1	Matrix metalloproteinase-9	245	MMP9_F	CGAGGAGACTGGAGAACTGG
			MMP9_R	CATCAAAGAGGCGGGTAAAA
House keeping genes				
FJ975145	β actin	200	b-actin_F	CGACATCCGTAAGGACCTGT
			b-actin_R	GCTGGAAGGTGGACAGAGAG
TRINITY_DN1960_c1_g1	Elongation factor 1α	242	EF-1α_F	ACGTGTCCGTCAAGGAAATC
			EF-1α_R	GACAAATTTGGGTGCGTCTT

The sequence for β actin was cited from DDBJ database with the accession number.

Table 2 RSIV genes in group 1 of the heatmap in Fig. 2

Accession no.	Description	ORF no in AB104413	TMM values	
			Day3	Day5
BAZ95703	RING-finger domain-containing protein	ORF 543R	108.3 ± 52.3	3156.7 ± 2237.4
BAZ95702	hypothetical protein	ORF 539R	80.9 ± 8.7	2697.4 ± 2041.3
BAZ95622	hypothetical protein	ORF 037R	43.3 ± 8.3	2066.3 ± 1541.4
BAZ95650	hypothetical protein	ORF 226R	55.3 ± 9.6	2736.9 ± 2528.5
BAZ95658	laminin-type epidermal growth factor-like domain	ORF 291L	46.0 ± 16.6	1922.2 ± 1766.2
BAZ95616	hypothetical protein	ORF 016L	20.3 ± 2.1	1587.0 ± 1376.4

The reads were mapped onto the genes in RIE12-1 (AP017456). For the mapping step, the top 2,000 genes highly expressed in the transcriptomic analysis were used for normalization among the samples. The TMM values are represented as average ± standard deviation.

Table 3 Immuno-related genes on the heatmap in Fig. 3

Gene ID	Description	Group	GO terms
TRINITY_DN6606_c0_g1	ATP-binding cassette sub-family B member 9	1	P:immune system process
TRINITY_DN29945_c0_g1	C-C motif chemokine 19	1	P:immune system process
TRINITY_DN82_c0_g1	C-X-C motif chemokine 6-like	1	
TRINITY_DN2968_c0_g4	grass carp reovirus (GCRV)-induced gene 2e	1	P:defense response to other organis
TRINITY_DN487_c0_g2	interferon regulatory factor 6 isoform X2	1	P:immune system process
TRINITY_DN1493_c0_g1	interferon-induced GTP-binding protein Mx-like	1	P:immune system process
TRINITY_DN2132_c2_g1	interferon-induced protein with tetratricopeptide repeats 5-like	1	P:defense response to other organism
TRINITY_DN30738_c0_g1	interleukin-12 subunit beta	1	
TRINITY_DN329_c0_g1	probable ATP-dependent RNA helicase DHX58	1	P:immune system process, P:defense response to other organism
TRINITY_DN301_c1_g1	radical S-adenosyl methionine domain-containing protein 2	1	P:immune system process
TRINITY_DN739_c0_g1	signal transducer and activator of transcription 1-alpha/beta-like isoform X1	1	P:immune system process
TRINITY_DN3499_c0_g1	stimulator of interferon genes protein	1	P:immune system process, P:defense response to other organism
TRINITY_DN2113_c0_g1	suppressor of cytokine signaling 1	1	P:immune system process, P:defense response to other organism
TRINITY_DN2106_c1_g1	tapasin-related protein	1	P:immune system process
TRINITY_DN2355_c0_g1	basic leucine zipper transcriptional factor ATF-like	2	P:immune system process, P:defense response to other organism
TRINITY_DN6030_c1_g1	basic leucine zipper transcriptional factor ATF-like 3	2	P:immune system process
TRINITY_DN4302_c0_g1	C-X-C motif chemokine 13	2	
TRINITY_DN1092_c0_g3	heat shock protein HSP 90-alpha 1	2	P:immune system process, P:defense response to other organism

