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Protective Effects of Long Double-Stranded RNA with Different CpG Motifs against *Miamiensis avidus* and Viral Hemorrhagic Septicemia Virus (VHSV) Infections in Olive Flounder (*Paralichthys olivaceus*)

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Abstract: The South Korean aquaculture industry has incurred considerable production losses due to various infectious diseases. Artificially synthesized polyinosinic–polycytidylic acid (poly I:C), structurally similar to double-stranded RNA (dsRNA) and cytidine–phosphate–guanosine oligodeoxynucleotides, can enhance immune responses and protect against diseases. Here, we investigated dsRNA molecules with different cytidine–phosphate–guanosine (CpG) motifs (dsRNA-CpGMix) as fused agents to treat *Miamiensis avidus* and viral hemorrhagic septicemia virus (VHSV) infection in olive flounders. We further investigated the efficacy of specific sequence motifs in dsRNA in modulating immunostimulatory effects. Fish treated with poly I:C or dsRNA-CpGMix exhibited higher survival rates than the control group. Olive flounder leukocytes stimulated with poly I:C or dsRNA-CpGMix showed increased scuticocidal activity in the presence of inactivated immune sera. dsRNA with CpG motif sequences induced higher resistance against *M. avidus* and VHSV infections than dsRNA without CpG motif sequences, and the dsRNA-CpGMix group showed upregulated *ISG15* or *Mx* compared to the dsRNA-GFP group. Thus, dsRNA containing CpG motifs can be used as effective immunostimulants to enhance resistance against viral and parasitic diseases in olive flounder. The specific sequences of the CpG motifs in dsRNA may be important for enhancing immune responses and resistance against *M. avidus* and VHSV infections in olive flounders.

Keywords: CpG motifs; *Miamiensis avidus*; immunostimulant; polyinosinic–polycytidylic acid

Key Contribution: The dsRNA harboring CpG motifs exhibit potential as potent immunostimulants, augmenting the defense mechanisms against viral and parasitic pathogens in olive flounder. The arrangement of CpG motifs within dsRNA appears crucial for enhancing immune reactions and fortifying resilience against infections induced by *M. avidus* and VHSV in olive flounder.

Citation: Choi, H.-J.; Choi, D.-Y.; Park, J.-J.; Jung, H.J.; Kim, B.-S.; Hong, S.-C.; Kim, J.-H.; Kang, Y.J. Protective Effects of Long Double-Stranded RNA with Different CpG Motifs against *Miamiensis avidus* and Viral Hemorrhagic Septicemia Virus (VHSV) Infections in Olive Flounder (*Paralichthys olivaceus*). *Fishes* **2024**, *9*, 227. https://doi.org/10.3390/fishes9060227

Academic Editor: Maria Angeles Esteban

Received: 30 April 2024
Revised: 5 June 2024
Accepted: 11 June 2024
Published: 13 June 2024

1. Introduction

Production losses caused by infectious diseases in cultured fish in South Korea have been estimated to be approximately 25–30% [1]. However, there is a lack of effective

control measures for most infectious diseases. With the implementation of stringent regulations on the use of chemotherapeutic agents, alternatives based on biopharmaceuticals have been proposed. Immunoprophylaxis with vaccines or immunostimulants can be used to control infectious diseases without chemotherapy.

The innate and adaptive immune systems enable vertebrates to protect themselves against foreign pathogens. As fish also have both innate and adaptive immune systems, they can acquire specific immunity through vaccines and non-specific immunity via immunostimulants [2]. However, the non-specific or innate immune systems of fish, rather than their adaptive immune systems, are activated in response to an infection. Developing vaccines against various infectious diseases is economically expensive, making development prospects limited and inefficient. Therefore, immunostimulant-mediated protection against all infectious diseases is more advantageous than vaccine-mediated protection against single diseases.

Fish cells have various Toll-like receptors (TLRs) homologous to mammalian TLRs. Agonist molecules recognized by TLRs as pathogen-associated molecular patterns (PAMPs) have been utilized as immunostimulants in vertebrates, including fish. Unmethylated cytidine–phosphate–guanosine (CpG) motifs flanked by two 5′ purines and two 3′ pyrimidines are recognized by TLR9 and are immunostimulatory in mammals [3–5]. As both CpG suppression and CpG methylation occur, the CpG motifs are rarely present in eukaryotic genomes; however, they are common in prokaryotic genomes [6,7].

Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs mimic the activity of bacterial DNA and are recognized as danger signals by mammalian immune cells [3,4,8,9]. The recognition of CpG DNA by TLR9 triggers the recruitment of the adaptor molecule MyD88, which activates a signaling cascade that produces various cytokines and chemokines, leading to type I interferon (IFN) transcription [10,11]. Artificially synthesized CpG oligodeoxynucleotides (CpG-ODNs) can also be recognized by TLR9 and may be categorized into classes A, B, and C based on their structural and functional characteristics [12–14]. The immunostimulatory effects of each CpG-ODN class are class-specific [10]. We previously demonstrated the protective effects of CpG-ODN 2216 (class A) and CpG-ODN 1668 (class B) against viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* infections, respectively [15]. We further demonstrated that olive flounders (*Paralichthys olivaceus*) administered plasmids harboring mixed CpG motifs from classes A, B, and C exhibited remarkably improved survival rates against VHSV and *M. avidus* infections compared to the controls [16]. However, information on the biological effects of CpG-ODNs in fish is limited.

Synthetic CpG-ODNs enhance the activation of non-specific cytotoxic cells and non-specific immune responses, including phagocytic respiratory burst and nitro blue tetrazolium activity in head kidney phagocytes and serum lysozyme activity, in catfish (*Ictalurus punctatus*) and carp (*Cyprinus carpio, Ctenopharyngodon idellus*) [17–19]. Additionally, plasmid DNA harboring synthetic CpG-ODNs enhances antibody responses, as determined using recombinant proteins, in tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*) and induces the production of IFN-like cytokines and interleukin (IL)-1β in leukocytes of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [13,20–22]. CpG-ODN1668 reportedly protects against rock bream iridovirus (RBIV) infection in rock bream due to increased innate immunity [23]. Additionally, as an agent that can protect against influenza virus infection in humans, CpG-ODNs have been demonstrated to be excellent immune stimulators with the potential to protect against viral infections in a non-specific manner [24–29]. CpG motifs introduced in plasmids exhibit a potent immunostimulatory capacity similar to that of CpG-ODNs and are studied actively as adjuvants for DNA vaccines in mammals and fish [16,30,31].

Artificially synthesized polyinosinic–polycytidylic acid (poly I:C), an analog of double-stranded RNA (dsRNA) [32], stimulates antiviral immune responses via the induction of type I IFN responses in mammals [33–35] and fish [36–41]. dsRNA, a by-product of viral

replication, is recognized by TLR3 as a PAMP, which triggers a strong proinflammatory response in vertebrates [32,42,43]. The binding of TLR3 to dsRNA results in the recruitment of the adaptor protein TRIF36 and activation of transcription factors such as IRF3, NF-κB, and AP-1, which in turn induce the transcription of type I IFN and inflammatory cytokines [44–47]. The secreted IFN regulates the expression of numerous genes encoding antiviral proteins, including dsRNA-dependent eukaryotic initiation factor kinase and Mx proteins [48]. A similar dsRNA recognition mechanism involving TLR3 occurs in fish [49]. The poly I: C agent reportedly protects against fish nodavirus (RGNNV) infection in sevenband grouper Epinephelus septemfasciatus [50]. In addition, poly I: C could protect against influenza virus infection in humans [51,52]. However, information on the ability of dsRNA to promote the resistance of fish to diseases caused by extracellular parasites is limited.

Furthermore, it is unclear if specific sequence motifs in dsRNA can modulate immunostimulatory effects. Thus, we aimed to determine whether dsRNA enhances the resistance of olive flounders against *M. avidus* infection and whether CpG motifs in dsRNA modulate resistance against VHSV and *M. avidus* infections in fish.

