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DNA vaccine encoding myristoylated membrane protein (MMP) of rock bream iridovirus (RBIV) induces protective immunity in rock bream (*Oplegnathus fasciatus*)

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ABSTRACT

Rock bream iridovirus (RBIV) causes severe mass mortalities in rock bream (*Oplegnathus fasciatus*) in Korea. In this study, we investigated the potential of viral membrane protein to induce antiviral status protecting rock bream against RBIV infection. We found that fish administered with ORF008L (myristoylated membrane protein, MMP) vaccine exhibited significantly higher levels of survival compared to ORF007L (major capsid protein, MCP). Moreover, ORF008L-based DNA vaccinated fish showed significant protection at 4 and 8 weeks post vaccination (wpv) than non-vaccinated fish after infected with RBIV (6.7×10^5) at 23 °C, with relative percent survival (RPS) of 73.36% and 46.72%, respectively. All of the survivors from the first RBIV infection were strongly protected (100% RPS) from re-infected with RBIV (1.1×10^7) at 100 dpi. In addition, the MMP (ORF008L)-based DNA vaccine significantly induced the gene expression of TLR3 (14.2-fold), MyD88 (11.6-fold), Mx (84.7-fold), ISG15 (8.7-fold), PKR (25.6-fold), MHC class I (13.3-fold), Fas (6.7-fold), Fas ligand (6.7-fold), caspase9 (17.0-fold) and caspase3 (15.3-fold) at 7 days post vaccination in the muscle (vaccine injection site). Our results showed the induction of immune responses and suggest the possibility of developing preventive measures against RBIV using myristoylated membrane protein-based DNA vaccine.

1. Introduction

Iridoviruses are large double-stranded DNA viruses with icosahedral morphology [1]. The family is divided into five genera, *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, *Ranavirus* and *Megalocytivirus*. Rock bream iridovirus (RBIV), which is a member of the *Megalocytivirus* genus [2], causes mass mortality in rock bream (*Oplegnathus fasciatus*) [3]. The virus affected fish exhibited the enlarged basophilic cells in the gills, liver, kidney, heart and spleen [3]. Specifically, RBIV-infected fish are characterised by enlargement of spleen [3], and spleen size may reflect disease status after virus infection [4,5]. RBIV is known for very strong pathogenicity against rock bream [6–9]. Some of studies have focused on the mortality and virus replication-dependent immune gene responses in rock bream under RBIV infection [10–13]. However, immune defense mechanisms of rock bream against RBIV is largely unknown. Hence, RBIV remains an important health problem in rock bream aquaculture industry.

Vaccination is an important fish disease control measure and essential for good farming practices [14–16]. Effective formalin inactivated virus vaccine for red seabream iridovirus (RSIV) disease are currently available for red seabream (*Pagrus major*), striped jack (*Pseudocaranx dentex*) and other fish species belonging to the genus *Seriola* in Japan. However, the vaccine is not effective for rock bream [2]. DNA vaccines offer numerous advantages over conventional vaccines. The DNA vaccine can induce both cellular and humoral immune response [17,18]. Previous studies demonstrated that DNA plasmid encoding an immunogenic antigen was very effective in fish against viral diseases [19–21]. The DNA vaccine against infectious haematopoietic necrosis virus (IHNV) is commercially available (since 2005), and so far, there have been no reports of IHNV outbreaks in vaccine administered group [22].

Genomic sequence analysis of megalocytivirus such as RBIV [23], infectious spleen and kidney necrosis virus (ISKNV) [24] and RSIV [25] suggest that these viruses share high levels of sequence identities. Based on complete genome of RBIV, structural protein ORF007L or ORF008L showed similarity to the major capsid protein (MCP) or myristoylated membrane protein (MMP), respectively, and have homology with the ISKNV and RSIV. Viral envelope membrane proteins play a crucial role in the course of antigen

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recognition, immune responses, and virus-host interaction. MCP gene is known as the main immunogenic protein of megalocytivirus, that has been widely used as an immunogen in DNA vaccination trials [26–28]. Myristoylated viral proteins are associated with various biological functions including the assembly of viruses [29–32]. The myristoylated proteins are also known as viral envelope membrane proteins of iridoviruses [33,34]. In the genus *Ranavirus*, myristoylated proteins is essential for replication of frog virus 3 (FV 3) [35] and the antibodies specific to these proteins can serve as valuable tools for grouper iridovirus (GIV) detection [36]. However, there are no reported regarding antigenic effects of MMP against megalocytivirus.

Currently, there are no reports on the relationship between the viral membrane protein-based DNA vaccine-induced antiviral effect and the immune responses in rock bream against RBIV infection. Hence, in this study, we evaluated ORF007L (MCP) and ORF008L (MMP)-based DNA vaccines for their potential to protect rock bream from RBIV infection. Furthermore, the gene expression levels of pattern recognition (TLRs), inflammatory cytokines (ILs and TNFα), interferon responses (Mx, ISG15 and PKR), antigen presentation (MHC class I and CD8) and apoptosis (perforin, granzyme, Fas, Fas ligand and caspases) were assessed in ORF008L (MMP) vaccinated rock bream.