TRINITY_DN10448_c0_g3	interferon beta-like	2	
TRINITY_DN9305_c0_g1	interferon regulatory factor 2	2	P:immune system process
TRINITY_DN37626_c0_g1	lysozyme g isoform X1	2	
TRINITY_DN3743_c0_g1	monocyte chemotactic protein 1B	2	P:immune system process
TRINITY_DN359_c0_g1	suppressor of cytokine signaling 1	2	P:immune system process, P:defense response to other organism
TRINITY_DN1299_c0_g1	5-aminolevulinate synthase, nonspecific, mitochondrial	3	P:immune system process, P:defense response to other organism
TRINITY_DN1706_c0_g1	solute carrier family 4-member 1b (Diego blood group)	3	P:immune system process
TRINITY_DN2078_c0_g3	stabilin-1 isoform X1	3	P:inflammatory response
TRINITY_DN1572_c0_g1	stromal cell-derived factor 1 isoform X2	3	P:immune system process, P:inflammatory response
TRINITY_DN815_c0_g1	C-X-C chemokine receptor type 1-like	4	P:immune system process, P:defense response to other organism
TRINITY_DN937_c0_g1	CD166 antigen homolog A isoform X1	4	P:immune system process
TRINITY_DN37447_c0_g1	cis-aconitate decarboxylase	4	P:inflammatory response, P:defense response to other organism
TRINITY_DN3399_c0_g1	collagenase 3	4	P:immune system process
TRINITY_DN8657_c0_g1	complement component C6	4	P:immune system process, P:defense response to other organism
TRINITY_DN1568_c0_g1	eosinophil peroxidase-like	4	P:inflammatory response
TRINITY_DN1818_c0_g1	granzyme-like protein 1	4	
TRINITY_DN1222_c0_g1	low choriolytic enzyme-like	4	P:defense response to other organism
TRINITY_DN1255_c1_g1	matrix metalloproteinase 9	4	P:immune system process, P:inflammatory response

Information and sequences of the DEGs are available as supplemental materials.

