
**UNIVERSITI PUTRA MALAYSIA**

# *EVALUATION OF TWO DEVELOPED RECOMBINANT CELL VACCINES EXPRESSING OMPK AND DNAJ AGAINST VIBRIOSIS IN ASIAN SEABASS, Lates Calcarifer (Bloch, 1790)*

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# EVALUATION OF TWO DEVELOPED RECOMBINANT CELL VACCINES EXPRESSING OMPK AND DNAJ AGAINST VIBRIOSIS IN ASIAN SEABASS, *Lates Calcarifer* (Bloch, 1790)

**By**

**SANTHA A/P SILVARAJ**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

**February 2021**

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**February 2021**

**Chair : Assoc. Prof. Ina Salwany Md Yasin, PhD**
**Institute : Bioscience**

Aquaculture is among the fast-growing food-producing sectors in the world. However, its growth is suppressed by various disease problems, including bacterial infection. An outer membrane protein (OMPs) of Gram-negative pathogenic bacteria holds an important role in the interaction between bacterium–host and a potential candidate for vaccine development against fish diseases. Thus, in this study, recombinant cell vaccines expressing OmpK and DnaJ of *Vibrio* spp. were developed to protect fish against vibriosis.

The OmpK genes of *V. alginolyticus* strain VA2 and DnaJ genes *V. harveyi* strain VH1 were inserted into pET-32 Ek/LIC vector and cloned into the *E. coli* BL21 (DE3) expression host. The recombinant fusion proteins of *r-OmpK* and *r-DnaJ* were further analysed by SDS PAGE, western blotting, restriction enzyme analysis, and sequencing. The tertiary structure of *r-OmpK* and *r-DnaJ* and the stability of the recombinant protein were studied using bioinformatic tools. The immunogenicity of the developed recombinant vaccine antigen was evaluated via several *in vivo* experiments.

In the first *in vivo* experiment, this study focuses on a vaccination trial on juvenile Asian seabass ($\pm$5 cm; $\pm$20 g) for a period of 15 days by using the developed recombinant cell vaccines. In this experiment, Group 1 was exposed intraperitoneally (IP) to the recombinant cell vaccine containing *r-OmpK*, Group 2 to recombinant *r-DnaJ*, Group 3 to formalin-killed *V. harveyi,* Group 4 to BL21 cells, and Group 5 to PBS. Samples were collected accordingly for all the downstream analyses, such as for analysis of targeted gene of OmpK and DnaJ in selected tissue samples, serum bactericidal analysis, agglutination test, and qPCR analysis which are converging on evaluation for prime

vaccination study. Even though IP injection did not induce an increase in antibody titer levels in the skin mucus, there was an apparent significant increase ($p<0.05$) of antibody production in the serum and gut lavage of the *r-OmpK* vaccine. Further, the results showed a significant ($p<0.05$) up-regulation for gene TLR2, MyD88, and MHCI in kidney and intestine tissue of *r-OmpK* vaccinated fish compared to the other four groups examined. Also, the vaccination triggered a higher significant expression ($p<0.05$) level at the post-prime-immunisation level of interleukin IL-10, IL-8, IL-1β in the spleen, intestine, and kidney *r-OmpK* compared to *r-DnaJ*. The expression of gene encoding pro-inflammatory cytokine IL-1β also increased in the spleen, intestine, and kidney tissues of vaccinated fish than in the control group at post challenge of *V. harveyi, V. alginolyticus, and V. parahaemolyticus*. The same expression patterns were observed for IL-8, IL-1β, IL-10, and CCL4 at 24 h, 48 h, and 72 h post-challenge. The fish vaccinated with *r-OmpK* protein was completely protected with a relative per cent of survival (RPS) of 90% against pathogenic *V. harveyi* infection and 100% against *V. alginolyticus* and *V. parahaemolyticus* infection. This study found that juvenile Asian seabass was well protected when challenged with *multiple Vibrio strains* after vaccination with *r-OmpK* compared to *r-DnaJ* protein.

