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Modulatory Effect of Inducing Iridovirus DNA Vaccine Combining Different CpG ODN Adjuvant in the Systemic Immune Mechanism Underlying the Protection Against Grouper Iridovirus of Asian Seabass (*Lates calcarifer*)

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ABSTRACT

Grouper iridovirus (GIV) is a major causative agent of disease in Asian seabass (*Lates calcarifer*), often leading to high mortality. However, information on vaccine development against GIV infection remains limited. This study investigated the potential of DNA vaccines combined with unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) to enhance the immune response of Asian seabass against GIV. A DNA vaccine was constructed by inserting the cloned GIV major capsid protein (MCP) gene into the pCDNA3.1 plasmid, which was subsequently introduced into *Escherichia coli* DH5α for propagation. Juvenile Asian seabass were intramuscularly injected with four treatments: PBS sterile (Control), DNA vaccine alone (V1), DNA vaccine combined with 20 nM CpG ODN (V2), and DNA vaccine combined with 2 µM CpG ODN (V3). Results showed that groups V1 and V3 exhibited strong protective effects against GIV infection. Both groups significantly enhanced the expression of several innate and adaptive immune-related genes compared to the control group. A separate challenge experiment further confirmed that vaccinated fish in the V1 and V3 groups had markedly higher survival rates than the control group. Notably, the V3 vaccine demonstrated superior efficacy, enhancing innate immunity, adaptive responses, and survival without any negative impact on growth performance. This study represents the first successful report of a DNA vaccine combined with CpG ODN adjuvant against GIV infection in Asian seabass. These findings highlight the potential of CpG-adjuvanted DNA vaccines as a promising strategy for improving disease resistance in aquaculture.

INTRODUCTION

The Asian seabass (*Lates calcarifer*) holds significant importance in aquaculture due to its favorable biological characteristics, including high fecundity, tolerance to high stocking densities, and adaptability across a wide range of salinity levels **(Vij *et al.*, 2014; Khang *et al.*, 2018)**. However, during the early growth phases, external factors such as aquatic environmental conditions pose challenges, often leading to reduced survival rates

and relatively slow growth **(Athauda & Anderson, 2014)**. In addition, several pathogens threaten Asian seabass at different life stages, including viral nervous necrosis (VNN), grouper iridovirus (GIV), scale drop disease virus (SDDV), infectious spleen and kidney necrosis disease, vibriosis, and streptococcosis **(Creeper & Buller, 2006)**. Among these, piscine iridoviruses—such as *Lymphocystivirus*, *Ranavirus*, and *Megalocytivirus*—are considered the most alarming causative agents of disease in bony fish, particularly in Asian seabass, due to the severe damage they inflict on the host **(Li *et al.*, 2014)**.

Grouper iridovirus (GIV), also known as sleepy grouper disease, is a viral infection that primarily targets the spleen and kidney of estuarine rock cod, grouper, and Asian seabass. Taxonomically, GIV is classified into two genera: *Ranavirus* and *Megalocytivirus* **(Ma *et al.*, 2016)**. In Taiwan and Singapore, *Ranavirus*-associated GIV outbreaks have raised increasing concern over the past decade **(Lai *et al.*, 2000; Qin *et al.*, 2003)**. Notably, a GIV outbreak in Singapore may have been introduced through diseased grouper larvae imported from Taiwan **(Qin *et al.*, 2003)**. GIV has also been detected in other marine species, such as Asian seabass and largemouth bass **(Chao *et al.*, 2002)**. Because Asian seabass serves as a host species for GIV, outbreaks can result in mass mortality. This underscores the urgent need to develop effective strategies to mitigate its spread.

In response to the growing prevalence of viral diseases in aquaculture, preventive strategies have been increasingly emphasized. Vaccination remains the most effective and widely adopted approach **(Zhang *et al.*, 2021)**. The evaluation of vaccine efficacy is commonly measured using the relative percentage survival (RPS), with several studies reporting encouraging protection rates under moderate to severe infection pressure **(Hazreen-Nita *et al.*, 2019)**. Fish are particularly susceptible to environmental changes due to their continuous contact with the aquatic environment, leaving their external surfaces constantly exposed to a wide range of microbes. Therefore, both innate and adaptive immune responses play a critical role in initiating protective defenses against pathogens.

Given this, the implementation of effective vaccine delivery strategies capable of eliciting strong immunological responses is essential for preventing and managing fish infections. However, knowledge regarding GIV vaccine development for Asian seabass remains limited. To the best of our knowledge, the present research represents the first effort to develop a vaccine specifically targeting GIV in Asian seabass.

Previous studies have identified the major capsid protein (MCP) as a prominent structural protein in iridoviruses, widely used for identification, differentiation, and classification of *Ranaviruses* **(Lin *et al.*, 2014)**. Importantly, the MCP of Taiwan grouper iridovirus (TGIV) has been shown to be a highly effective antigen, capable of eliciting targeted immune responses in grouper species **(Zhang *et al.*, 2021)**. In addition, CpG oligodeoxynucleotides (CpG-ODNs) have demonstrated immunostimulatory properties as vaccine adjuvants or immune protective agents in Asian seabass **(Kittichon *et al.*, 2016)**.