2. Materials and Methods

2.1. Vector Construction

A green fluorescent protein (GFP; open reading frame, 717 bp) fragment sequence was ligated into the Litmus 28i vector (New England Biolab [NEB], Ipswich, MA, USA), pre-digested with EcoRI and HindIII. The resulting plasmid was designated pLit-GFP. A fragment (ODN Mix6) corresponding to six randomly arranged copies of each CpG-ODN sequence (CpG-ODN 1668, 2216, and 2395) was synthesized and inserted into the pUC57 vector (pUC57-ODN Mix6) (Cosmo Genetech, Seoul, Republic of Korea). The sequences of CpG-ODN 1668, 2216, and 2395 were 5′-TCCATGACGTTCCTGATGCT-3′ as class B type, 5′-GGGGGACGATCGTCGGGGGG-3′ as class A type, and 5′-TCGTCGTTTTCGGCGCGCGCCG-3′ as class C type. After being digested with BglII and BamHI, the ODN Mix6 fragment was ligated into the Litmus 28i vector, which was pre-digested with the same restriction enzymes. The resultant plasmid was designated as pLit-ODN Mix6 (Figure 1). The pLit-GFP and pLit-ODN Mix6 plasmids, designed for dsRNA expression, were transformed into *Escherichia coli* HT115 (ΔRNase III).

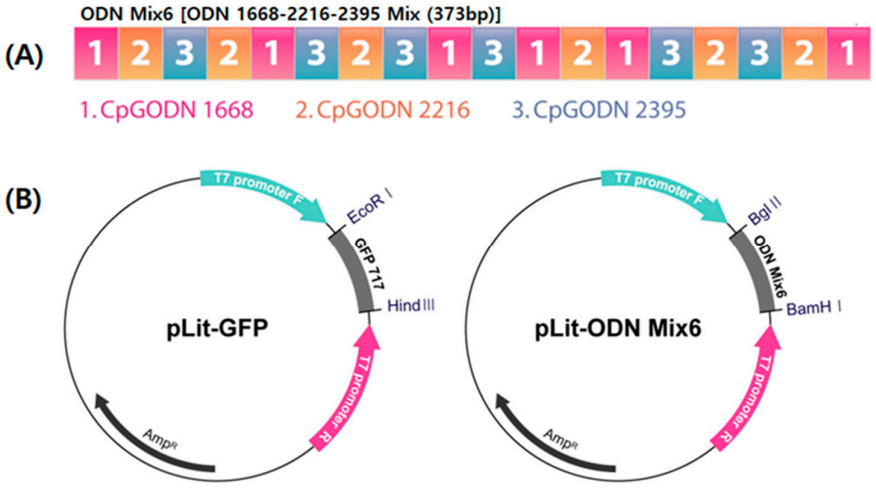

Figure 1. Map of vectors with double T7 promoter-driven long double-stranded RNA expression cassette in which GFP or ODN Mix6 was inserted. (**A**) ODN Mix6 is a fragment with six randomly arranged copies of each CpG-ODN sequence (CpG-ODN 1668, 2216, and 2395). (**B**) Plasmids ligated into the Litmus 28i vector (GFP 717 and ODN Mix6).

2.2. dsRNA Transcription and Isolation

Long dsRNAs encoding GFP or ODN Mix6 DNA (dsRNA-GFP or dsRNA-CpGMix) were extracted from pLit-GFP or pLit-ODN Mix6 harboring *E. coli* HT115 (ΔRNase III). To produce long dsRNA, *E. coli* HT115 (ΔRNase III) was transformed with the constructed pLit-GFP or pLit-ODN Mix6 vectors, inoculated in 200 mL of LB broth supplemented with 0.1 mg/mL ampicillin in a 300 mL flask, and incubated overnight at 37 °C. The cultured samples were centrifuged at 5900 rcf for 10 min at 4 °C. The pellet was resuspended in 0.5% SDS at 95 °C for 2 min. After 10 min of incubation on ice, the lysates were digested with RNase (Promega, Madison, WI, USA) and DNase (ELPIS) at 37 °C for 10 min. The dsRNAs were purified using RNAiso (TakaraBio, Shiga, Japan), electrophoresed on 1% agarose gel, stained with ethidium bromide (EtBr), and imaged using Gel Doc XR (Bio-Rad Laboratories, Hercules, CA, USA) (Figure 2).