In the second experiment, the immunoprotection ability of the recombinant vaccine *r-OmpK* was determined at two different dosages ($10^7$ and $10^9$ cfu/mL) against *V. harveyi* infection in juvenile Asian seabass ($\pm 5$ cm; $\pm 20$ g). In this experiment, Group 1 was exposed intraperitoneally (IP) to the recombinant cell vaccine containing *r-OmpK* of $10^7$ cfu/mL, Group 2 to recombinant *r-OmpK* of $10^9$ cfu/mL, Group 3 to PBS, and Group 4 undisturbed juvenile Asian seabass. Cumulative mortality of 10% was observed in the group vaccinated with *r-OmpK* of dose $10^9$ cfu/mL, which significantly ($p<0.05$) differs from *r-OmpK* of dose $10^7$ at 120 h post-infection. At post-vaccination, the group vaccinated with $10^9$ cfu/mL of *r-OmpK* showed a significant ($p<0.05$) increase of antibody in serum, body mucus, and gut lavage fluid compared to group $10^7$ cfu/mL of *r-OmpK*. At post-infection, the agglutinating antibody titer of serum, mucus, and gut lavage fluid was developed for *r-OmpK* groups. In this study, the TLR2 expression was highly induced in mucosal intestine tissue of $10^9$ cfu/mL *r-OmpK* group during the post-vaccination period. These results confirmed that *r-OmpK* was recognised at the main mucosal tissues of juvenile Asian seabass and triggered a MyD88 in the intestine, and MHC1 has significantly arisen following vaccination with *r-OmpK* of $10^9$ cfu/mL in the intestine. In general, all immune-related genes (IL-1β, IL8, IL10, and CCL4) were constitutively expressed in groups vaccinated with *r-OmpK*. Moreover, vaccination of *r-OmpK* of $10^9$ cfu/mL also affects some hematological parameters positively in juvenile Asian seabass. Histopathological examination of the most important organs (spleen, kidney, and intestine) was studied in the *r-OmpK* of $10^9$ cfu/mL vaccinated and infected juvenile Asian seabass. The *r-OmpK* of $10^9$ cfu/mL and PBS vaccinated seabass showed histological alterations in the vital organs compared to healthy groups. The severity increased throughout the challenge experiment for fishes in the PBS challenge group. This study suggested that the increased dosage of the vaccine may influence an increased level of defense in juvenile Asian seabass at vaccination state.

Finally, to better understand the vaccine effects on juvenile Asian seabass immune-related gene expression, the present study assessed the transcriptomic head-kidney responses. The head kidney transcriptome of PBS control and *r-OmpK* of $10^9$cfu/mL vaccinated fish against *V. harveyi* were successfully sequenced using the Illumina platform, and paired-end reads were generated. Approximately 89.5 million and 10.9 billion reads were obtained, trimmed, and assembled into 21 678 unigenes for control PBS and *r-OmpK* groups. These unigenes sequences were annotated and classified by performing Gene Ontology (GO), Cluster of Orthologous Groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional classifications. Comparison of the gene expression profiles of the two examined vaccines revealed some differences. The total number of genes expressed for control PBS was around 21.18 %, and 13.41 % were expressed in *r-OmpK*. In addition, some differentially expressed genes were associated with defense mechanisms and immune systems processes. In total, about 226 genes were identified related to defense mechanisms, and 1,879 genes were related to immune systems. The study has indicated that the introduction of antigen in juvenile Asian seabass has given rise to the alterations in the expression of some important pathways such as CAMs, antigen processing and presentation signaling, and coagulation cascade after the *V. harveyi* challenge. The presence of a large number of immune-related genes and pathways similar to other vertebrates suggests that innate and adaptive immunity in vaccinated juvenile Asian seabass are conserved.