The purpose of this study was to develop a DNA vaccine derived from the MCP gene (MCP-pcDNA3.1), supplemented with CpG-ODN, to enhance the immune response of Asian seabass against GIV. Specifically, gene expression in the spleen and head kidney was analyzed, and post-challenge survival rates were evaluated. Collectively, this study provides new insights into the protective mechanisms of DNA vaccination and its role in enhancing immune responses in Asian seabass against GIV.

MATERIALS AND METHODS

1. Fish stock and husbandry practice

A total of 500 juvenile Asian seabass (*Lates calcarifer*, 3–4 g) were obtained from Kaohsiung, Taiwan. Prior to experimental grouping, the fish were acclimatized in composite tanks under controlled laboratory conditions. During acclimatization, fish were fed twice daily with a commercial diet at approximately 3% of body weight. No mortality occurred during this period. Daily water exchange was performed at 25% volume replacement, and water quality parameters were consistently maintained as follows: temperature 26– 28°C, salinity ratio 7:3 (seawater:freshwater), pH 7–8, and dissolved oxygen > 6mg/ L, following the conditions described by **Fotedar (2016)**. After acclimatization, fish were transferred to 1000-L indoor fiber tanks for experimentation.

2. DNA vaccine and CpG ODN

The open reading frame (ORF) of the GIV major capsid protein (MCP) gene was inserted into the eukaryotic expression vector pcDNA3.1 (Invitrogen) to construct a plasmid capable of expressing MCP. The recombinant plasmid was introduced into *Escherichia coli* DH5α and purified from bulk bacterial culture using the FavorPrep™ Endotoxin-Free Plasmid Extraction Midi Kit (Favorgen), according to the manufacturer's instructions. The purified plasmid was used as the DNA vaccine. A type A CpG ODN with a specific sequence design was synthesized and purified by high-performance liquid chromatography (HPLC) (Sigma) as previously described by **Chen *et al.* (2015)**.

3. Experimental design

A total of 200 juvenile Asian seabass (5.5–6 g) were randomly distributed into four indoor fiber tanks (50 fish per tank), each representing one experimental group: control (PBS sterile, C), DNA vaccine alone (V1), DNA vaccine with 20 nM CpG ODN (V2), and DNA vaccine with 2 μM CpG ODN (V3). The study comprised three components:

1. Gene expression analysis of multiple immune-related genes on days 1, 3, 7, and 14 post-immunization.
2. Viral challenge test to evaluate immune parameters and relative percent survival (RPS) on days 1, 3, and 7 post-challenge.
3. Growth performance evaluation conducted over 56 days, with biweekly sampling.

Throughout the experiment, fish were fed a commercial diet three times daily (09:00, 15:00, and 21:00) at approximately 3% of body weight. The overall experimental design is presented in Fig. (1).

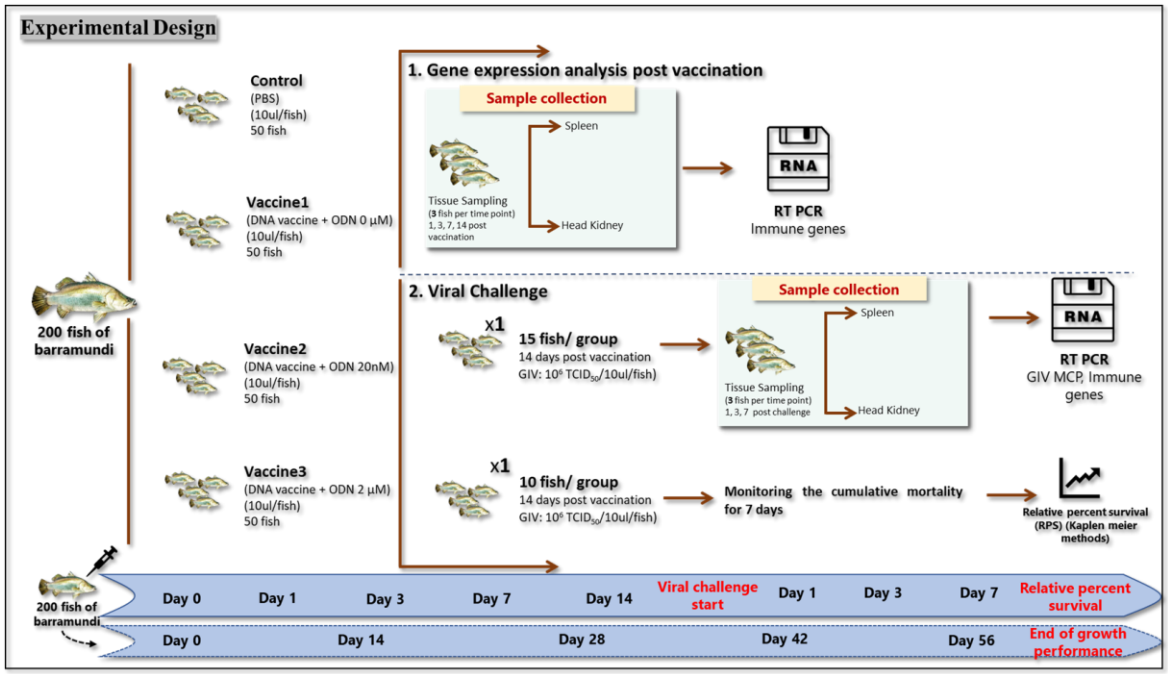

Fig. 1. Experimental design for the investigation of the DNA vaccine protection efficacy against grouper iridovirus on Asian seabass (*Lates calcarifer*)

2. Vaccination

Three experimental groups were administered intramuscular injections (10 µL/fish) of a DNA vaccine (1 µg per g of fish body weight) combined with CpG ODN at three doses: 0 (V1), 20 nM (V2), and 2 µM (V3). The control group (C) underwent a mock vaccination and received phosphate-buffered saline (PBS) to serve as a reference for untreated fish. Each treatment group consisted of 50 fish.

