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Effect of miR-155 as a molecular adjuvant of DNA vaccine against VHSV in olive flounder (*Paralichthys olivaceus*)

Hyun Ju Lim[a], Najib Abdellaoui[b], Ki Hong Kim[a,*]

[a] Department of Aquatic Life Medicine, Pukyong National University, Busan, 48513, South Korea
[b] Department of Microbiology, Pukyong National University, Busan, 48513, South Korea

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ABSTRACT

Rhabdoviral G protein-based DNA vaccines have been recognized as a useful way to protect cultured fish from rhabdoviral diseases. In Korea, viral hemorrhagic septicemia virus (VHSV) genotype IVa has been the primary culprit of high mortalities of cultured olive flounder (*Paralichthys olivaceus*). In this study, we inserted a miR-155-expressing cassette into the VHSV's G protein-based DNA vaccine, and analyzed the effects of miR-155 on the antiviral activity and on the vaccine efficacy in olive flounder. Olive flounder fingerlings were intramuscularly (i.m.) immunized with 10 μg/fish (1st experiment) or 1 μg/fish (2nd experiment) of DNA vaccine plasmids. However, there were no significant differences in mortalities and serum neutralization titers between fish immunized with 1 μg and 10 μg plasmids/fish, suggesting that i.m. injection with 1 μg plasmids/fish would be enough to induce effective adaptive immune responses in olive flounder fingerlings. In survival rates, as fish immunized with just G protein expressing plasmids showed no or too low mortalities, the adjuvant effect of miR-155 was not discernible. Also, in the serum neutralization activities, although G gene or G gene plus miR-155 expressing DNA vaccines induced significantly higher activities than control vaccines (PBS and vacant vector), no significant differences were found between G gene alone and G gene plus miR-155 expressing DNA vaccines. In the serum virucidal activity, fish immunized with G gene plus miR-155 expressing DNA vaccine showed significantly higher activity against hirame rhabdovirus (HIRRV) at 3 days post-immunization (d.p.i.) compared to other groups, suggesting that miR-155 produced from the vector can enhance innate immune responses in olive flounder. The significantly enhanced serum virucidal activities against VHSV especially at 28 d.p.i. in the groups immunized with G gene alone and G gene plus miR-155 expressing DNA vaccines reflect the increased antibodies against G protein, which could activate the classical complement pathway and subsequent viral inactivation. As the available information on the DNA vaccines in olive flounder is not sufficient, more diverse researches on the protective efficacy of DNA vaccines are needed to make more practical use of DNA vaccines in olive flounder farms.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV) has been one of the main causes of mass mortalities in cultured fish around the world, mainly during the low water temperature period [1,2]. VHSV can be divided into 4 genotypes based on the glycoprotein (G) gene sequence, and each genotype shows a specific geographical distribution and host species [3–5]. Therefore, the development of control measures that are suitable for each genotype and host species in the geographical region are needed to obtain the maximum efficacy. In Korea, VHSV genotype IVa has been the primary culprit of high mortalities of cultured olive flounder (*Paralichthys olivaceus*) [6], however, still there are no commercial therapeutics or vaccines.

DNA vaccines have been recognized as a useful way to protect cultured fish from rhabdoviruses, such as infectious haematopoietic necrosis virus (IHNV), spring viraemia of carp virus (SVCV) and VHSV [7,8]. In olive flounder, Byon et al. [9] and Lazarte et al. [10] reported the protective potential of intramuscularly injected (i.m.) DNA vaccines encoding G protein of VHSV, however, there were considerable differences in the dose of plasmids used for the vaccination between the two papers (10 μg/fish and 1 μg/fish, respectively). Moreover, Lazarte et al. [10] additionally used an expression cassette for DDX41 gene in the DNA vaccine vector to enhance adaptive immune responses. As the available information on the DNA vaccines in olive flounder is not sufficient, more diverse researches on the protective efficacy of DNA vaccines are needed to make more practical use of DNA vaccines in

* Corresponding author.
E-mail address: khkim@pknu.ac.kr (K.H. Kim).