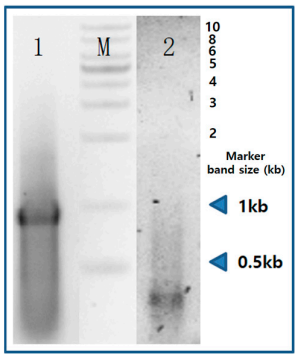

Figure 2. Purification of dsRNA in *Escherichia coli* HT115 harboring dsRNA-producing vectors. Lane 1 is dsRNA of GFP, lane 2 is dsRNA of ODN Mix6, and lane M is 1-kbp ladder (ELPIS-BIOTECH, Daejeon, Republic of Korea).

2.3. Immunostimulation and Challenge

Olive flounder fingerlings weighing 4–5 g were obtained from a local fish farm, divided into twelve 50 L tanks (18 fish/tank), and acclimated for at least two weeks before the study. The fish in each experimental group were divided into two subgroups: *M. avidus*- and VHSV-challenged groups.

For the *M. avidus* challenge, fish from eight tanks were divided into two groups, with each group consisting of four subgroups. The fish in the four subgroups were intraperitoneally (i.p.) injected with 50 μL of phosphate-buffered saline (PBS), 50 μg/50 μL/fish of poly I:C, or 50 μg/50 μL/fish of dsRNAs (dsRNA-GFP or dsRNA-CpGMix). The water temperature was maintained at 21–22 °C throughout the experiment. Three fish in each tank were randomly sampled to collect blood for serum separation at 3 d post-injection. The remaining fish in one group (15 fish) were intraperitoneally challenged with 2×10^4 ciliates/50 μL and the other group was challenged with 2×10^5 ciliates/50 μL/fish. Mortality was monitored for 14 d, and the dead fish were necropsied to confirm the presence of ciliates (Figure 3A).

The VHSV challenge experiment had the same groups as the *M. avidus* challenge experiment. The fish in four groups were intraperitoneally (i.p.) injected with the abovementioned treatment agents. The water temperature was maintained at 15 °C. At 24 h post-injection, three fish from each tank were sampled, and their kidneys were isolated. The remaining fish were intramuscularly (i.m.) challenged with VHSV (KJ2008) at 10^3 PFU/50 μL/fish, and mortality was monitored for 14 d (Figure 3B).

All experiments were conducted per the institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee of Sun Moon University (approval number: SM-2021-04-01).