Sampling was conducted for 14 days post-vaccination (dpv) at four time points: day 1, day 3, day 7, and day 14. At each time point, three fish (biological triplicates) were randomly selected from each group, yielding a total of 15 fish per group for the gene expression study. Spleen and head kidney tissues were collected for the quantification of immune-related genes, specifically TLR9, TLR21, and IL-1β. All samples were flash-frozen in liquid nitrogen at –200 °C and subsequently stored at –80 °C until RNA extraction.

3. Viral challenge

Two viral challenge experiments were conducted to evaluate the protective efficacy of the DNA vaccine against GIV infection. Fourteen days after vaccination, 25 fish from each group were intraperitoneally injected with GIV at a dose of 10^6 $TCID_{50}$.

Experiment 1: Gene expression and viral load

A total of 15 fish (five per group) were sampled across three time points: day 1, day 3, and day 7 post-challenge. At each time point, three fish (triplicate biological replicates) were randomly selected for spleen and head kidney collection. Expression of immune-related genes (GATA-3, IgM, and IgT) and viral load quantification were performed. All samples were flash-frozen in liquid nitrogen (–200°C) and stored at –80 °C for subsequent RNA extraction.

Experiment 2: Relative percent survival (RPS)

The remaining 10 fish from each group were monitored daily for mortality following GIV exposure. Survival data were used to calculate the relative percent survival (RPS) according to the formula described by **Chen *et al.* (2015)**. Each treatment group included 10 biological replicates to ensure statistical robustness.

$$\text{RPS (\%)} = [1 - \frac{\text{\% loss immunized}}{\text{\% loss control}}] \times 100$$

3. Growth performance

The growth performance experiment was carried out throughout the experimental period, spanning from day 0 to day 56 after vaccination, with the aim of assessing the potential adverse effects of the immunization. A total of 10 fish in each group were used in this study. Weight measurements of the fish were recorded at biweekly intervals throughout the duration of the experiment in order to assess their growth performance. The growth performance characteristics were computed using the equations outlined in the study by **Cárdenas *et al.* (2015)**.

$$\text{WG} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

$$\text{SGR (\%/ day)} = \frac{\ln[\text{final weight (g)}] - \ln[\text{initial weight (g)}]}{\text{time (days)}} \times 100$$

$$\text{FCR} = \frac{\text{Total feed intake}}{\text{final weight (g)} - \text{initial weight (g)}} \times 100$$

4. Immune gene expression

Gene expression in the collected tissues was analyzed using reverse transcription–quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from spleen and head kidney tissues using TRIzol reagent (Invitrogen), following the manufacturer's protocol. To remove DNA contamination, RNA samples were treated with DNase I (Roche, Germany) prior to the second step of TRIzol purification.

First-strand cDNA synthesis was carried out using 3µg of total RNA and the HiScript I First Strand cDNA Synthesis Kit (Bio-Novas, Invitrogen), according to the manufacturer's instructions. Quantitative PCR was performed with the 2× qPCRBIO SyGreen Blue Mix HI-ROX reagent (PCR Biosystems) on a T Professional Thermal Cycler (Biometra). Gene-specific primers (Table 1) were used for amplification.

All reactions were performed in triplicate (technical replicates) for each of the three biological replicates per group. Gene expression levels were normalized against the housekeeping gene β-actin, and relative expression values were calculated using the control group (C) at each time point as the reference (set to 1.0)

Table 1. Primers used for immune-related gene expressions

	Gene	Forward (5'→3')	Reverse (5'→3')	Source
Housekeeping gene	β-actin	CTGGACTTCGAGCAGGAGAT	GTTGTAGGTGGTCTCGTCGA	Picchietti *et al.*, 2017
Pattern recognition receptor	Toll Like Receptor 9 (*TLR9*)	CACTGTTCTGCCCCTACTGA	AGTCTCTCACAGCCTGGTTC	PRJNA345597
	Toll Like Receptor 21 (*TLR21*)	CTGCGGTTGTGATCTTCCTG	ACGCATTTCCCTCCATGTTG	PRJNA345597
Inflammatory cytokines	Interleukin-1β (*IL-1β*)	CCTGTCGCATTTCAGTACGG	ATTTCCACCGGCTTGTTGTC	Zeynali *et al.*, 2020
T cell response	GATA-3	CCCTGGCCGAGAGTATGAAA	TCAGGCTACTTGGTGGGAAG	Zoccola *et al.*, 2017
Antibody genes	IgM	GTACAGCCTCTGGATTAGACATT	CTGTTGTCTGTGGAGATGGT	Scapigliati *et al.*, 2010
	IgT	TGTGTCAAAGTCTGCCTGGGATTCA	CTTAGGAGGTGGAGGAGGCTTTT	Scapigliati *et al.*, 2010
Viral load	GIV-McP	CTGCGGATTGGCTCTGCCCACCGT	AGCCCGCCGGCTATGTCGGTAGCA	Stephen *et al.*, 1997

5. Data analysis

All statistical analyses were performed using IBM SPSS Statistics, Version 24.0 (IBM Corp., Armonk, NY, USA). Gene expression data were analyzed using one-way analysis of variance (ANOVA) followed by Student's *t*-test for pairwise comparisons. Statistical significance was defined as $P< 0.05$, while $P< 0.01$ and $P< 0.001$ were considered highly significant () and extremely significant (*), respectively.