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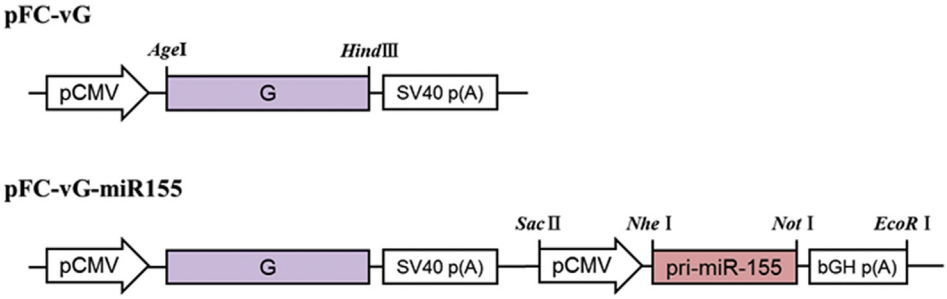

Fig. 1. Construction of VHSV DNA vaccine vectors. (A) VHSV G gene was inserted in pFC vector (pFC-vG). (B) Primary miR-155 expressing cassette was inserted behind the G gene expressing cassette (pFC-vG-miR155).

olive flounder farms.

The use of appropriate molecular adjuvants can be a method to enhance immunogenicity of DNA vaccines. Initially, unmethylated CpGs in plasmid vectors were recognized as an immunostimulatory and adjuvant molecule [11–14]. Recently, cassettes expressing various cytokine genes and interferon-related genes are built in the DNA vaccine vector as molecular adjuvants to enhance adaptive immune responses [15–18].

MicroRNAs (miRNAs) are small, non-coding RNAs, and most of miRNAs are transcribed through RNA polymerase II. Initially transcribed miRNAs (primary miRNAs) in the nucleus are processed by Drosha to generate pre-miRNAs that are transported to the cytoplasm. Pre-miRNAs in the cytoplasm re-processed by Dicer to produce 20–22 nt RNA duplexes which incorporate into the RNA induced silencing complex (RISC), and functional single-stranded mature miRNAs guide the RISC to the complement sequence of target mRNA (usually 3′UTR) to inhibit the translation of the target mRNAs [19]. Several different mRNAs can be targets of one kind of miRNA, and vice versa, one kind of mRNA can be a target of several different miRNAs, which allows complex net-workings in the regulation of genes by miRNAs [19]. Numerous miRNAs are involved in immune responses [20,21]. Among those miRNAs, miR-155 is known to involve in the regulation of not only innate immune reactions but also adaptive immune responses in mammals [22–24]. Furthermore, in our previous studies [25,26], miR-155 was significantly up-regulated by the infection of VHSV in olive flounder and Epithelioma papulosum cyprini (EPC) cells.

Recently, Izzard et al. [27] reported that neutralizing antibody levels of mice immunized with live influenza A virus vaccine were significantly enhanced by the incorporation of miR-155 into the viral genome. However, the adjuvant effect of miR-155 in DNA vaccines has not been reported. In this study, we inserted a miR-155-expressing cassette into the VHSV's G protein-based DNA vaccine, and analyzed the effects of miR-155 on the antiviral activity and on the vaccine efficacy in olive flounder.

2. Materials and methods

2.1. Cell culture and virus

Epithelioma papulosum cyprini (EPC; ATCC CRL-2872) cells were grown at 28 °C in Leibovitz's L-15 medium (L-15, Sigma) containing 10% fetal bovine serum (FBS, WELGENE) and 1% penicillin-streptomycin (WELGENE). For preparation of the viral hemorrhagic septicemia virus (VHSV) KJ2008 and hirame rhabdovirus (HIRRV) CA9703, 2×10^6 EPC cells were cultured on T-25 cell culture flasks in L-15 with 10% FBS and 1% antibiotics. The cells were incubated overnight at 28 °C and moved to 20 °C, and inoculated with virus at multiplicity of infection (MOI) of 1.0 in L-15 medium containing 1% antibiotics. When a complete cytopathic effect (CPE) was observed, virus was collected after removal of cell debris by centrifugation at 4000 rpm for 5 min, and viral titer was measured by plaque assay.