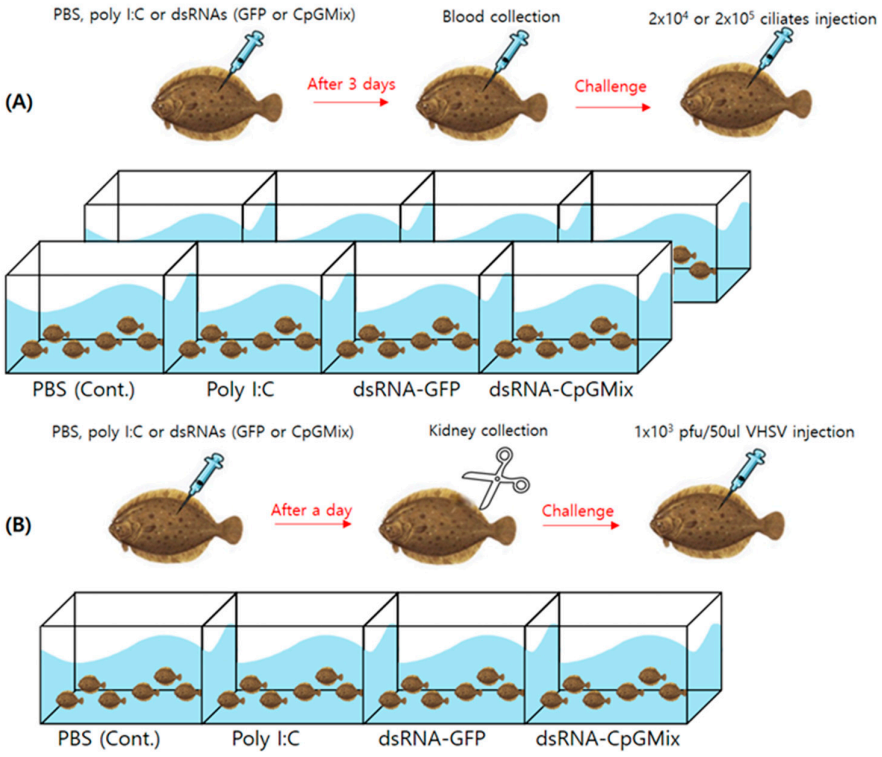

Figure 3. Schematic diagram of in vivo experiments. Olive flounder fingerlings were divided into twelve 50 L tanks (18 fish/tank). (**A**) For the *M. avidus* challenge experiment, fish from eight tanks were divided into two groups. Each group was further divided into four subgroups. Fish in the four groups were intraperitoneally (i.p.) injected with 50 μL of phosphate-buffered saline (PBS, control), 50 μg/50 μL/fish of poly I:C, or 50 μg/50 μL/fish of dsRNAs (dsRNA-GFP or dsRNA-CpGMix). At 3 d post-injection, blood was collected from three random fish in each. The remaining fish in one group (15 fish/tank) were i.p. challenged with 2×10^4 ciliates/50 μL and the fish in the other group were challenged with 2×10^5 ciliates/50 μL/fish. (**B**) The VHSV challenge experiment had four groups. Fish were injected with the abovementioned treatment agents via i.p. At 24 h post-injection, three fish from each tank were sampled, and their kidneys were isolated. The remaining fish (15 fish/tank) were intramuscularly (i.m.) challenged with VHSV (KJ2008) at 10^3 PFU/50 μL/fish.

2.4. Serum Scuticocidal Activity

Sera obtained from the fish in the *M. avidus* challenge group were used to examine scuticocidal activity. The assay was performed using 96-well flat-bottomed plates. All serum samples were serially diluted 2-fold, with the dilutions ranging from 1/4 to 1/4096, in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO, USA). Fifty microliters of diluted sera was dispensed into each well of the plate. The ciliates from the depleted Chinook salmon embryo (CHSE) cells were centrifuged at 200× *g* for 5 min, collected in a culture vessel, and washed three times with HBSS. The ciliates were then added to the wells (1×10^2 ciliates/well); the plates were incubated at 20 °C and observed hourly for 24 h to analyze scuticocidal activity. The titer of each serum sample was observed with an inverted microscope at 40–100× magnification and at the final dilution concentration at which the ciliates were 100% lysed or non-motile at 12 h.

2.5. Semi-Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA from the kidneys of each group was extracted using RNAiso plus reagent (TakaraBio) at 24 h post-immunization. Complementary DNA (cDNA) was synthesized from the total RNA through RT-PCR using M-MLV reverse transcriptase and Oligo(dT) 15 primers (Promega). The *Mx* gene or IFN-stimulated gene 15 (*ISG15*) was amplified using PCR with the primer sets MxF (5′-AACAGCCAAGGCAAAGATTG-3′) and MxR (5′-

AATGTCCAGCTCCTCCTTCA-3′) or ISG15F (5′-CTCCATGTAATCTGCAGCAA-3′) and ISG15R (5′-AGATCTAGTGCAGGTGTGA-3′), respectively. Two primers, 18SF (5′-AGTTGCTGCAGTTAAAAAGC-3′) and 18SR (5′-TGGCATCGTTTACGGTCGGAACTA-3′) were used to amplify the 18S ribosomal RNA (18S rRNA) gene in olive flounders. The PCR was performed using the 2× Prime Taq Premix (GeNet Bio, Daejeon, Republic of Korea). The amplification procedure included one 4 min cycle at 95 °C, sixteen (for 18S rRNA) or thirty (for *Mx* and *ISG15*) 30 s cycles at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and a final 7 min extension step at 72 °C. The PCR samples were electrophoresed on a 1% agarose gel, stained with EtBr, and imaged using Gel Doc XR. *Mx* and *ISG15* expression was determined relative to 18S rRNA gene expression using Quantity One software (Bio-Rad Laboratories).

2.6. Statistical Analyses

A one-way analysis of variance with Tukey's multiple comparisons test was used to analyze the significance of differences between the groups. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS (version 9.0; SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation.