Relative percent survival (RPS) was evaluated using Kaplan–Meier survival analysis, and differences among groups were assessed with the Log-Rank test, followed by pairwise multiple comparisons. Growth performance parameters were analyzed using

one-way ANOVA with Tukey's honestly significant difference (HSD) test for post hoc comparisons. A significance level of $P< 0.05$ was applied.

RESULTS

1. Expression profiles of immune-related genes post vaccination

In the spleen, no statistically significant differences ($P> 0.05$) in TLR9 expression were observed among the experimental and control groups on day 1 post-vaccination (dpv) (Fig. 2A). However, by day 3, the V3 group exhibited a modest but significant up-regulation ($P< 0.01$) compared to the control group. On day 14, all vaccinated groups showed significant increases in TLR9 expression, with the strongest response in V3 ($P< 0.001$). Similarly, TLR21 expression showed a highly significant up-regulation ($P< 0.001$) in the V3 group on day 14 (Fig. 2C). Notably, V3 displayed a continuous increase across all time points compared to the control, with fold-change values of 0.03 for TLR9 and 0.02 for TLR21. For IL-1β, significant up-regulation was observed in V1 and V2 on day 14 ($P< 0.001$) relative to the control (Fig. 2E). Among all treatments, V3 demonstrated the most pronounced induction of the innate immune response.

In the head kidney, TLR9 expression increased significantly as early as day 1 in the vaccinated groups, with V1 and V3 showing a steady upward trend throughout the experimental period (Fig. 2B). The highest expression was observed in V3 on day 14. In contrast, V2 showed no significant differences compared to the control across all time points ($P > 0.05$). Similarly, TLR21 expression in V1 and V3 increased steadily from day 1 to day 14 post-vaccination (Fig. 2D). For IL-1β, the V1 and V3 groups exhibited very strong up-regulation ($P < 0.001$) on day 14, while V2 showed only a modest increase ($P< 0.05$) on day 3 (Fig. 2F).

Overall, groups V1 and V3 induced the most consistent and significant up-regulation of innate immune-related genes, highlighting their superior immunostimulatory effects compared with V2.