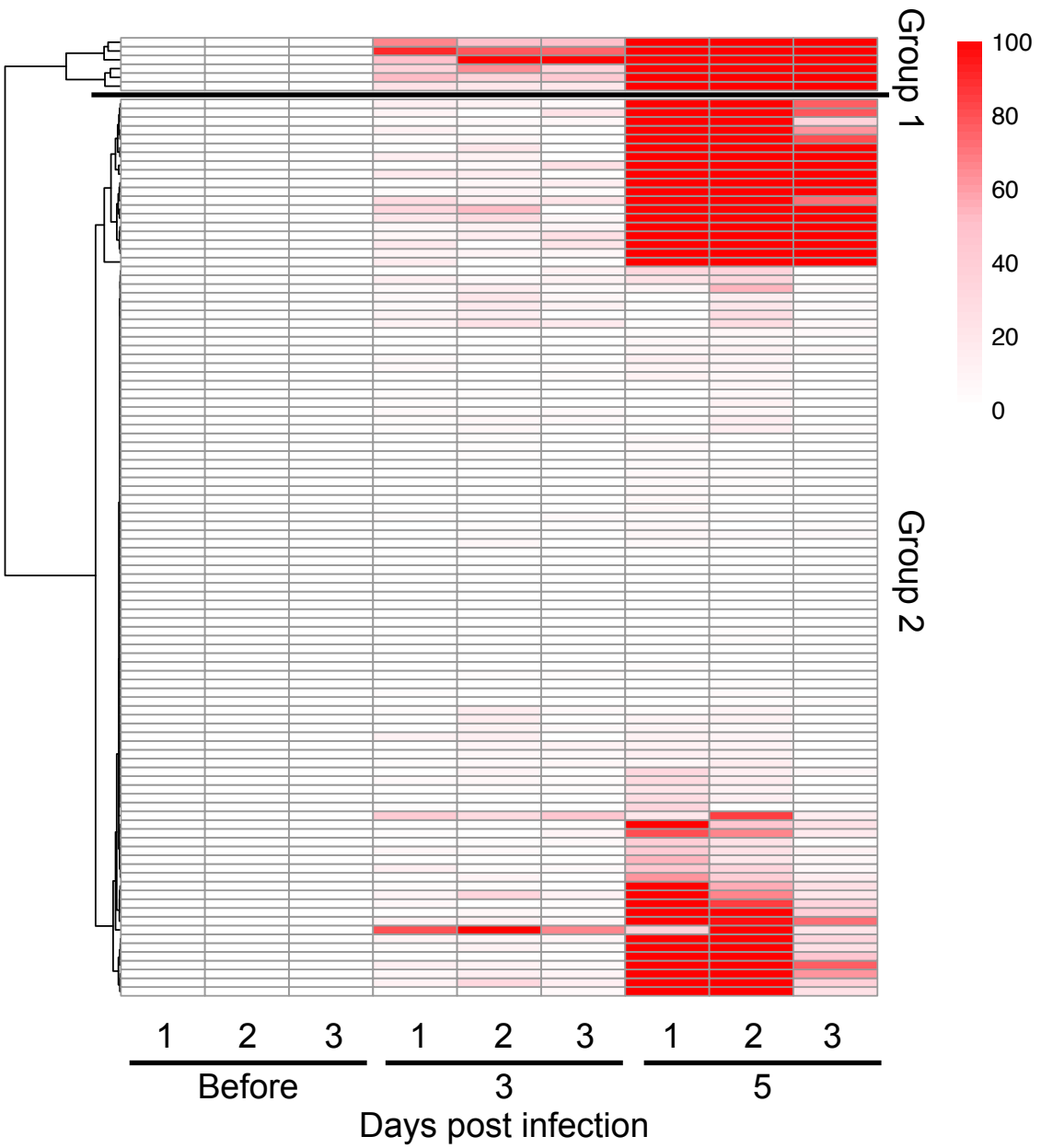

Fig. 1. Heatmap of RSIV gene expression. The reads were mapped onto the genes in RIE12-1 (AP017456). For the mapping step, the top 2,000 genes highly expressed in the transcriptomic analysis were used for normalization among the samples. Values in the colored chart represent TMM values.

