Investigation of bacterial antigenic fragments OMPs as a potential vaccine candidate against vibriosis in TGGG

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ABSTRACT

Aquaculture holds a crucial position in the economies of developing nations, making significant contribution to both food security and income. Despite its importance, aquaculture encounters challenges including bacterial infections threats like vibriosis that caused by *Vibrio* spp. Concerns regarding antibiotic resistance have prompted a shift towards usage of vaccine as a sustainable alternative treatment method. This study focused on the development of a fusion fragment protein GADPH-OmpK as a potential vaccine candidate against vibriosis in hybrid TGGG Grouper (*Epinephelus fuscoguttatus x E. lanceolatus*) and the impact it has on the expression of immune genes. The fusion fragment protein was successfully overexpressed and used to intraperitoneally immunize hybrid TGGG grouper. The immunogenetic expression of Interleukin-2, Interleukin-6 and Interferon-ϒ in the results indicated that the vaccine elicits a considerable immune response in the fish, demonstrating its potential in enhancing aquaculture sustainability. Overall, this research presents a promising avenue for advancing aquaculture practices and mitigating antibiotic resistance issues in fish farming since recombinant protein vaccine offer various advantages.

Keywords: aquaculture, TGGG hybrid grouper, vaccine, immune response

INTRODUCTION

Aquaculture is an essential source of aquatic food and revenue in developing countries, as this sector offers financial benefits and also valuable species like farmed shrimp, marine fish, and freshwater fish for the export market (Ina-Salwany et al., 2018). Cole et al. (2009) stated that the marine culture industry has produced about 40% of the world's seafood, resulting this sector supplanting conventional marine capture commodities (Kurniawan et al., 2021). Nevertheless, high-density species environments employed in the aquaculture industry accelerate parasite

and disease transmission, necessitating monitoring quality, safety, and microbiological indicators for a sustainable future. Some chemicals including antibiotics, insecticides, herbicides and hormones have been used to treat and control disease outbreaks however, studies found that they can cause environmental contamination and antibiotic resistance, potentially leading to more unintended species and diseases emergence (Okeke et al., 2022).

One of the common bacterial infections is vibriosis caused by *Vibrio* spp, a prevalent pathogenic bacterium such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* has been known to negatively affect various shellfish and marine fish, causing hindrance for sustainable growth for marine vertebrates (Tall et al., 2013; Baker-Austin et al., 2018). Vibriosis has been reported to infect groupers (*Epinephelus* spp.) at all growth stages with about 66.7% of cases and be the cause of death rates of up to 50% (Liao and Leao., 2008; Chong et al., 2011; El-Galil and Mohamed, 2012). Therefore, this study delves into the development and evaluation of a fusion fragment protein as a potential vaccine candidate targeting vibriosis in hybrid groupers.

The immune system in fish is composed of adaptive and innate components that respond to exogenous or endogenous stimuli as well as defend against foreign substances like malignant cells, microorganisms, or toxins. The natural killer cells (granulocytes, monocytes, macrophages), the complement system, the antimicrobial enzymes, the interleukins, and the interferon are all examples of organic defense mechanisms (Biller-Takahashi & Urbinati, 2014). Previous research done by Monir et al. (2022) demonstrated that monovalent vaccine has a limited immunogenicity profile while bivalent vaccine shows a promising spectrum in achieving strong immunocompetent effects. Hence, this study looked at the efficacy of fusion fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and outer membrane protein K (OmpK) as a potential recombinant vaccine candidate and their immunogenicity was evaluated by analyzing the quantitative expression of gene encoding Interleukin-2 (IL-2), Interleukin-6 (IL- 6) and Interferon-gamma (IFN-ϒ).