This study provides deep-sequence data of juvenile Asian seabass head kidney transcriptome, and infection with *V. harveyi* resulted in a large number of DEGs in the head kidney of juvenile Asian seabass. Furthermore, the DEGs identified were involved in many pathways of bacterial pathogenesis. The rapid and significant response of the *r-OmpK* immune signaling pathways following *V. harveyi* infection may be associated with their involvement in the immune process.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENILAIAN DUA SEL REKOMBINAN VAKSIN OMPK DAN DNAJ TERHADAP VIBRIOSIS DALAM IKAN SIAKAP, *Lates Calcarifer* (Bloch, 1790).**

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Akuakultur adalah antara sektor pengeluaran makanan yang berkembang pesat di dunia. Namun, pertumbuhannya ditekan oleh pelbagai masalah penyakit, termasuk jangkitan bakteria. Protein membran luar (OMP) bakteria patogen Gram-negatif memainkan peranan penting dalam interaksi antara bakteria hosya, dan merupakan calon yang berpotensi untuk pengembangan vaksin terhadap penyakit ikan. Oleh itu, dalam kajian ini, vaksin sel rekombinan OmpK dan DnaJ dari *Vibrio* spp. diklon untuk melindungi ikan daripada *vibriosis*.

Gen *OmpK* daripada bakteria *V. alginolyticus* strain VA2 dan gen *DnaJ* daripada *V. harveyi* strain VH1 dimasukkan ke dalam vektor pET-32 Ek / LIC dan diklon ke dalam host ekspresi *E. coli* BL21 (DE3). Protein fusi rekombinan *r-OmpK* dan *r-DnaJ* dianalisis lebih lanjut dengan 'SDS PAGE', pemblotan 'Western', analisis enzim pembatasan dan analisis jujukan. Struktur tersier *r-OmpK* dan *r-DnaJ*, dan kestabilan protein rekombinan dikaji menggunakan sesawang bioinformatik. Imunogenisiti antigen vaksin rekombinan yang dikembangkan telah dinilai melalui beberapa eksperimen secara '*in vivo*'.

Dalam percubaan '*in vivo'* pertama, kajian ini memfokuskan pada percubaan vaksinasi pada ikan siakap (±5 cm; ±20 g) selama 15 hari dengan menggunakan vaksin sel rekombinan yang dikembangkan. Dalam eksperimen ini, Kumpulan 1 divaksinkan secara intraperitoneal (IP) dengan vaksin sel rekombinan yang mengandungi *r-OmpK*, Kumpulan 2 dengan rekombinan *r-DnaJ*, Kumpulan 3 dengan *V. harveyi* yang selnya dibunuh menggunakan formalin, Kumpulan 4 adalah *E. coli* (BL21) dan Kumpulan 5 adalah PBS. Sampel dikumpulkan dengan sewajarnya untuk semua analisis seperti untuk analisis gen OmpK dan DnaJ yang disasarkan dalam sampel tisu terpilih, analisis bakterisida serum, ujian aglutinasi dan analisis qPCR, berfokus

eksperimen adalah pada sesi awal vaksinasi. Walaupun suntikan IP tidak menyebabkan peningkatan kadar titer antibodi pada lendir kulit, terdapat peningkatan yang ketara ($p<0,05$) dalam antibodi serum dan usus ikan yang divaksinkan dengan *r-OmpK*. Selanjutnya, hasilnya menunjukkan pengaturan yang signifikan ($p<0,05$) untuk gen TLR2, MyD88 dan MHCI pada tisu ginjal dan usus ikan yang divaksinkan dengan rekombinan *r-OmpK* dibandingkan dengan empat kumpulan lain yang diperiksa. Vaksinasi juga mencetuskan tahap ekspresi signifikan yang lebih tinggi ($p<0.05$) pada tahap imunisasi pasca perdana interleukin IL-10, IL-8, IL-1β pada limpa, usus dan ginjal siakap yang divaksinasikan dengan *r-OmpK* berbanding dengan *r-DnaJ*. Ekspresi gen yang melibatkan sitokin pro-inflamasi IL-1β juga meningkat pada tisu limpa, usus dan ginjal ikan yang divaksin dan dijangkiti *V. harveyi*, *V. alginolyticus* dan *V. parahaemolyticus*. Corak ekspresi yang sama diperhatikan untuk IL-8, IL-1β, IL-10, dan CCL4 pada 24 jam, 48 jam dan 72 jam selepas cabaran. Siakap yang diberi vaksin dengan protein *r-OmpK* dilindungi sepenuhnya dengan peratus kelangsungan hidup relatif (RPS) 90% terhadap jangkitan patogen *V. harveyi* dan 100% terhadap jangkitan *V. alginolyticus* dan *V. parahaemolyticus*. Kajian ini mendapati bahawa siakap dapat dilindungi dengan baik apabila dicabar dengan pelbagai jenis *Vibrio* setelah vaksinasi dengan *r-OmpK* berbanding dengan protein *r-DnaJ*.