2. Materials and methods

2.1. Virus preparation and pre-challenge studies

The virus used in the present study was originally isolated from RBIV-infected rock bream in 2010 as described earlier [10]. The MCP gene copies of the original RBIV in the supernatant preparations, quantified by absolute quantitative real time-polymerase chain reaction (qRT-PCR) was 7.5×10^7/100 μl and at 10, 100 and 1000-fold serially diluted virus solutions containing 1.1×10^7, 1.2×10^6 and 6.7×10^5/100 μl MCP gene copies, respectively, as described before [10]. The virus challenge dose was confirmed with a pre-challenge study using ten naïve fish of each group of which 100% died at a dose of 1.1×10^7, 1.2×10^6 and 6.7×10^5 MCP gene copies at 23 °C.

2.2. Plasmid construction

The two genes encoding ORF007L (major capsid protein, MCP) and ORF008L (myristoylated membrane protein, MMP) of RBIV have been deposited in GenBank of NCBI; (i) ORF007L, which encodes a protein of 453 amino acid residues, (ii) ORF008L, which encodes a protein of 485 amino acids. The primers used in this study are listed in Table 1. The 1299 bp (MCP) and 1453 bp (MMP) of the PCR products were ligated to the TA cloning vector pEGFP-N2 (Clontech). The recombinant plasmids were digested with *Eco*RI/*Bam*HI, and the fragments containing the vaccine genes were retrieved. The plasmid was isolated using AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Korea). The empty pEGFP-N2 plasmid was used as a control. The integrity of the plasmid DNA was assessed by 1.2% agarose gel electrophoresis and quantified using a NanoDrop-1000 spectrophotometer (Thermoscientific, USA). The vaccine plasmid was diluted in sterile phosphate-buffered saline (PBS) to 20 ng/μl and stored at −20 °C.

2.3. Vaccine effect in relation to dose and virus infection dose (experiment I)

RBIV-free rock bream was obtained from a local farm. Two vaccines (ORF007L and ORF008L) were examined for antiviral potentials at 3 different concentrations (Table 2). Ten fish in each group (10.2 ± 1.1 cm, 18.2 ± 1.5 g) were administered intramuscularly (i.m.) with each of the 2 vaccine (ORF007L and ORF008L) at 10, 100 and 200 ng/100 μl/fish and 2 empty vector (ORF007L and ORF008L) or with phosphate-buffered saline (PBS) as a control. At 4 weeks post vaccination, the five fish were injected with RBIV (6.7×10^5 MCP gene copies, 100 μl/fish) and the other 5 fish were injected with RBIV (1.2×10^6 MCP gene copies, 100 μl/fish). Because RBIV is known for very strong pathogenicity against rock bream [4–9] and all the fish died at virus doses of 6.7×10^5–1.1×10^7 MCP gene copies in this experiment, we evaluated vaccine efficacy at different virus doses to obtain survivors. Each group was maintained in an aquarium containing 30 L of UV-treated seawater at 23 °C.

2.4. Vaccination (experiment II)

Fish (12.2 ± 1.5 cm, 27.2 ± 2.0 g) were separated into four groups; (i) ORF008L (MMP) vaccine-injected group (n = 50); (ii) virus-injected control group (n = 50); and (iii) Naive (n = 50). Experimental fish group (i) was injected i.m. with 200 ng/100 μl/fish of ORF008L vaccine. PBS (100 μl/fish) was injected for virus-injected control group (ii), and a naive group (iii) without injection was also maintained. Fish were maintained at 21–23 °C in tanks (200 L) with continuous supply of seawater and aeration.

At 4 and 8 weeks post vaccination (wpv), vaccinated or non-vaccinated fish were intraperitoneally (i.p.) injected with RBIV (100 μl/fish) containing 6.7×10^5 MCP gene copies. Each group (15 fish) were maintained in aquaria containing 30 L of UV-treated seawater at 23 °C. To determine the virus gene copy number, spleens were collected from all the dead fish at each time points or all the survived fish at end of experimental period. Tables 3 and 4 summarize the experimental conditions.

The relative percent survival (RPS) was calculated according to the following formula, as reported previously [37]: RPS = [1 − (% mortality of vaccinated group/% mortality in control] × 100. For all the artificial infection experiment, log rank test was carried out using GraphPad Prism5 Software (GraphPad Prism, USA) for survival analysis.

2.5. Re-infection of survivors from experiment II

Survived fish at 100 days post infection (dpi) from 4 and 8 wpv, respectively, were re-infected with RBIV (1.1×10^7 MCP gene copies, 100 μl/fish). Fifteen fish (16.1 ± 1.2 cm, 40.1 ± 1.2 g) from virus-injected control group were infected with RBIV in the same manner as the survivors (Table 3). Experimental fish groups were kept at 23 °C in aquarium containing 30 L of UV-treated seawater. To determine the virus copy number, spleens were collected from all the experimental fish.

2.6. Quantitative expression of immune genes

For the analysis of gene expression, at 7 days post ORF008L (MMP) vaccination, the muscle (injection site), spleen, kidney and liver were collected from five fish from vaccinated or PBS-injected groups and then flash frozen in liquid nitrogen. The total RNA extraction was performed using the RNAiso Plus reagent (TaKaRa, Japan) followed by treatment of recombinant DNase I (RNase free; TaKaRa, Japan) and cDNA synthesis using ReverTra Ace qPCR RT Kit (Toyobo, Japan) according to the standard protocol. qRT-PCR was carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using an AccuPre®2x Greenstar qPCR Master mix (Bioneer, Korea). The reaction condition was an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C) for 15 s and annealing (58–60 °C) for 45 s, as previously described [10]. The gene specific primers

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