3. Results

3.1. Effects of Long Double-Stranded RNA Containing the ODN Mix6 Motif against VHSV Infection in Olive Flounders

The cumulative mortality rate of the control group was 100% after the VHSV challenge at 10^3 PFU/fish (Figure 4A). The groups administered dsRNA-GFP exhibited a lower mortality rate than the control group. The groups injected with dsRNA-CpGMix or poly I:C showed increased survival rates compared to the control group ($p < 0.05$).

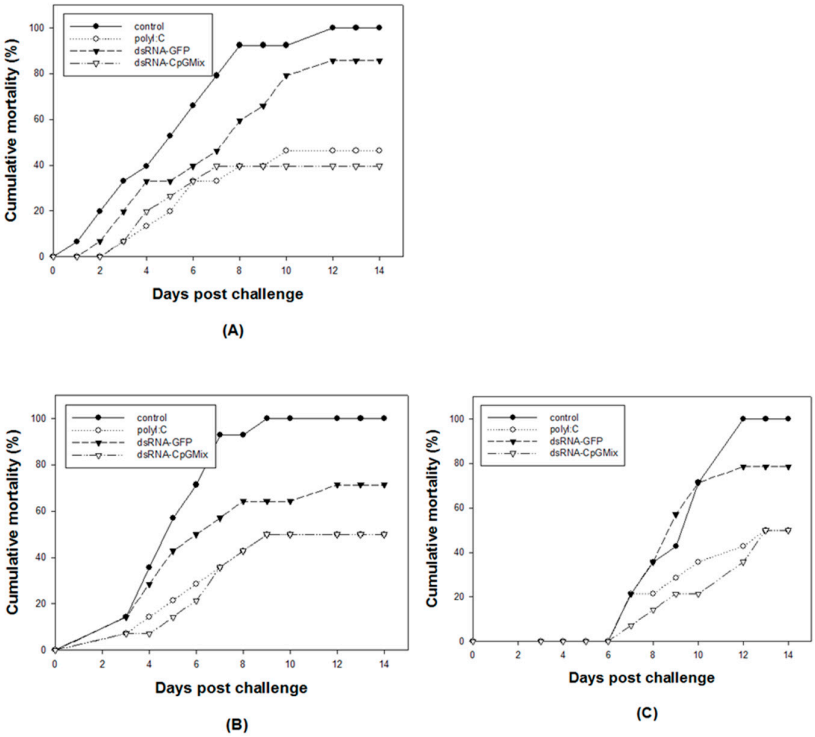

Figure 4. Cumulative mortality rates of olive flounder (*Paralichthys olivaceus*) fingerlings intraperitoneally (i.p.) injected with phosphate-buffered saline (control), poly I:C, dsRNA-GFP, or dsRNA-CpGMix against VHSV. Three days after injections, (**A**) the remaining fish in each group were intramuscularly (i.m.) challenged with VHSV (KJ2008) at 10^3 PFU/fish. Mortality was recorded daily for 14 d. (**B**) The fish in each group were i.p. challenged with 2×10^5 ciliates. (**C**) The fish in

each group of the other replicate groups were challenged with 2 × 10^4 ciliates. Mortality was monitored for 14 d. These data are derived from a single unreplicated experiment.

3.2. Effects of Long Double-Stranded RNA Containing CpG Motifs against M. avidus Infection in Olive Flounders

In the challenge experiments with 1 × 10^4 ciliates (low level) or 2 × 10^5 ciliates (high level)/50 μL, the cumulative mortality rate of the fish injected with dsRNA was approximately two times lower than that of fish injected with PBS alone. The groups injected with dsRNA-CpGMix and poly I:C showed the highest survival rates (Figure 4B,C). The ciliates from the internal and external organs of all dead fish were also examined.

3.3. Gene Expression in Olive Flounders

The effects of each dsRNA on type I IFN response in olive flounders were investigated by analyzing *Mx* and *ISG15* expression 24 h post-administration. The fish administered poly I:C or dsRNA-CpGMix showed higher expression of *Mx* and *ISG15* than those in the other groups. The fish injected with dsRNA-CpGMix showed higher *ISG15* expression (approximately 20 times higher) ($p < 0.05$) than those injected with PBS alone (Figure 5).