Dalam eksperimen kedua, keupayaan imunoproteksi vaksin rekombinan *r-OmpK* ditentukan pada dua dos yang berbeza ($10^7$ dan $10^9$ cfu / mL) terhadap jangkitan *V. harveyi* pada siakap (∓5 cm; ∓20 g). Kematian kumulatif 10% diperhatikan pada kumpulan yang diberi vaksin dengan *r-OmpK* dos $10^9$ cfu / mL, yang secara signifikan ($p <0.05$) berbeza daripada *r-OmpK* dos $10^7$ pada jam 120 selepas jangkitan. Selepas vaksinasi, kumpulan yang diberi vaksin dengan $10^9$ cfu / mL *r-OmpK* menunjukkan peningkatan antibodi yang signifikan ($p <0,05$) dalam serum, lendir badan dan cairan lavage usus berbanding dengan kumpulan $10^7$ cfu/mL *r-OmpK*. Selepas jangkitan *V. harveyi*, titer antibodi penggumpalan serum, lendir badan dan cairan lavage usus dikesan untuk kumpulan *r-OmpK*. Dalam kajian ini, ekspresi TLR2 diperhatikan meningkat pada tisu usus $10^9$ kumpulan cfu/mL *r-OmpK* selama tempoh selepas vaksinasi. Hasil ini mengesahkan bahawa *r-OmpK* dikenali pada tisu mukosa ikan siakap dan gen MyD88 di kesan di usus, dan MHC1 menigkat dengan signifikasi yang ketara setelah vaksinasi dengan *r-OmpK* sebanyak $10^9$ cfu / mL di usus. Secara umum, semua gen yang berkaitan dengan imun (IL-1β, IL8, IL10 dan CCL4) diekspresi secara konsumatif dalam kumpulan sikap yang diberi vaksin dengan *r-OmpK*. Selain itu, vaksinasi dengan *r-OmpK* sebanyak $10^9$ cfu/mL juga mempengaruhi beberapa parameter hematologi secara positif pada ikan siakap. Pemeriksaan histopatologi organ-organ terpenting (limpa, ginjal dan usus) dikaji dalam *r-OmpK* $10^9$ cfu/mL yang divaksinasi dan dijangkiti *V. harveyi*. Vaksin *r-OmpK* dengan kepekatan $10^9$ cfu/mL dan siakap yang diberikan vaksin PBS menunjukkan perubahan histologi pada organ vital jika dibandingkan dengan kumpulan yang sihat dan keparahannya meningkat sepanjang eksperimen cabaran untuk ikan dalam kumpulan cabaran PBS. Kajian ini menunjukkan bahawa peningkatan tahap pertahanan terhadap siakap pada keadaan vaksinasi mungkin dipengaruhi oleh peningkatan dos vaksin yang digunakan.

Akhirnya, untuk lebih memahami kesan vaksin terhadap ekspresi gen yang berkaitan dengan imun ikan siakap, kajian ini menilai tindak balas transkritomik ginjal. Transkrip ginjal yang divaksi dengan PBS dan *r-OmpK* $10^9$cfu/mL yang dijangkiti dengan patogen *V. harveyi* berjaya dianalisis menggunakan platform Illumina dan 'pair end read'. Lebih kurang 89.5 juta dan 10.9 bilion bacaan diperoleh, dianalisis dan dikumpulkan menjadi 21, 678 gen dari kumpulan PBS dan *r-OmpK*. Urutan unigen ini diberi penjelasan dan diklasifikasikan dengan melakukan klasifikasi fungsional Gene Ontology (GO), Cluster of Orthologous Groups (KOG) and Encyclopedia of Genes and Genomes (KEGG). Perbandingan profil ekspresi gen dari dua vaksin yang diperiksa menunjukkan beberapa perbezaan. Jumlah gen yang dinyatakan untuk PBS kawalan adalah sekitar 21,18% dan 13,41% gen dinyatakan dalam *r-OmpK*. Beberapa gen yang dinyatakan berbeza dikaitkan dengan mekanisme pertahanan dan proses sistem kekebalan tubuh. Secara keseluruhan kira-kira 226 gen dikenal pasti berkaitan dengan mekanisme pertahanan dan 1,879 gen berkaitan dengan sistem imun. Kajian ini menunjukkan bahawa pengenalan antigen dalam ikan siakap telah menyebabkan perubahan dalam ekspresi beberapa jalur penting seperti CAM, pemprosesan antigen dan pemberian persembahan, dan lata pembekuan setelah cabaran *V. hareyi*. Kehadiran sebilangan besar gen dan laluan yang berkaitan dengan kekebalan tubuh serupa dengan vertebrata lain menunjukkan bahawa kekebalan bawaan dan adaptif dalam ikan siakap yang divaksin dipelihara.