2.2. Vector construction

The G gene ORF of VHSV was amplified with PCR using a primer pair: G-For (5′-ACCGGT**GCCACCATGG**AATGGAATACTTTTTTCTTGGTGATTCTGG-3′) containing *Age*I (underlined) and Kozak sequence (underlined and bold), G-Rev (5′-AAGCTTT-CAGACCATCTGGCTTCTGGAGAAC-3′) containing *Hind*III (underlined). The PCR product was cloned into pGEM-T easy vector (Promega), then, the cloned plasmids were digested with the restriction enzymes. The G gene fragment was inserted in the downstream of CMV promoter of pFC vector (System Bioscience, Korea), and designated as pFC-vG.

The miR-155 sequences of olive flounder and fathead minnow (*Pimephales promelas*) [25,26] were identical to the zebrafish miR-155 sequence (miRBase; accession: MI0002023). The precursor of miR-155 was retrieved by blasting mature miR-155 against fathead minnow genome deposited in NCBI database. The retrieved sequence was analyzed using MFOLD RNA server with default parameters to predict secondary structure. Furthermore, the predicted hairpin structure was submitted to iMiRNA-SSF web-server which classifies pre-miRNA hairpin sequences as real or pseudo-pre-miRNA. The region encompassing precursor miR-155 plus 200 nt of flanking regions (left and right) was cloned from EPC cells using primers containing *Nhe*I (5′-GCTAGCTGGTCCATTAATATGATTTATTGTC-CTTCT-3′) and *Not*I (5′-GCGGCCGCGAAACATTATTACTTTACCCTGTTTGTGATC-3′) restriction enzyme sites. The cloned primary miR-155 was inserted into the downstream of CMV promoter of pcDNA 3.1 (+) (Invitrogen) that was pre-digested with *Nhe*I and *Not*I enzymes. This miR-155 expression cassette (pCMV-miR155-bGHp(A)) was subcloned into pGEM-T easy vector using a primer pair (5′-CCGCGGGACATTG-ATTATTGACTAGTTATTAATAGT-3′ *Sac*II and 5′-GAATTCCCATAGAGCCCACCGCATCC-3′ *Eco*RI). Finally, the pCMV-miR155-bGHp(A) cassette was inserted into pFC-vG vector that was pre-digested with *Sac*II and *Eco*RI enzymes, and designated as pFC-vG-miR155 (Fig. 1).

2.3. In vitro quantification of miR-155

EPC cells (ATCC no. CRL-2872) were seeded into 35 mm dishes (1×10^6/dish) and incubated overnight at 20 °C in L-15 medium supplemented with 10% FBS and 1% antibiotics. When confluency reached 80–90%, cells were transfected with 2 μg of pFC-vG or pFC-vG-miR155 using FuGENE HD transfection reagent (Promega) according to the manufacturer's protocol. Mock-control cells were treated with the transfection reagent alone. At 48 h post-transfection, small RNA was isolated using Hybrid-R™ miRNA (GeneAll, Korea) following manufacturer's instructions. HB miR Multi Assay Kit™ system II (HeimBiotek, Korea) was used to synthesize cDNA and to quantify miR-155. Amplification of small nucleolar U6 used as a reference gene and miR-155 was done using specific primers provided by HeimBiotek. Real-time PCR was performed with 2 μl of the cDNA using Light Cycler 480 (Roche). Amplification was done as follows: initial activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 10 s and