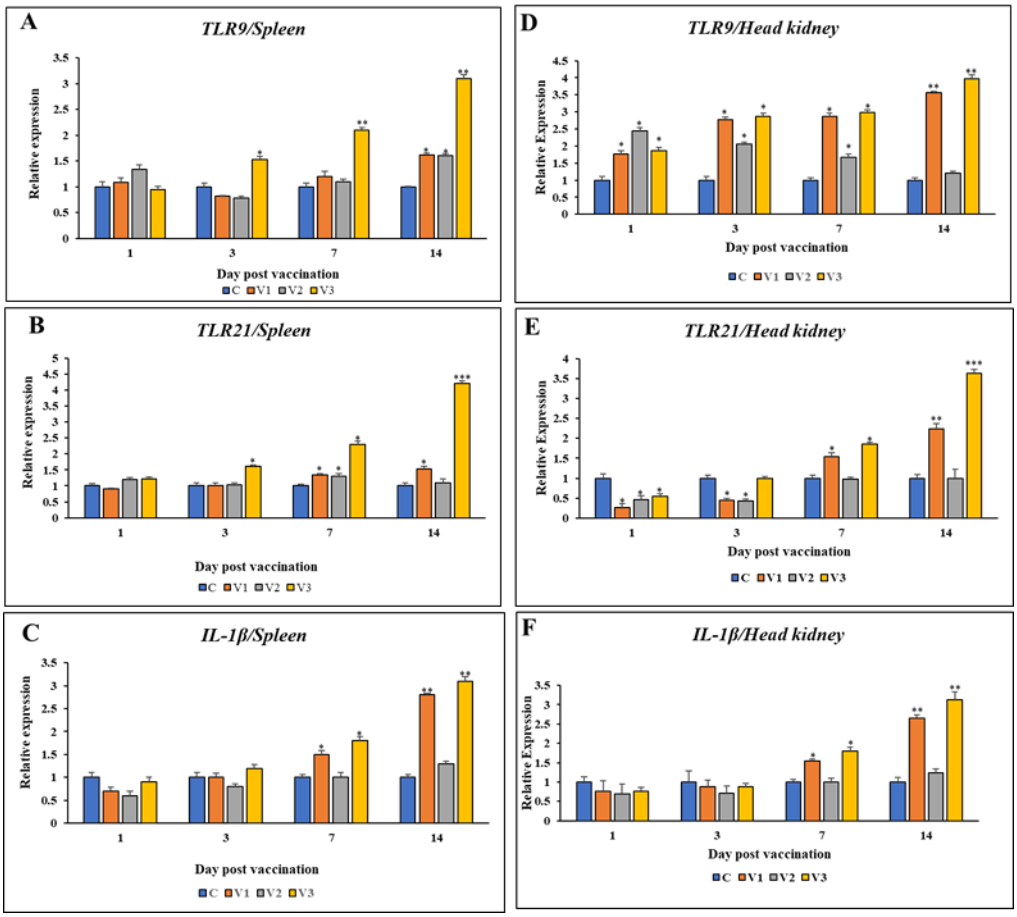

Fig. 2. The gene relative expression level of innate immune response in Asian seabass post-vaccination. The juvenile Asian seabass was administered a DNA vaccine at a dosage of 1µg per gram of body weight in conjunction with CpG ODN at several doses: 0 (V1), 0.1 µM (V2), and 10 (V3). The control group received an injection of a sterile phosphate-buffered saline (PBS) solution. The relative expression levels of TLR-9 (A, B), TLR-21 (C, D), and IL-1β (E, F) genes were assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in the spleen (A, B, C) and head kidney (D, E, F) tissues under various treatment conditions. Tissue samples were taken from three fish in each treatment group at each designated time point for further study. The experiment was conducted using triplicates of each sample, and the relative expression of each gene was standardized by normalizing it against the housekeeping gene β-actin. The relative expression levels of each gene in the control group (C) at each time point were assigned an arbitrary value of 1. Statistically significant differences are denoted by the symbols *, **, and *** when the p-value is less than 0.05, 0.01, and 0.001, respectively.

2. Clinical sign and expression profiles of immune-related genes following challenge with GIV

The infected fish exhibited clinical signs, including a loss of appetite, bilateral lens opacity, and swollen spleens (Fig. 3). In the 7 dpv, there were no fish in the control group. In the spleen after the challenge, the relative expression of GATA-3 showed up-regulated in all treatments in the vaccinated group on day 1 compared to the control (Fig. 4A). The peak of expression level was observed on 14 dpv in the V3 group. Moreover, the expression level of IgM demonstrated no significant differences ($P > 0.05$) in all treatments vaccinated group compared to the control group on 1 dpv (Fig. 4C), as well as the expression level of IgT (Fig. 4E). However, the vaccinated group V1 and V3 were observed starting to increase since 3 dpv and final at 7 dpv, with the fold change to 0.08 on IgT expression level.

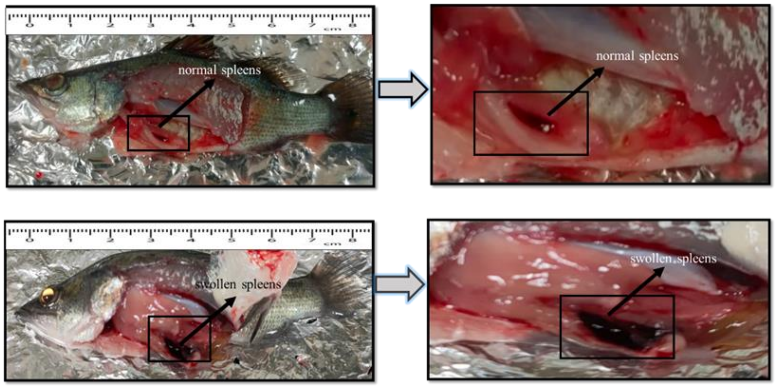

Fig. 3. The clinical signs of GIV infection. The spleen of healthy juvenile Asian seabass (Left). The juvenile Asian seabass infected with GIV 10^6 $TICD_{50}$ (10 µL/fish) shows a swollen spleen (Right)

In the head kidney, GATA-3 expression was significantly up-regulated in the V1 and V3 groups on day 3 post-challenge ($P < 0.01$) compared with the control group (Fig. 4B). By day 7, both V1 and V3 exhibited extremely significant up-regulation ($P < 0.001$). Similarly, IgM expression was markedly elevated in V1 and V3 on day 7 ($P < 0.001$) (Fig. 4D). In addition, IgT expression was significantly up-regulated in the V1 and V3 groups on both days 3 and 7 post-challenge compared with the control (Fig. 4F).

In contrast, the V2 group consistently showed expression levels comparable to those of the control group, with no statistically significant differences detected.

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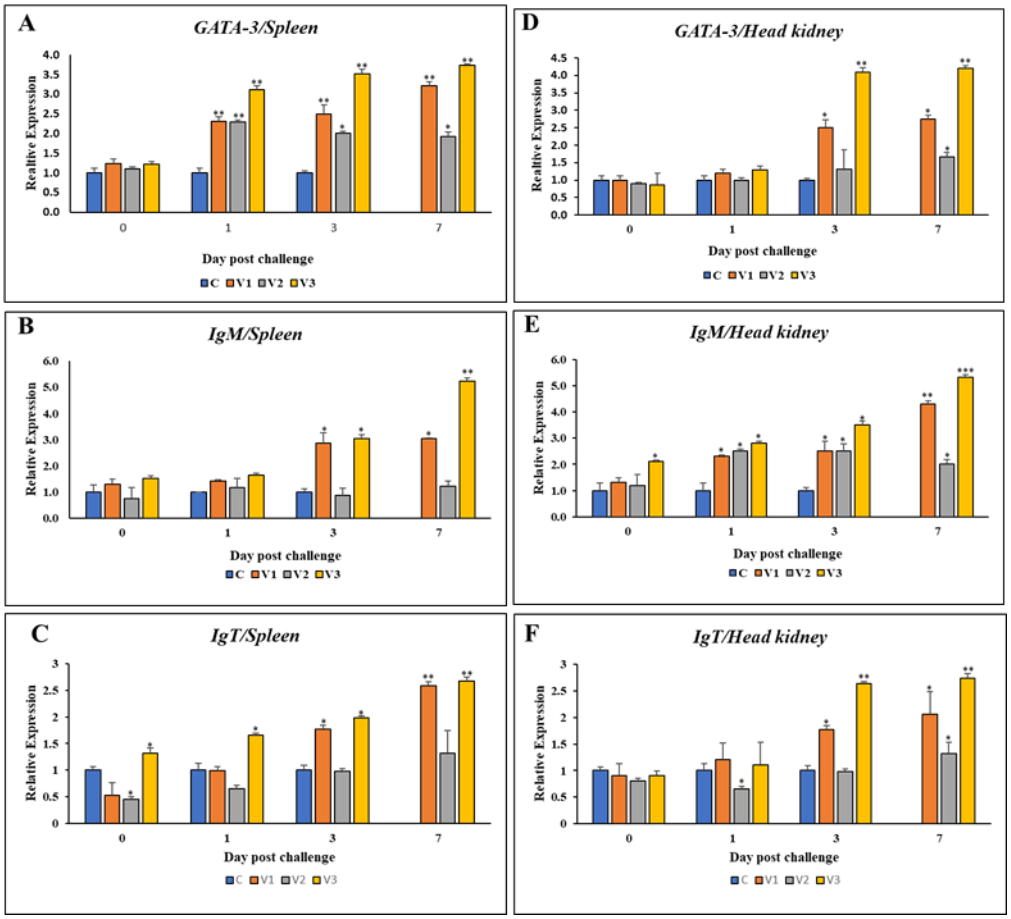