The objective of this study was 1) to overexpress bacterial antigenic fusion fragment of OMP as a vaccine candidate, 2) to immunize the hybrid TGGG grouper (*Epinephelus fuscoguttatus x E. lanceolatus*) with the vaccine candidate and 3) to evaluate the immune response of unvaccinated and vaccinated fish via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

MATERIALS AND METHODS

Vaccine Candidate Development

Overexpression of a Fusion Fragment Protein

In this study, the plasmid system harboring the recombinant protein of GAPDH-OmpK employed was attained from previous research done by Budiman et al. (2022). An overexpression was conducted following the protocol established by Zhang et al. (2007). Briefly, 3mL of pre-cultured *Escherichia coli* Rosetta carrying GAPDH-OmpK was inoculated into 100mL Terrific Broth (TB) containing 100 µL of kanamycin antibiotic at a ratio of 1:100 (v/v). The culture flask was vigorously shaken and incubated at 37°C until an optical density (OD600) of 0.6 was reached, later induced with 100 µL of isopropyl – D –

thiohalactopyranocide (IPTG). The culture further incubated overnight (12 – 16 hours) at 18°C. The cell was harvested by centrifugation for 10 min at 8000 rpm at 4°C and the supernatant was discarded. The pellet was washed and resuspended with 10 mM Tris-HCl buffer (pH 7.6) containing 1mM EDTA and proceeded with sonication. The soluble fraction was then separated from the cell debris by centrifugation at 35,000x g for 30 min at 4ºC. The expressed protein was analyzed on 15% SDS–polyacrylamide gel electrophoresis.

Vaccination of Hybrid TGGG Grouper

TGGG Acclimatization and Vaccination

This research involving animal experimentation of hybrid TGGG grouper was approved by the University of Malaysia Sabah Animal Ethics Committee with reference number AEC 0018/2023.

Groups of 5-inch length of hybrid grouper weighing 40 ± 6 g were bought and acclimatized for 1 week at the UMS Fish Hatchery in a controlled environment (28 ppt salinity, 6.0 mg/L dissolved oxygen, 30ºC and pH of 7.9). The fish resided in 100 L fiberglass-reinforced plastic (FRP) tanks filled with aerated recirculating saltwater. TGGG fish daily diet was fed twice with commercial dry pellets containing crude protein (50%), crude fat (>8.4%), crude fiber (<2.9%), ash content (<16%) and moisture (<10%). Daily assessments of fish health and regular monitoring of water quality in each tank were conducted.

2 groups of 5-inch hybrid TGGG grouper (n=42) were selected for the vaccination procedure. The first group (n=21) (vaccinated) was immunized with pure recombinant protein vaccine, GADPH-OmpK at a dosage of 50 µL/100 g while another group (n=21) (unvaccinated) served as a control injected with 50 mM Tris HCl (pH 7.5) at a dosage of 50µL/100g through intraperitoneal injection (IP) using 23G needle. This procedure was performed at the UMS Fish Hatchery and guided by the trained research assistant from Borneo Marine Research Institute (BMRI). Fish sampling was done at day 7 and day 14 post-vaccination using 250 mg/L l−1 of MS-222 (Tricaine®- S, Western Chemical) for euthanizing.

Immune-related Gene Expression Analysis by qRT-PCR

Extraction of RNA

The spleen of the fish was harvested following the protocol used by Zhang et al. (2007) with slight modifications. A fish from each tank was lethally anesthetized with 250 mg/L l−1 of MS-222 (Tricaine®-S, Western Chemical) for 20 minutes. Using a scalpel, fish spleen was excised under RNase-free conditions. The sample was then homogenized using mortar and pestle on ice. Approximately 3 volumes of RNAlater ™ solution (Invitrogen by Thermo Fisher Scientific) was subsequently added to 1 volume of the homogenized sample for storage at -80ºC. This method was done for day 0, day 7 and day 14 post-vaccination.

The RNA extraction method was done according to the RNeasy® Plus Mini Kit protocols with some modifications. Sample pooling was performed from unvaccinated and

vaccinated groups of treatments, accordingly. The RNA integrity and concentration were determined using the Nanodrop spectrophotometer. The total RNA synthesized samples were preserved at −80°C until further use.

qRT-PCR Analysis of Immune-related Genes

A quantitative real time–polymerase chain reaction (qRT-PCR) procedure was performed using the ViPRimePLUS One Step AtTaq RT-qPCR Green Master Mix I (SYBR® Green Dye) kit. A standard total of 20 µL reaction mix was prepared for all samples (10 µL AtTaq one Step RT-qPCR Green Master Mix I, 1 µL forward primer, 1 µL reverse primer followed by 5 µL of RNA sample and 3 µL of distilled water. Amplification was performed using the C100™ Thermal Cycler (Bio-Rad) with the following program conditions: reverse transcription cycle at 42°C for 10 minutes followed by 95°C for 8 minutes (enzyme activation), 95°C for 10 seconds (denaturation) and 60°C for 60 seconds (data collection). All data were normalized to the reference gene, β-actin (Yang et al., 2022). A non-template control sample (NTC) was included in the qPCR test to confirm the master mix has no contamination.