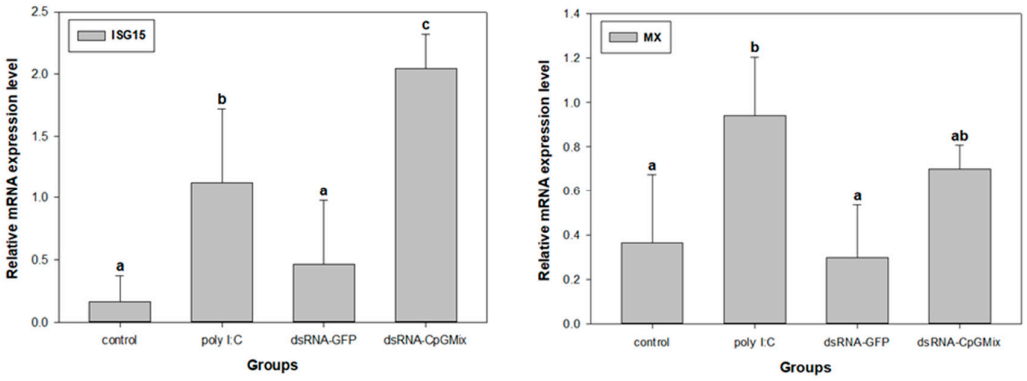

Figure 5. RT-PCR amplification of *ISG15* and *Mx* in olive flounders (*Paralichthys olivaceus*) i.p. injected with PBS (control), poly I:C, dsRNA-GFP, or dsRNA-CpGMix (50 μg/fish). Total RNA was isolated from the kidneys of olive flounders 24 h post-injection. *ISG15*, *Mx*, and 18S ribosomal RNA (18S) were PCR-amplified from cDNA samples using primers for each gene. Values (inverse of serum dilutions) are presented as mean, and the T-bars indicate standard deviation. Different letters on the bars indicate significant differences ($p < 0.05$). These data are based on three replicated experiments.

3.4. Scuticocidal Activity

The fish injected with dsRNA-CpGMix showed significantly higher serum scuticocidal activity than those injected with PBS alone or dsRNA-GFP (Figure 6). The serum scuticocidal activity in the fish injected with dsRNA-CpGMix was similar to that in the fish injected with poly I:C. Scuticocidal activity was not observed in the wells containing heat-inactivated serum or HBSS alone.

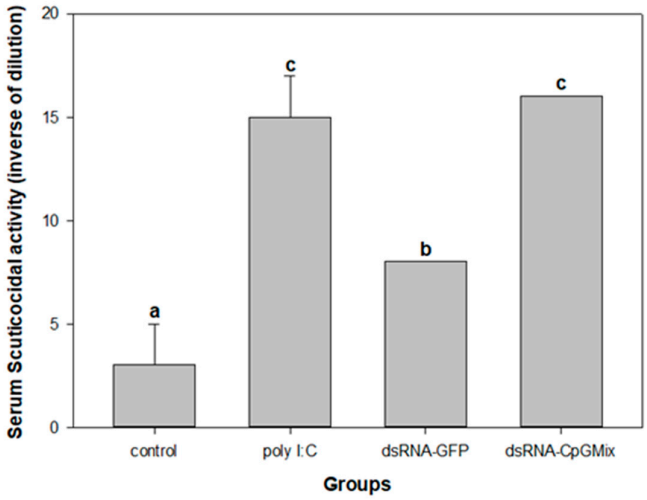

Figure 6. Scuticocidal activity in sera obtained from olive flounders i.p. injected with PBS (control), poly I:C, dsRNA-GFP (dsGFP), or dsRNA-CpGMix. Values (inverse of serum dilutions) are presented as mean, and the T-bars indicate the standard deviation. Different letters indicate significant differences ($p < 0.05$). These data are based on three replicated experiments.

4. Discussion

In mammalian cells, endosomal TLR3 recognizes dsRNA [32,42,43] and recruits TRIF by interacting with its TIR domains [53]. TRIF then indirectly activates transcription factors, ISG15 and Mx, which modulate the transcription of IFNs and inflammatory cytokines [44,46,47,49]. These responses include synthesizing and secreting IFNs and lysozymes and producing cytotoxic T cells, as well as phagocytic processes [54]. IFNs are potent against infectious diseases [55].