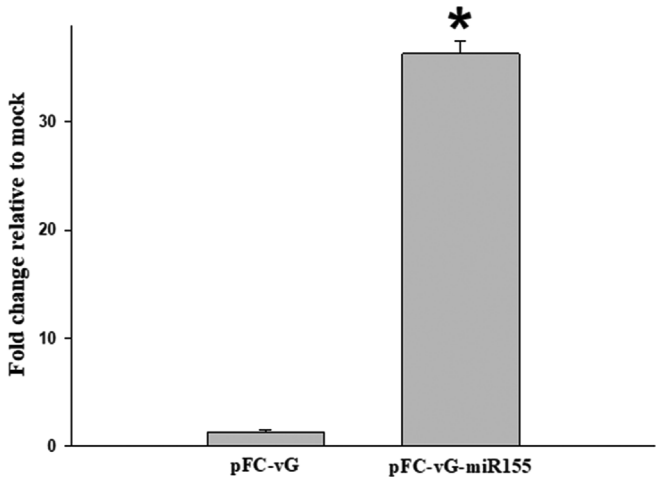

Fig. 2. Quantification of miR-155 in Epithelioma papulosum cyprini (EPC) cells using real-time RT-PCR. Relative expression of miR-155 in EPC cells transfected with pFC-vG or pFC-vG-miR155 was compared to mock-control cells using Ct method. Fold changes (means ± SE based on triplicate assay) were represented in histograms. The U6 gene was used as an internal control. The asterisk on the bar represents a significant difference at $P < 0.05$.

annealing-extension step at 60 °C for 40 s. The results were analyzed using the comparative threshold method ($2^{-\Delta\Delta Ct}$).

2.4. In vivo experiment

2.4.1. 1st experiment (10 μg of plasmids)

Olive flounder fingerlings (mean weight 2.94 g) were obtained from a local olive flounder hatchery and acclimated for 1 week at 20 °C. Before experiment, randomly selected 10 fishes were dissected to confirm the absence of pathogens including VHSV. Prior to immunization and challenge infection, olive flounders were anesthetized with 75 ppm MS-222 (Sigma). The fishes were randomly divided into four experimental groups with 45 fishes (PBS, pFC empty vector, pFC-vG, and pFC-vG-miR155) and intramuscularly injected with 10 μg of plasmids in 50 μl PBS or same volume of PBS alone. At 3, 14 and 28 d post-immunization (d.p.i), blood was sampled from 6 fishes per group for serum isolation. Four weeks post immunization (w.p.i), water temperature was gradually down to 13 °C over 3 days, and 2 replicates (12–13 fish/replicate) of each group were challenged by i.m. injection of 10^2 and 10^3 VHSV, respectively. Mortality was recorded daily for 3 weeks post-challenge.

2.4.2. 2nd experiment (1 μg of plasmids)

The procedures before immunization were the same as those in the 1st experiment. Olive flounder fingerlings (mean weight 3.62 g) were randomly divided into four experimental groups with 30 fishes (PBS, pFC empty vector, pFC-vG, and pFC-vG-miR155) and i.m. injected with 1 μg of plasmids in 50 μl PBS or same volume of PBS alone. At 28 d.p.i, blood was sampled from 5 fishes per group for serum isolation. Four weeks post immunization, water temperature was gradually down to 13 °C over 3 days, and 2 replicates (12–13 fish/replicate) of each group were challenged by i.m. injection of 5×10^3 and 5×10^4 VHSV, respectively. Mortality was recorded daily for 3 weeks post-challenge.

2.5. Virucidal activity of serum

The isolated sera were serially diluted with L-15 containing 1% antibiotics in 96-well round-bottomed plate (60 μl/well), then same volume of either VHSV or HIRRV (1×10^3 PFU/well) were mixed with serially diluted sera. Each serum-virus mixture was incubated at 4 °C for 1 h, and added to triplicate wells containing EPC cells monolayer in 96-well flat-bottomed plate. The plates were incubated at 15 °C and observed CPE every day. The titer of each serum was the last dilution at which CPE was not observed.