Kajian ini memberikan data urutan mendalam transkriptom ginjal siakap dan jangkitan dengan *V. harveyi* mengahasilkan sebilangan besar DEG di ginjal siakap. Selanjutnya, DEG yang dikenal pasti terlibat dalam banyak patogenesis bakteria. Tindak balas yang cepat dan ketara dari laluan isyarat imun *r-OmpK* berikutan jangkitan *V. harveyi* mungkin berkaitan dengan penglibatan mereka dalam proses imun.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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| | |
|---|---|
| % | percentage |
| µl | microlitre |
| µM | micromolar |
| ® | Registeredp |
| ™ | Trademark |
| º | Degree celcius |
| Asp (D) | Aspartate |
| BLAST | Basic Local Alignment Search Tool |
| Bp | base pair |
| $CaCl_2$ | Calcium chloride |
| CFU | colony forming units |
| dATP | deoxyadenosine triphosphate |
| dNTP | deoxynucleotide triphosphate |
| DNA | deoxyribonucleic acid |
| ECPs | Extracellular products |
| *E. coli* | *Escherichia coli* |
| *L. calcarifer* | *Lates calcarifer* |
| GMO | Genetically modified organism |
| H&E | haematoxylin and eosin |
| His (H) | Histidine |
| Hpi | Hour post infection |
| Ig | immunoglobulin |
| IP | intraperitoneal |
| Kb | kilobase pair |

| | |
|---|---|
| LB | Luria Bertani |
| LPS | Lipopolysaccharide |
| M | molar |
| Mg | milligram |
| $MgCl_2$ | magnesium chloride |
| mM | Millimolar |
| MS-222 | tricaine methanesulfonate solution |
| NaCl | sodium chloride |
| NGS | Next Generation Sequencing |
| OMPs | Outer membrane proteins |
| PAMPs | Pathogen-associated molecular patterns |
| PBS | Phosphate uffered saline |
| PCR | Polymerase chain reaction |
| RE | restriction enzyme |
| RNA | ribonucleic acid |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

The aquaculture growth is towards increased intensification and commercialisation of aquatic production. At present, with an average annual growth rate of 8.9% from 1970, aquaculture is the speediest growing food-producing sector compared to 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems (Ye et al., 2017). In Malaysia, marine aquaculture has expanded significantly over the last two decades, contributing to about 70 % of the total aquaculture production (Islam et al., 2016). Various species of marine, brackish water finfish are produced using the cages system, including Asian seabass (*Lates calcarifer*), snappers (*Lutjanidae*), grouper (*Epinephelus fuscoguttatus*), cobia (*Rachycentron canadum*), and red tilapia (*Oreochromis* spp).