Oligonucleotide Primers

The oligonucleotide primers used in this study are Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interferon-gamma (IFN-□) and β-actin as a housekeeping gene. The genes are listed in Table 1 below.

Table 1: Primers used in qPCR

No.	Genes	Sequence (5'-3')	Reference
1	β-actin	F: TGCGTGACATCAAGGAGAAGC R: TCTGGGCAACGGAACCTCT	Yang et al., 2022
2	Interleukin-2 (IL-2)	F: GCCGACCTGGTTGTAATCCTCA R: ATCTCAAAGCCTGTCTCATTGGT	He et al., 2021
3	Interleukin-6 (IL-6)	F: AGGAAGGTCTGGCTGTCAGGA R: GCCCTGAGGCCTTCAAGATT	He et al., 2021
4	Interferon-γ (IFN-γ)	F: CCACCAACATGGAGGCTAAC R: CTGCCACCTCACCATTGCT	He et al., 2021

RESULTS

Vaccine Candidate Development

Overexpression of a Fusion Fragment Protein

The expression of Escherichia coli Rosetta containing the fusion fragment protein GADPH-OmpK was assessed on 15% SDS-PAGE. The results of after and before induction by IPTG were visualized in Lanes 1 and 2, respectively, as depicted in Figure 1. The solubility of the protein was also observed by examining the appearance of bands for supernatant in Lane 3 and pellet in Lane 4 of the protein after ultracentrifugation. The observed band size was ~23kDa which corresponds to the study done by Budiman et al. (2022).

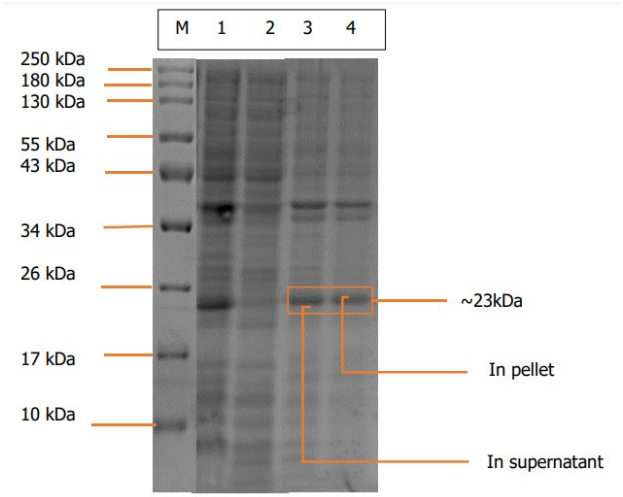

Figure 1: SDS-PAGE of the recombinant protein GADPH-OmpK expression in Escherichia coli Rosetta. The M lane represent marker, band in Lanes 1 and 2 represent the pre and post IPTG induction. Lanes 3 and 4 represent the soluble and insoluble fusion fragment protein obtained after sonication.

Immune-related Gene Response Analysis

As seen in Figure 2, the expressions of IL-2 and IL-6 were observed to be downregulated both at day 7 and day 14 post-vaccination. In contrast to IFN-ϒ where the gene was seen to be upregulated at day 7 and day 14 post-vaccination. The fold changes were calculated according to the Livak method (Livak & Schmittgen, 2001).