2.6. Serum neutralization test

The isolated sera of 28 d.p.i from fishes injected with 10 and 1 μg of plasmids were heat-inactivated at 56 °C for 30 min to inactivate complement, and 10 fold diluted with L-15 containing 1% antibiotics. Diluted sera were mixed with equal volume of 10 fold diluted fresh serum of naïve olive flounder, and serially diluted with L-15 containing 1% antibiotics in 96-well round-bottomed plate (60 μl/well). Then, the same volume of VHSV (1×10^3 PFU) was mixed with serially diluted sera. Each serum-complement-virus mixture was incubated at 4 °C for 1 h, and added to triplicate wells containing EPC cells monolayer in 96-well flat-bottomed plates. The plates were incubated at 15 °C and observed CPE every day. The titer of each serum was the last dilution at which CPE was not observed.

2.7. Statistical analysis

Statistical significance was analyzed using SPSS for Windows (Chicago, IL, USA). Data were analyzed by using one-way ANOVA followed by Tukey HSD post-hoc test. Kaplan-Meier method was used to the data on cumulative mortality, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. In vitro quantification of miR-155

EPC cells transfected with plasmids containing miR-155 expressing cassette (pFC-vG-miR155; Fig. 1) showed over 36 folds of miR-155 expression at 48 h of post-transfection (h.p.t), while cells transfected with pFC-vG showed no statistical difference in miR-155 quantity compared to that of mock-control cells (Fig. 2).

3.2. Serum virucidal activity

Virucidal activity against VHSV and HIRRV was analyzed with the sera from fishes injected with 10 μg of plasmids. In the results of virucidal activity against VHSV, the pFC-vG and pFC-vG-miR155 groups showed higher activities than the control groups (PBS and pFC) at all analyzed times, and showed steeply increased activities at 28 d.p.i. (Fig. 3A). On the other hand, in HIRRV, only pFC-vG-miR155 group showed a significantly higher level than the other groups only at 3 d.p.i. (Fig. 3B).

3.3. Serum neutralization activity

Neutralization test against VHSV was analyzed with the sera sampled at 28 d.p.i from fishes injected with 10 and 1 μg of plasmids. The serum neutralizing antibody titers of pFC-vG and pFC-vG-miR155 groups (both 10 and 1 μg of plasmids) were significantly higher than that of PBS and pFC groups (Fig. 4A and B). However, there were no significant differences in the serum neutralization activity between pFC-vG and pFC-vG-miR155 groups.

3.4. Protective efficacy

In the 1st experiment (10 μg of plasmids), no mortality occurred in pFC-vG and pFC-vG-miR155 groups when challenged with 1×10^2 PFU of VHSV, while PBS and pFC groups showed more than 65% mortality (Fig. 5A). When challenged with 1×10^3 PFU of VHSV, PBS and pFC groups showed 66.7 and 83.3% mortality, respectively, while pFC-vG and pFC-vG-miR155 groups showed 16.7 and 8.3% mortality,