The cage culture areas have increased from 27,000 to 1,741,000 $m^2$ between 1982 and 2009, leading to the upsurge of production from 413 to 22,521 MT during the same period in Malaysia. There were about 3,258 farmers involved in cage aquaculture practices in 2009 (DOF, 2011). However, the growth of the aquaculture industry has been overwhelmed with its share of diseases and other aquaculture management problems. This situation can be attributed to various multi-faceted and highly interconnected factors such as the increased globalisation of trade in live aquatic animals and their products (Subasinghe et al., 2001; Lindland et al., 2019; Mitchell et al., 2019). Further on, the output and productivity of Malaysian cage farming may be affected by external factors such as marine pollution, climate change, and other environmental factors beyond the culturists' control. Studies found an increased risk of disease occurrence within cage reared fish and the potential risk of transfer of diseases to and from natural fish populations (Islam et al., 2016). The impacts of the disease have been estimated in socio-economic terms (e.g. losses in production, income, employment, market access or market share, investment and consumer confidence; food shortages; industry failure or closure of business or industry).

Vibriosis is one of the most severe infectious bacterial diseases affecting fish in tropical and subtropical zones of the world. Its pathogens mainly include *Vibrio harveyi, V. parahaemolyticus* and *V. alginolyticus* and other *Vibrio* sp. Romi (2016) reported that vibriosis occurs in more than 14 countries, ravaging approximately 48 marine fish species. In Malaysian aquaculture, grouper farmers have reported vibriosis as a key disease since the 1960s, where high mortality was recorded in fish pens at Penang (Chiew et al., 2019). Even if

*vibriosis* was reported to occur mainly during the hatchery and grow-out phases; yet, current studies specify that adults' fish might also be affected.

Meanwhile, for the Asian sea bass, an outbreak was reported in open-net cages in Sabah, where the causal agent was identified as *V. harveyi* (Chiew et al., 2019). According to Istiqomah et al. (2020), v*ibriosis* has been reported in Indonesia marine fish culture since the 1990s. Although it was mostly recounted in grouper and shrimp farming, still there were reports on snapper.

## 1.2 Problem statement

The usage of antibiotics can be an effective treatment against vibriosis, but unfortunately, long-term handling may affect the environment. Furthermore, due to the gradual spread of drug resistance, effective vaccine development is urgently required to enhance existing Vibrio measures (Ningqiu et al., 2010; Ina-Salwany et al., 2019). Thus, the prominent ways to overcome these problems are through vaccination. However, although numbers of highly efficacious vaccines have been established for aquaculture, those covering a broad range of bacterial diseases are still limited. Furthermore, the diversity of pathogens and their serotypes causes the progress in vaccine development against *vibriosis* to slow down, and no commercial vaccine is presently obtainable in this precinct (Ningqiu Li et al., 2010; Rosli et al., 2016).

Besides, a vaccine for a facility with a specific disease problem must be manufactured much more frequently (Toranzo et al., 2009). Therefore, more efforts are in need to develop a safe and effective subunit vaccine. Even so, the inconvenience of vaccination application hindered its successful development as an effective vaccine against the deadly infection by *vibriosis* in fish (Ruyra *et al*., 2013 and Vimal *et al*., 2013). Therefore, there is a need for optimisation of several factors such as the choice of antigen delivery systems whether to use intra- or extra-cellular delivery as well as whether to use oral, immersion or injectable vaccine delivery systems and to develop prime vaccination regimes that confer the highest protection throughout the fish production cycle (Toranzo et al., 2009). In the present study, a recombinant cell vaccine encoding the OmpK gene of *V. alginolyticus* and the DnaJ gene of *V. harveyi* was constructed and expressed *in vitro* and *in vivo*. The immune protective efficacy was tested after multiple *vibrio* strains challenge in juvenile Asian seabass model intraperitoneally (IP).

## 1.3 Justification of the study

The outer membrane protein (OMP) frequently works as virulence features for scavenging nutrients and resisting host defensive mechanisms in pathogenic organisms (Silhavy et al., 2010). In addition, OMPs possess epitopes essential for binding to B and T lymphocytes, rendering them ideal vaccine candidates

for both extra and intra-cellular replicating bacteria (Maiti et al., 2020). Furthermore, OMP has been classified as a promising vaccine candidate identified as an immunogenic antigen for some *Vibrio* sp. According to Maiti et al. (2020), OMPs conserved across serotypes could be used as potential candidates in vaccine development. Several studies have demonstrated their efficacy and potential as vaccine candidates.