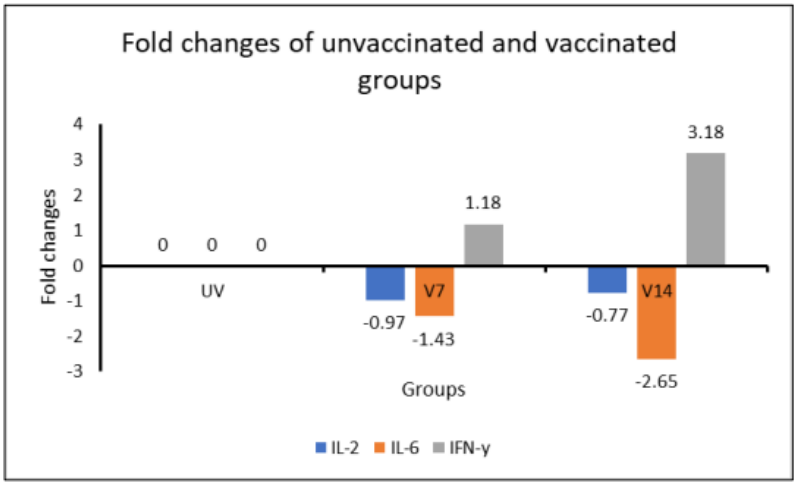

Figure 2: Fold changes of different genes with unvaccinated and vaccinated treatments. The data above showed downregulation for gene expressions of IL-2 and IL-6 at day 7 and day 14, but displayed upregulation for gene expression of IFN-ϒ at day 7 and day 14, respectively.

DISCUSSION

This study reported the possibility of GAPDH-OmpK as a recombinant protein vaccine candidate that can induce immunoprotection in hybrid TGGG grouper (Epinephelus fuscoguttatus x E. lanceolatus). This research focused mainly on 1) overexpression of bacterial antigenic fusion fragment of GAPDH and OmpK as a vaccine candidate, 2) immunization of the hybrid TGGG grouper with the vaccine candidate and 3) the immune response of unvaccinated and vaccinated fish to indicate regulation of fish immunity.

As seen in Figure 1 the overexpression of fusion fragment protein vaccine candidate was successfully performed under the induction of 1mM IPTG at 37°C. The protein size was approximated at ~23kDa however, according to Budiman et al. (2022) the size is slightly larger than the theoretical fusion size which is 20.29 kDa due to the presence of His-tag at the N–terminal fusion. This, nevertheless, confirmed that the band is a product of the expression of the GADPH–OmpK gene. The vaccination procedure of TGGG grouper was performed following animal ethics and guided by a trained person. Fish vaccination was done through intraperitoneal injection (IP) via the abdominal cavity since this method is believed to be the most successful (Vinitantharat et al., 1999).

In this research, the unvaccinated group served as control hence, the fold changes (ΔΔCq) were zero for all IL-2, IL-6 and IFN-ϒ genes as shown in Figure 2. Figure 2 displayed fold changes for both unvaccinated and vaccinated groups for day 7 and day 14. In quantitative gene expression analysis, fold changes of > 2 are accepted as significant expressions. Based on the results obtained, the IFN-ϒ gene showed a significant upregulation by 8 fold in the vaccinated day 14 group as compared to the unvaccinated group. In contrast, the IL-6 gene was significantly downregulated by -2.65 in the vaccinated day 14 group as compared to the unvaccinated group. Downregulation expression of the IL-2 and IL-6 genes in the vaccinated day 7 group occurred but were not significantly different with -0.97 and -1.43 fold respectively as compared to the unvaccinated group. The IL-2 gene exhibited insignificant downregulation expression in the vaccinated day 14 group as compared to the unvaccinated group.

According to the study done by Chen et al. (2017), cytokines modulate the immune response to infection or inflammation and regulate inflammation itself via a complex network of interactions. This could be because the immune system modifies both IL-2 and IL-6 expression after the initial activation to prevent excessive inflammation or immune dysregulation. In other words, downregulation after vaccination could indicates a regulatory mechanism to prevent excessive inflammation. Vaccination may induce an early immune response, leading to a slight upregulation at day 7 and a more pronounced and significant upregulation at day 14. It concurred with Makesh et al. (2023) who observed that substantially higher antibody levels were generated by the recombinant vaccine at 50 and 100 μg/fish, and an immunological response was shown as soon as one week after immunization. This implies that the vaccination is successful in promoting IFN-ϒ expression.

In summary, vaccination with the recombinant protein has successfully evokes varied fold changes in hybrid grouper immunegenetic response, thus indicating the immunoprotective potential of GAPDH-OmpK as a fish vaccine.

CONCLUSION

In conclusion, this study lays the foundation for the development of a potential bivalent vaccine against vibriosis in hybrid grouper fish as the GAPDH-OmpK vaccine candidate showed varied expression patterns of the fish immune-related genes, demonstrating a dynamic response to immunization. Further investigations and improvements in the experimental design would contribute more to the advancement of fish farming practices, addressing challenges related to bacterial infections as well as promoting sustainable aquaculture developments.

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