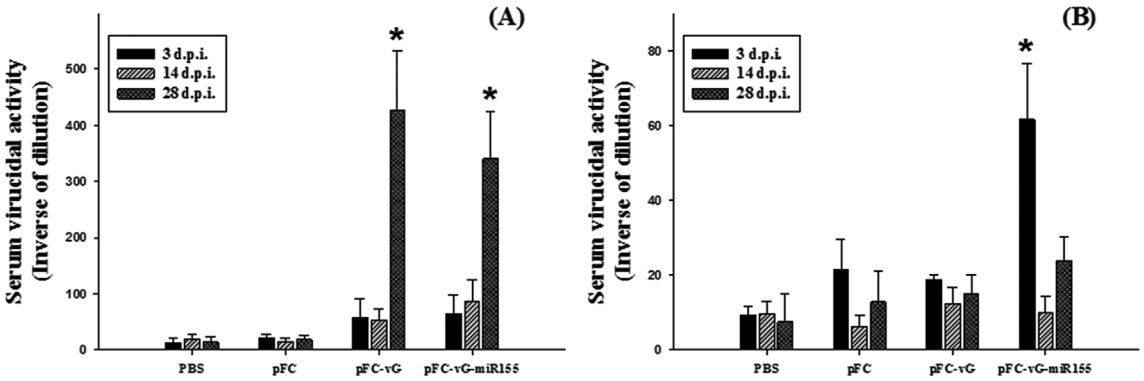

Fig. 3. Serum virucidal activity against (A) VHSV and (B) HIRRV. Olive flounder (*Paralichthys olivaceus*) fingerlings were immunized with 10 μg/fish of G gene expressing plasmids (pFC-vG) or G gene plus miR-155 expressing plasmids (pFC-vG-miR155). Control fish were injected with the same amount of vacant vector (pFC) or the same volume of phosphate buffered saline (PBS). At 3, 14 and 28 days post immunization (d.p.i), serum was isolated from randomly sampled fish, and used for the analysis of serum virucidal activity. Asterisks on the bar indicate the statistical significance from other samples at $p < 0.05$.

respectively (Fig. 5B). In the 2nd experiment (1 μg of plasmids), PBS and pFC groups showed 81–100% mortalities by a challenge with 5×10^3 or 5×10^4 PFU of VHSV, while pFC-vG and pFC-vG-miR155 groups showed 0–8.3% mortalities (Fig. 5C and D).

4. Discussion

There have been several reports on the use of cytokine genes as molecular adjuvants of DNA vaccines that were designed for the prevention of fish viral diseases; interleukin (IL)-1β to cyprinid herpesvirus-3 (CyHV-3) in carp [28], type I interferons to infectious salmon anemia virus (ISAV) in Atlantic salmon [29,30], and various cytokine genes to IHNV in rainbow trout [31]. Lazarte et al. [10] reported the enhanced survival of olive flounder against VHSV challenge by immunization with DNA vaccine plasmids co-expressing VHSV G protein and DDX41, a protein in the DEXD/H-box (DDX) family. However, there have been no reports on the use of microRNAs as molecular adjuvants of DNA vaccines in fish. In mammals, Wheatley et al. [32] developed a DNA vaccine vector for the prevention of human immunodeficiency virus type 1 (HIV-1), in which to increase the immunogenicity of HIV-1 envelope antigens, miR-155 scaffold-based artificial miRNA expression cassette that could inhibit protein kinase R-like endoplasmic reticulum kinase (PERK) was additionally inserted into the DNA vaccine vector. However, in that case, as the mature miR-155 sequence was removed from the miRNA expression cassette, it did not reflect the adjuvant effect of miR-155 but just suggested the possible use of miRNAs for molecular adjuvants of DNA vaccines.

In the present study, we aimed to assess the potential of miR-155 as a molecular adjuvant for VHSV G protein-based DNA vaccines. However, we could not observe any adjuvant effect of miR-155 from the experimental data of survival rates. As fish immunized with just G protein expressing plasmids showed no or too low mortalities, the adjuvant effect of miR-155 was not discernible. Also, in the serum neutralization activities, although G gene or G gene plus miR-155 expressing DNA vaccines induced significantly higher activities than control vaccines (PBS and vacant vector), no significant differences were found between G gene alone and G gene plus miR-155 expressing DNA vaccines. There have been many reports demonstrating the high immunogenicity and antigenicity of VHSV G protein [33–35], which might cover the adjuvant effect of miR-155 in the present study. Therefore, to know exactly the adjuvant role of miR-155, the differential expression of immune factors in the immunized fishes should be further analyzed.

In the present results, there were no significant differences in mortalities and serum neutralization titers between fish immunized with 1 μg plasmids/fish and 10 μg plasmids/fish, suggesting that i.m. injection with 1 μg plasmids/fish would be enough to induce effective adaptive immune responses in olive flounder fingerlings. Consequently, to know the effects of molecular adjuvants in VHSV G gene-based DNA vaccines, experiments with lower doses of plasmids might be needed.