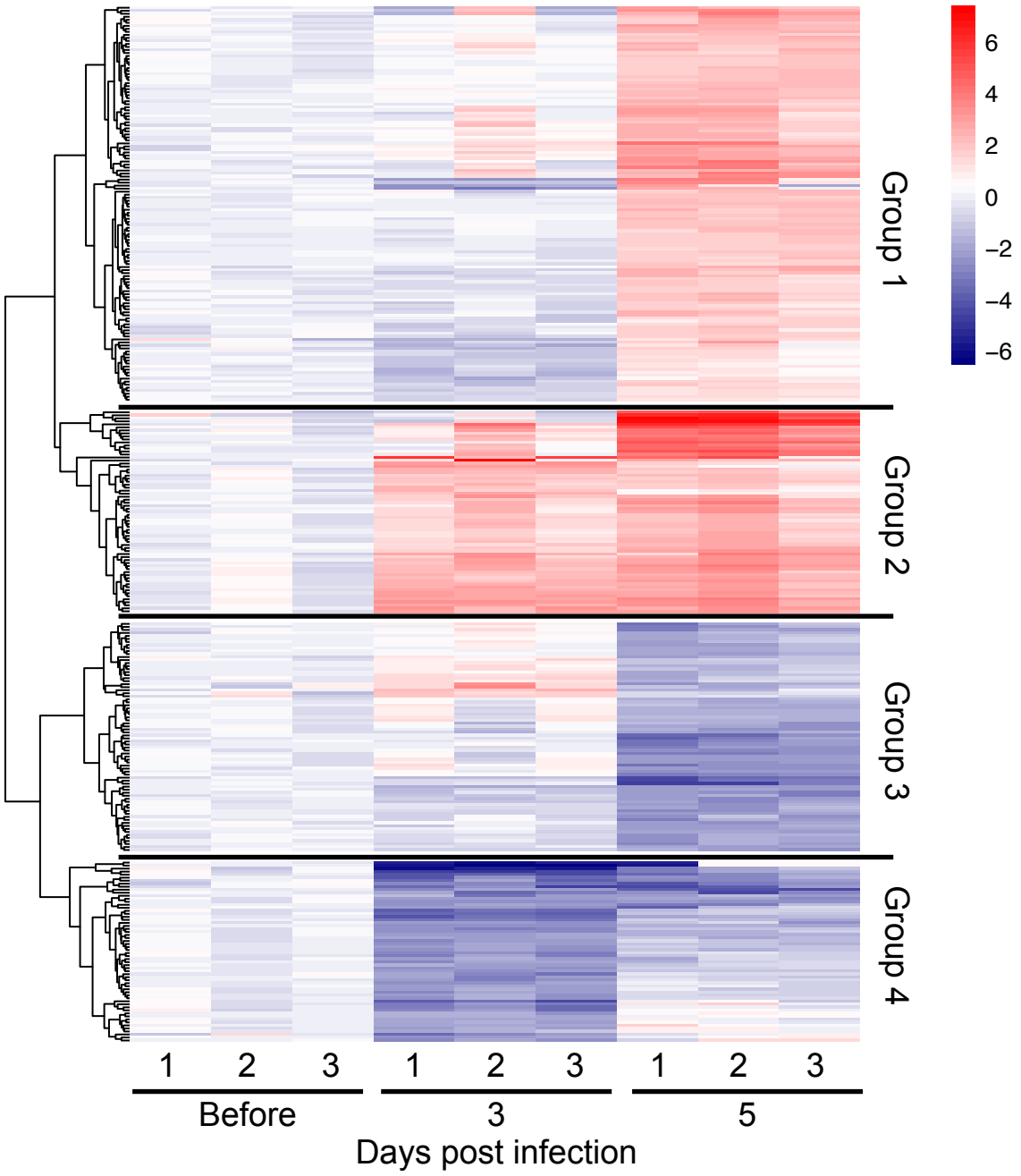

Fig. 2. Heatmap of host DEGs. Numbers at the bottom represent an individual same as those in Fig. 1. Values in the colored chart represent log2 fold change.

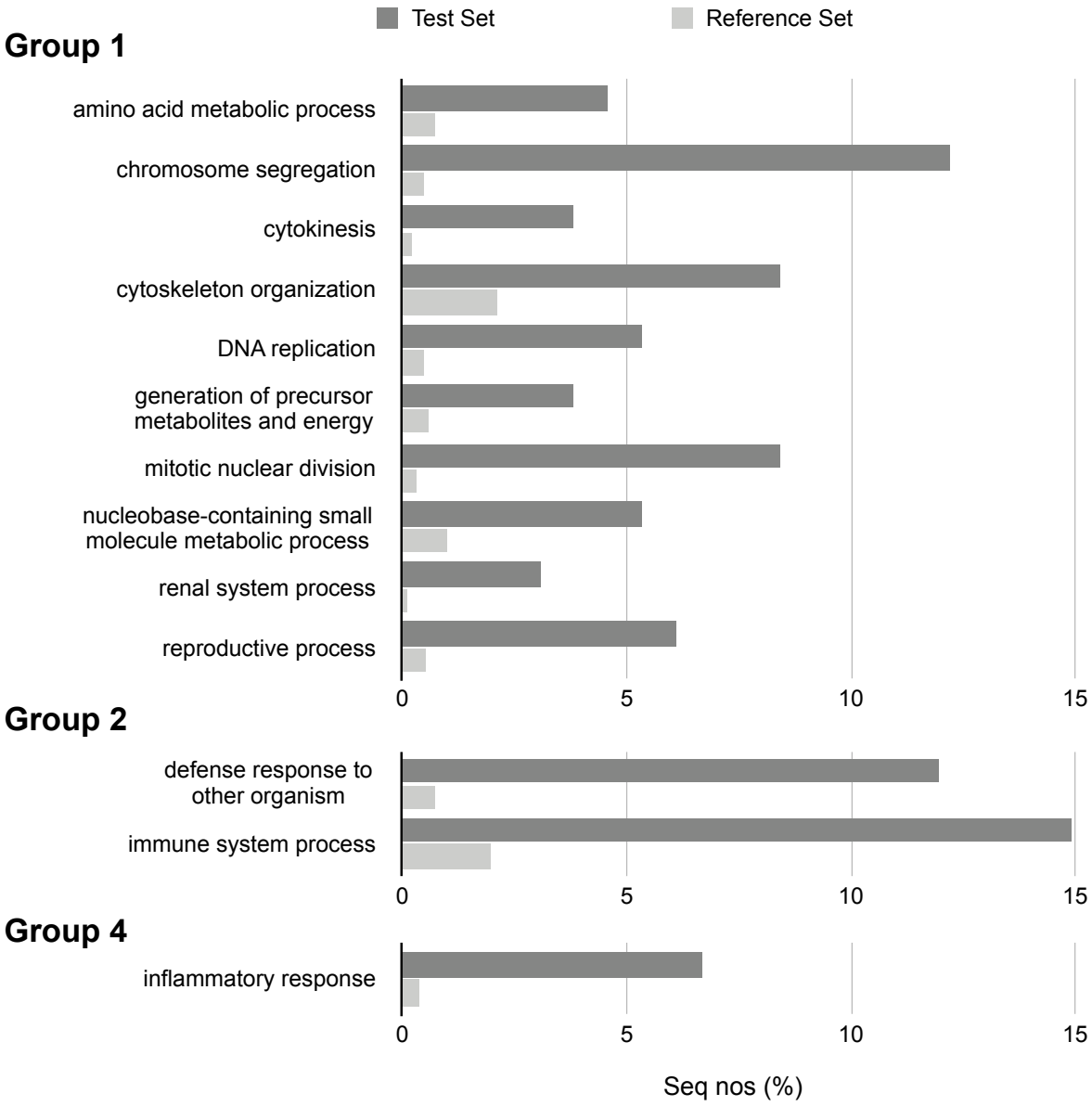

Fig. 3. Gene Ontology (GO) enrichment results of host DEGs. GO enrichment analyses were performed for each group. No GO was enriched in group 3.