Fig. 4. The gene relative expression level of adaptive immune response in Asian seabass post-challenged. The juvenile Asian seabass was administered a DNA vaccine at a dosage of 1 µg per gram of body weight, along with CpG ODN at several doses: 0 (V1), 0.1 µM (V2), and 10 (V3). The control group received an injection of a sterile phosphate-buffered saline (PBS). The relative expression levels of Gata-3 (A, B), IgM (C, D), and IgT (E, F) genes were assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in the spleen (A, B, C) and head kidney (D, E, F) tissues under various treatment conditions. Tissue samples were taken for examination from three fish in each treatment group at each designated time point. The experiment was conducted using triplicates of each sample, and the relative expression of each gene was standardized by normalizing it against the housekeeping gene β-actin. The relative expression levels of each gene in the control group (C) at each time point were assigned an arbitrary value of 1. Statistically significant differences are denoted by several levels of significance, namely $P < 0.05$ (significant), $P < 0.01$ (very significant), and $P < 0.001$ (extremely significant), represented by *, **, and ***, respectively.

3. Viral load of GIV in the Asian seabass tissues

In the spleen, the viral load of the GIV virus was lower detected in the vaccinated group compared to the control group (Fig. 5A). However, in the V2 group, it was observed to increase extremely significantly ($P< 0.001$) compared to other groups on 7 dpv. Moreover, the viral load of GIV in the head kidney showed an extremely significant increase ($P < 0.001$) only in the control group (Fig. 5B). Interestingly, in the head kidney, there was no viral load expression in the V2 group similar with other groups, indicating that the viral load is in the spleen organ.