In the serum virucidal activity, fish immunized with pFC-vG-miR155 showed significantly higher activity against HIRRV at 3 d.p.i. compared to other groups. The immunostimulatory activity of miR-155 has been well-demonstrated in mammals [36,37], and the present result

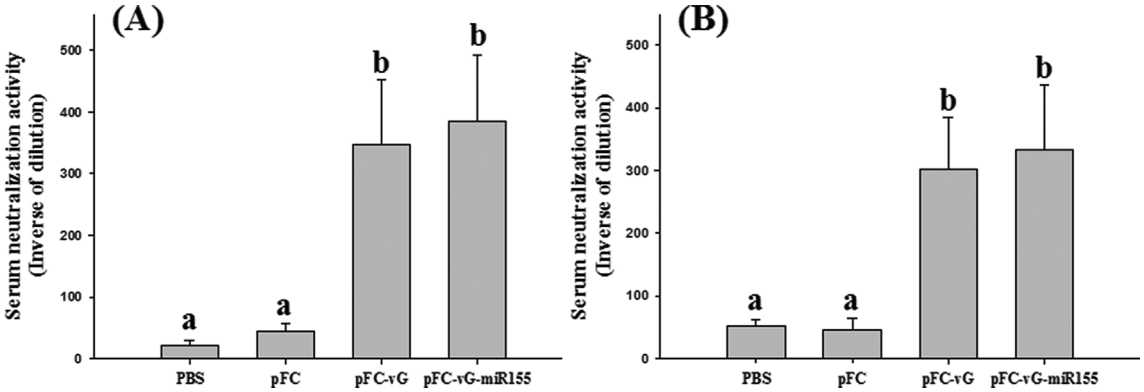

Fig. 4. Serum neutralization activity against VHSV. Olive flounder (*Paralichthys olivaceus*) fingerlings were immunized with 10 μg/fish (A) or 1 μg/fish (B) of G gene expressing plasmids (pFC-vG) or G gene plus miR-155 expressing plasmids (pFC-vG-miR155). Control fish were injected with the same amount of vacant vector (pFC) or the same volume of phosphate buffered saline (PBS). At 28 days post immunization (d.p.i), serum was isolated from randomly sampled fish, and used for the analysis of serum neytralization activity. Different letters on the bar represent the statistical significance at $p < 0.05$.