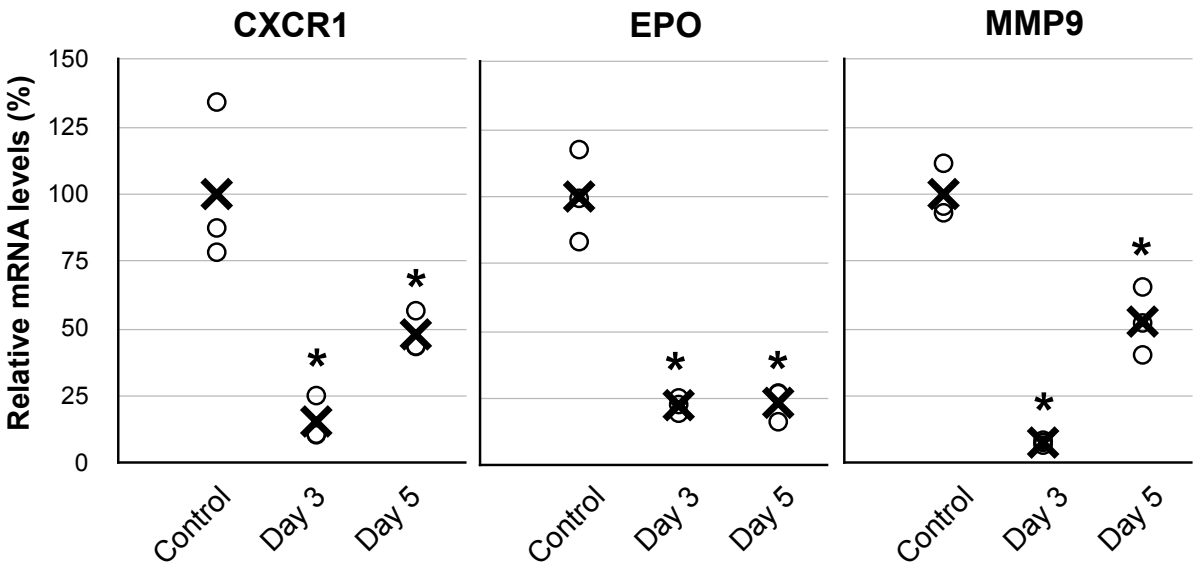

Fig. 4. The expression levels of CXCR1, EPO, and MMP9 in the spleen of the rock bream. The spleen was collected before, and 3 and 5 dpi. β-actin and EF-1α were used as internal controls. Circles and cross marks represent individual and average values, respectively. Asterisks indicate a significant difference in comparison with the control ($P < 0.05$).

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Chapter 4

General conclusion

The red sea bream iridovirus has had a significant negative impact on economic losses in several nations. However, discussions surrounding the fish immune response have highlighted the potential for protection through immunization. This thesis focuses on the plasmid DNA vaccine against RSIV disease and the mechanisms underlying its ability to protect fish from RSIVD.

Chapter 2 outlines the design of the vaccination trial experiment. The efficacies of the DNA vaccines encode ORF111R and ORF450L were not evaluated in the experiments. This is partly because the fish used in this study showed lower mortality than so far reported. Understanding the mechanisms underlying the virus's virulence in fish is needed to develop more efficient vaccines. The experiment in Chapter 3 was inspired by the findings of Chapter 2.

In chapter 3, rock bream was selected as one of the most susceptible fish to RSIV. To investigate the mechanisms of RSIV infection in fish, an experiment was designed, and the data were analyzed using transcriptome technology. The results showed that certain RSIV gene expressions and immune responses to the infection were comprehensively illustrated. Significant expression of six RSIV genes was considered of interest for vaccine development. In addition, the genes involved in the inflammatory response to viruses are required to combat the virus infection. In contrast, the downregulations of genes associated with inflammation were observed, suggesting that these changes are involved in host susceptibility to the virus.

Although the difficult challenge of understanding how to combat the virus and develop more effective vaccines remains, the results of this study are expected to help overcome this challenge in the future.