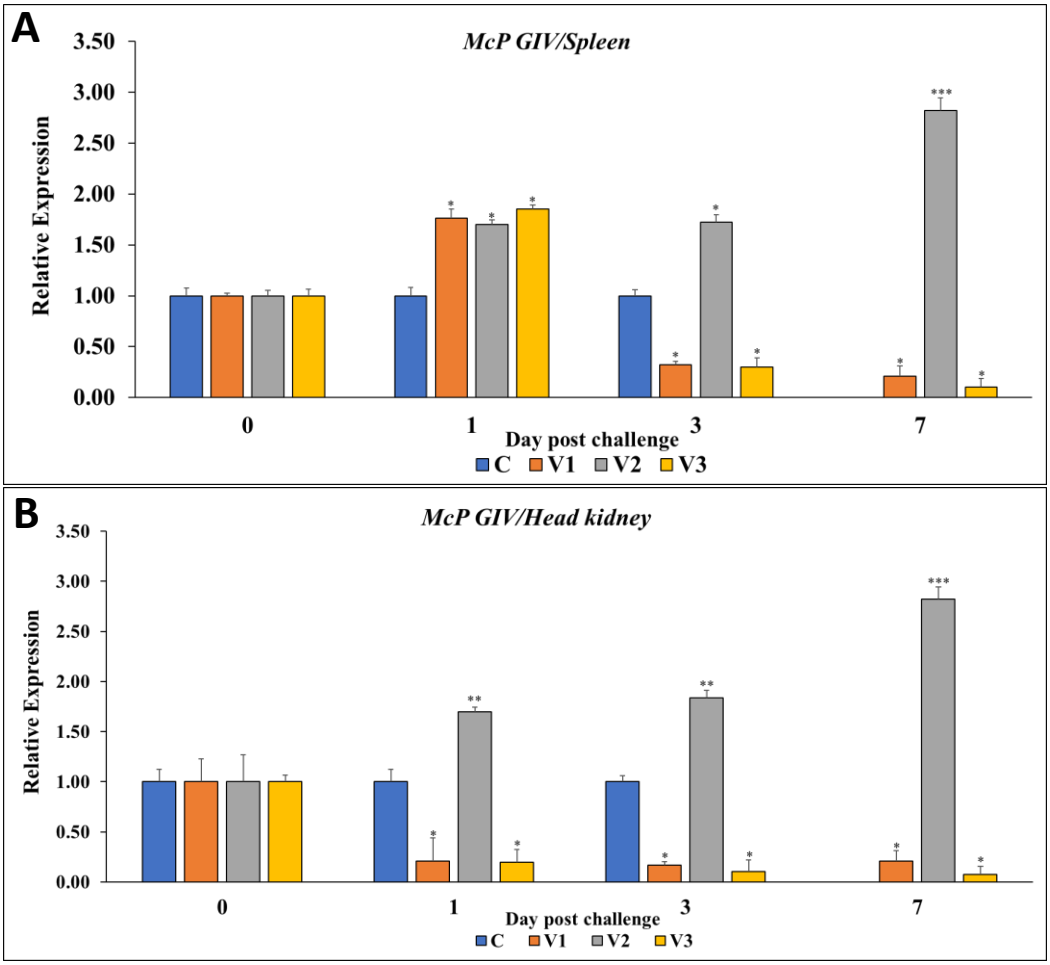

Fig. 5. Effect of different treatments on viral load of GIV in the spleens of Asian seabass after being challenged with GIV. The viral load present in the spleen (A) and head kidney (B) is being examined. The experiment was conducted using triplicates of each sample, and the relative expression of each gene was standardized by normalizing it against the housekeeping gene β-actin. The relative expression of each gene in the control group (C) at each time point was assigned a value of 1 as a reference point. Statistically significant differences are denoted by the symbols *, **, and *** for *P*-values less than 0.05 (significant), 0.01 (very significant), and 0.001 (extremely significant), respectively.

4. Protection efficacy of vaccine

The Kaplan Meier survival curve of the fish-vaccinated group was significantly different from those of the unvaccinated group (Fig. 6). Less mortality was observed in the vaccinated group over 7 days. By contrast, cumulative mortality was observed 100% after injection with GIV in the PBS-treated group as a control group. Asian seabass juveniles that received the vaccine had significantly higher survival rates after 7 days. At the end of the challenge experiment, the survival rate of fish was 70, 0, and 100% for the challenged V1, V2, and V3 groups, respectively.

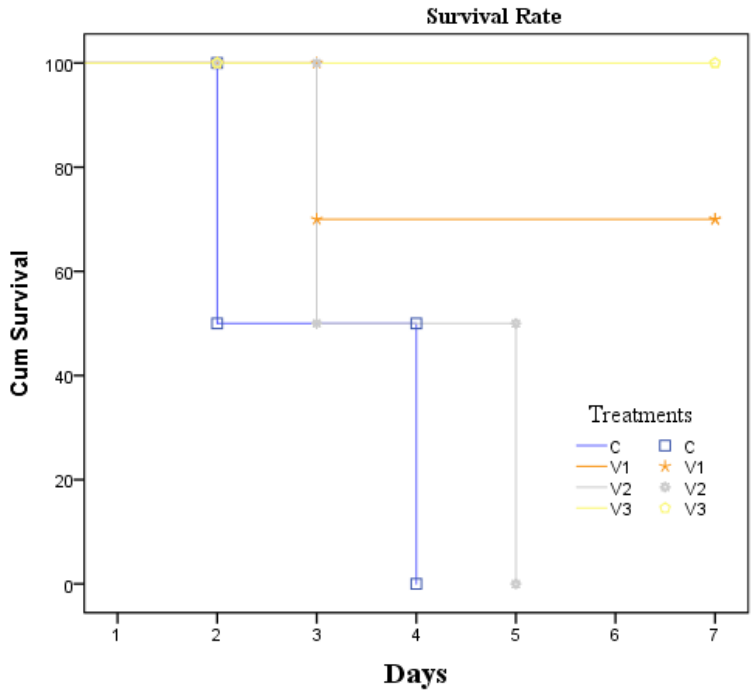

Fig. 6. Kaplan–Meier analysis of Asian seabass (n = 10) challenged with GIV after 14 days of vaccination. The juvenile Asian seabass were vaccinated with DNA vaccine (1 µg per g of body) in combination with CpG ODN at the dose of 0 (V1), 0.1 µM (V2), and 10 (V3). C: The control group was injected with a PBS sterile

5. Growth performance

When considering the results together, it is evident that the V1 and V3 groups had the most robust innate immune response. This suggests that the administration of both vaccinations may provide superior protection against GIV infection. Furthermore, no notable disparities were identified among the four experimental groups, including the control group, in terms of final weight, weight increase, specific growth rate, and feed conversion ratio (Table 2).

Table 2. Growth performance parameters and survival rate of Asian seabass fed with vaccinated feed for 8 weeks

Diet	Initial weight (g)	Final weight (g)	Weight Gain (g)	Specific Growth Rate (%)	Feed Conversion Ratio
Control	5.14 ± 0.85	18.08 ± 0.73	12.94 ± 0.64	9.07 ± 1.42	2.99 ± 0.64
Vaccine 1	5.07 ± 0.23	18.42 ± 0.39	13.35 ± 0.54	9.22 ± 0.37	2.92 ± 0.27
Vaccine 2	5.21 ± 0.37	18.34 ± 0.50	13.13 ± 0.71	9.01 ± 0.55	3.02 ± 0.18
Vaccine 3	5.16 ± 0.40	18.45 ± 0.31	13.29 ± 0.42	9.12 ± 0.47	2.91 ± 0.17

Values are shown as the mean ± SD (n=10). No significant differences $P > 0.05$. It means no side effect using DNA Vaccine combining CpG ODN adjuvant to growth performance parameters.