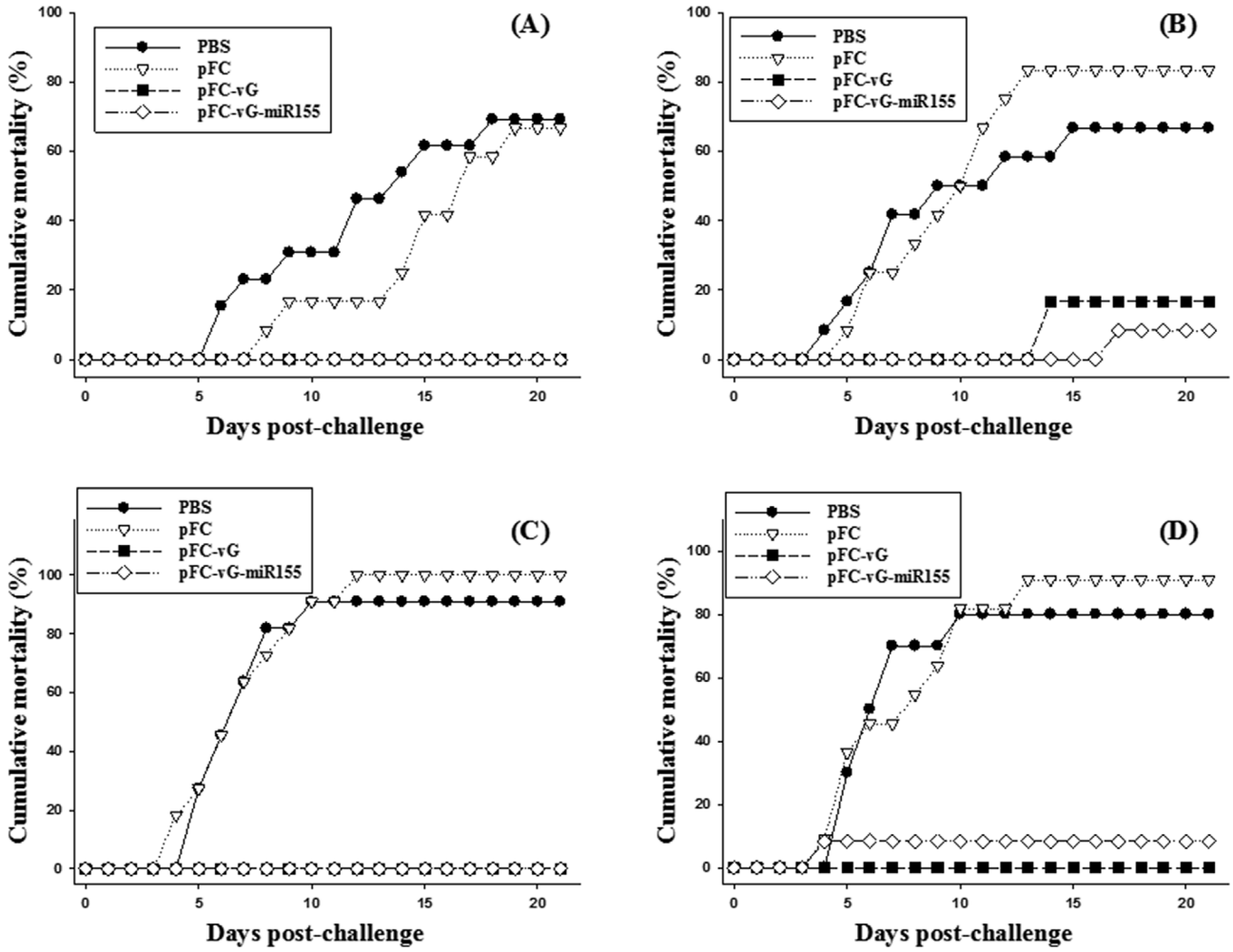

Fig. 5. Cumulative mortalities of olive flounder (*Paralichthys olivaceus*) fingerlings immunized with 10 μg/fish (A and B) or 1 μg/fish (C and D) of DNA vaccine plasmids and challenged with (A) 1×10^2, (B) 1×10^3, (C) 5×10^3 and (D) 5×10^4 PFU of VHSV.

suggests that miR-155 produced from the vector can enhance innate immune responses in olive flounder. However, the serum virucidal activity against VHSV was not significantly enhanced by the addition of a miR-155 cassette. In the results, the virucidal activity against VHSV was mainly related to the G protein, while the activity against HIRRV was mainly dependent on miR-155. The differences between VHSV and HIRRV in the susceptibility to serum factors induced by G protein and miR-155 should be further investigated. The significantly enhanced serum virucidal activities against VHSV especially at 28 d.p.i. in the groups immunized with pFC-vG and pFC-vG-miR155 reflect the increased antibodies against G protein, which could activate the classical complement pathway and subsequent viral inactivation.

Although high efficacy of DNA vaccines in the protection of fish from rhabdoviral diseases has been reported, the use of DNA vaccines in aquaculture farms has been limited due to mainly the categorization of DNA vaccinated fish as living modified organisms (LMOs) in many countries. However, recent permission of Clynav, a commercial DNA vaccine for the protection of salmon against alphavirus, in European Union (EU) has increased the expectation of further practical application of DNA vaccines for the control of fish diseases. Although we could not elucidate the adjuvant effect of miR-155 on a VHSV G protein-based DNA vaccine in this study, diverse tries to find out the optimum construct and effective administration scheme of DNA vaccines would be greatly helpful to develop practical DNA vaccines against VHSV in olive flounder.

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