In our preliminary study, we confirmed the effectiveness of CpG-ODN class types, CpG motif-harboring plasmids, and single RNA poly I:C in protecting olive flounders against viral and parasitic diseases. Treating olive flounders with CpG-ODN 1668 and 2216 enhanced their resistance against VHSV and *M. avidus* infections, respectively, suggesting that selecting a CpG-ODN according to the characteristics of a certain pathogen is crucial for inducing optimal defensive immune responses in fish. CpG-ODN 2395 showed an intermediate effect between the effects of CpG-ODN 1668 and 2216 [15,56–58]. Based on these preliminary findings, we artificially synthesized fragments corresponding to each of the six copy sequences randomly arranged as CpG-ODN 2216 in class A, 1668 in class B, and 2395 in class C and purified them by expressing dsRNA.

Single-stranded RNA expressing unmethylated CpG motifs and a poly(G) tail stimulated human monocytes and peripheral blood mononuclear cells to produce IL-6 and IL-12 and activate NF-κB, p38, and MAPK [59]. These effects were similar to the immunostimulatory effects of CpG-ODNs. However, there are no reports on the dsRNA CpG motifs that exert further immunostimulatory effects on hosts. Here, we demonstrated that dsRNA containing CpG motifs stimulates immune responses in olive flounders. Different types of mixed CpG motif-containing dsRNA increased the survival rates of fish infected with VHSV. While the survival rates were comparable to those of fish administered poly I:C, the GFP sequence-containing dsRNA did not protect the fish against VHSV. These results suggest that CpG motifs in dsRNA are recognized by certain receptor(s) in olive flounders, stimulating protective immune responses by activating type I IFN expression.

Here, the fish administered poly I:C or dsRNA with different CpG motifs (dsRNA-CpGMix) showed enhanced survival rates against *M. avidus* compared to the controls. In a previous study, the scuticocidal activity of leukocytes in olive flounders increased

following stimulation with artificially synthesized poly I:C or CpG-ODNs. These results suggest that IFN-mediated cellular immune responses actively protect fish against intruding scuticociliates. Moreover, the serum scuticocidal activity in fish administered poly I:C was stronger than that in the controls, suggesting that both cellular and humoral immune responses may be stimulated by poly I:C. The fish administered dsRNA-CpGMix also showed high survival rates against *M. avidus*, and their survival rates were similar to those of fish administered poly I:C. Therefore, the CpG motif sequences present in dsRNA may effectively protect olive flounders against infectious diseases.

The findings of this study indicate that dsRNA-CpGMix activates innate immunity and INF-related immune responses in olive flounders, corroborated by reports of immunomodulatory effects of poly I:C. Although the immune systems of fish can recognize dsRNA and unmethylated CpG DNA through TLR3 and TLR9, respectively, the receptors that bind to CpG motifs on dsRNA are unknown, even in humans. Furthermore, fish administered dsRNA encoding partial GFP showed higher survival rates than the control but presented lower survival rates than fish administered dsRNA containing CpG motifs. This finding suggests that differences in the sequences, structures, or presence of certain motifs in dsRNA may be important factors in modulating immune responses. In the future, we will conduct detailed studies to identify the cellular immunostimulatory mechanism of dsRNA-CpG.

5. Conclusions

The sequences of the CpG motifs in dsRNA may be important for enhancing immune responses and resistance against *M. avidus* and VHSV infections in olive flounders. Furthermore, the dsRNA-CpGMix-immunostimulated olive flounders showed immunity against VHSV and *M. avidus*.

Author Contributions: Formal analysis, H.-J.C., D.-Y.C., J.-J.P., H.J.J., and Y.J.K.; investigation, H.-J.C. and D.-Y.C.; conceptualization, S.-C.H., B.-S.K., and Y.J.K., methodology, S.-C.H. and B.-S.K.; writing—original draft preparation, J.-H.K. and Y.J.K., reviewing, J.-H.K.; editing, Y.J.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education [grant number NRF-2021R1I1A2045209].

Institutional Review Board Statement: All experiments in this study were conducted per the institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee of Sun Moon University (approval number: SM-2021-04-01).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available on request from the corresponding author.

Conflicts of Interest: Author Jung-Jin Park was employed by the company Samyang Anipharm Co., Ltd., Seoul City, South Korea. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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