DISCUSSION

The efficacy of vaccine-induced immune responses depends on the interplay between the innate and adaptive immune systems, which together provide protection against pathogen invasion **(Yao *et al.*, 2019)**. Previous studies on fish vaccination have largely focused on early immune responses following vaccine delivery by injection or immersion **(Hoare *et al.*, 2017)**. To gain further insight into the mechanisms of protection, the present study examined the expression of immune-related genes (IL-1β, TLR9, TLR21, GATA-3, IgM, and IgT) in the spleen and head kidney at different time points following vaccination and challenge with GIV.

The innate immune system in fish plays a critical role in the early defense against pathogens, largely through the recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) **(Li *et al.*, 2017)**. Among these PRRs, Toll-like receptors (TLRs) are key in initiating signaling cascades that lead to cytokine and chemokine production **(Chen *et al.*, 2023)**. TLR9, for instance, recognizes unmethylated CpG motifs in bacterial and viral DNA **(Lee *et al.*, 2015)**. In this study, TLR9 expression was significantly up-regulated in the spleen of V3 on day 14, while V1 and V3 showed consistent induction in the head kidney. These findings suggest that plasmid DNA in V1 and V3 can be recognized as antigenic material by TLR9.

Similarly, TLR21, which shares functional similarity with TLR9 and recognizes CpG motifs **(Gao *et al.*, 2021)**, was significantly up-regulated in V3 spleen samples, and moderately induced in both V1 and V3 head kidneys. In contrast, V2 showed no significant changes compared to the control, indicating poor immunostimulatory activity.

Together, these results suggest that co-administration with CpG ODN at 2 μM (V3) enhanced vaccine efficacy by activating both TLR9 and TLR21 pathways.

IL-1β, a pro-inflammatory cytokine central to balancing humoral and cellular immune responses **(Xu *et al.*, 2017)**, was strongly up-regulated in both spleen and head kidney of V1 and V3, with V3 showing the highest induction. Importantly, IL-1β expression correlated with TLR9 and TLR21 activity, underscoring the functional link between CpG-induced signaling and cytokine activation. Previous studies have also demonstrated that CpG ODNs enhance TLR9-mediated immunity in golden pompano **(Chen *et al.*, 2023)**, consistent with our findings in Asian seabass.

Following challenge with GIV, immune modulation extended into adaptive responses. GATA-3, a transcription factor driving Th2 cell differentiation **(Takizawa *et al.*, 2011)**, was significantly up-regulated in V1 and V3 groups post-challenge, indicating activation of Th2-associated pathways. This likely promoted cytokines such as IL-4, IL-5, and IL-13, which mediate humoral responses. Correspondingly, IgM and IgT were elevated in both spleen and head kidney tissues of V1 and V3 on day 7, with V3 showing the strongest expression. The enhanced Ig responses suggest efficient activation of both systemic and mucosal immunity, a crucial feature for durable protection. These results are consistent with reports in the tiger pufferfish, where CpG-adjuvanted vaccines elicited significantly higher IgM responses than conventional inactivated vaccines **(Yu *et al.*, 2023)**.

Interestingly, V2 showed partial induction of IgM and IgT in the head kidney but not in the spleen, where higher viral loads were detected. This suggests that a suboptimal CpG ODN concentration (20 nM) may have impaired antigen recognition or downstream signaling, resulting in weaker systemic immunity. It is possible that at low doses, CpG supplementation disrupts antigen processing or TLR binding efficiency, thereby reducing vaccine efficacy. Further research is warranted to clarify these mechanisms.

The challenge experiments confirmed the protective efficacy of vaccination. The control group (PBS) experienced 100% cumulative mortality by day 4 post-challenge, whereas vaccinated groups showed markedly lower mortality: V1 (30%), V2 (0%), and V3 (100%). Mortality in vaccinated fish began later and progressed more slowly, with V1 and V3 maintaining relatively low viral loads in the spleen and head kidney. These findings indicate that both V1 and V3 were effective in stimulating innate and adaptive immune responses, providing significant protection against GIV.

Overall, the results demonstrate that a DNA vaccine encoding MCP, particularly when combined with CpG ODN at 2 μM, strongly induces innate (TLR9, TLR21, IL-1β) and adaptive (GATA-3, IgM, IgT) immune responses in Asian seabass. V1 (DNA vaccine alone) also conferred protection, though less robust than V3. The poor performance of V2 highlights the importance of optimizing CpG dosage for effective immune stimulation. Together, these findings underscore the potential of CpG-adjuvanted DNA vaccines as a promising strategy for controlling GIV in aquaculture.

CONCLUSION

In the host fish species, an ideal vaccine should possess the capability to stimulate innate mechanisms, elicit an adequate antibody response, stimulate T-cell responses, and establish specific immune memory. In short, the results of this study demonstrated that the administration of a DNA vaccine combining CpG ODN could improve the innate immune responses, the adaptive immune response, and the survival rate against GIV infection. The treatment vaccine 3 in the Asian seabass provided the optimum combination of DNA Vaccine and CpG ODN to increase immune responses, expression of immune-related genes, and survival rate after being challenged with GIV. Moreover, there were no side effects from the administration of these vaccines.

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