A study by, Nehlah et al. (2017) showed high protection (Relative percentage survival RPS = 100%) in juvenile hybrid grouper (*Epinephelus fuscoguttatus Forsskal* × *Epinephelus lanceolatus*) vaccinated with a *rOmpK* subunit vaccine after challenge with *V. alginolyticus*. Similarly, Hamod et al. (2012) showed high protection (RPS = 67.8%) in adult rohu (*Labeo rohita*) vaccinated with a *rOmpK* subunit vaccine after a challenge with *V. anguillarum*. In another study, Amirah (2018) reported that the bioencapsulation of the inactivated recombinant vhDNAJ cells vaccine into *Artemia* sp. demonstrated that the vaccine could survive up to 83.3% after 36 h post encapsulation, signifying the vaccine was safe and might be beneficial to the host.

The outer membrane protein of the fish pathogen, *V. alginolyticus* and *V. harveyi*, which plays important roles in the interaction between bacteria and hosts, are potential candidates for the development of recombinant vaccines (Ningqiu et al., 2008; Nguyen et al., 2018; Maiti et al., 2020). It was reported that the outer membrane protein, O*mpK* and *DnaJ* could elicit immune protection in fish species against wide broad vibriosis infections (Cai et al., 2010). Despite so, the vaccination strategy also includes the decision on which diseases to be vaccinated against, the type of vaccines to be used, vaccine dosages, vaccination method and time sequence of vaccination (Toranzo et al., 2009). It is widely accepted that vaccination via injection gives higher protection than oral and immersion vaccination (Komar et al., 2004; Evensen, 2009; Maiti et al., 2020). Therefore, prime vaccination practice at the preliminary stage is necessary for developing an ideal vaccine (Natalia et al., 2014).

In this study, two candidates protective recombinant cells of outer membrane protein pET-32/LIC-*OmpK* (*r-OmpK*) and pET-32/LIC-*DnaJ* (*r-DnaJ*) were identified and analysed. Next, the vaccination via intraperitoneal injection on juvenile seabass using newly developed recombinant cell vaccines *r-OmpK* and *r-DnaJ* were compared with killed whole cells vaccine (Nehla et al., 2018). The protective efficacy of *r-OmpK* and *r-DnaJ* was examined against multiple Vibrio sp. strains: *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* in a single immunisation event with no booster applied (Natalia et al., 2014). Further, to elucidate the mechanism of immunoprotection, the expression level of pathogen-specific antibodies and immune-related genes were compared between vaccinated and non-vaccinated juvenile bass. Additionally, a haematological and histopathology analysis was studied to understand the right vaccine candidate of OMP used in juvenile Asian seabass. Finally, the Illumina sequencing technology for de novo reference transcriptome assembly was adopted using the head kidney of juvenile Asian seabass. The de novo

transcriptomes of two groups, vaccinated and challenged with *V. harveyi* pathogenic strain, were analysed.

## 1.4 Objectives

The main objectives of this study as listed below:

1. To construct and express two outer membrane proteins (OMPs) of *Vibrio alginolyticus* strain VA2 and *V. harveyi* strain VH in *Escherichia coli* BL21 (DE3).

2. To evaluate the immune responses of recombinant cells OmpK (*r-OmpK*) and DnaJ (*r-DnaJ*) following intraperitoneal vaccination in juvenile Asian sea bass.

3. To determine the efficacy of the developed recombinant vaccine (*r-OmpK*) using the intraperitoneal injection method at two different doses as post-prime vaccination and post-challenge of V*ibrio harveyi* in juvenile Asian seabass.

4. To determine the immune system regulation in terms of differentially expressed genes and regulations of specific immune response in the related pathway, using a *de novo* transcriptomic analysis in post vaccinated head kidney samples of Asian seabass.

## 1.5 Hypothesis

The hypothesis of the study is as below:

Hypothesis 1:

$H_0$: The outer membrane proteins (OMPs) of *Vibrio alginolyticus* strain VA2 and *V. harveyi* strain VH1 were not constructed and expressed in *Escherichia coli* BL21 (DE3).

$H_1$: The outer membrane proteins (OMPs) of *Vibrio alginolyticus* strain VA2 and *V. harveyi* strain VH1 were constructed and expressed in *Escherichia coli* BL21 (DE3).

Hypothesis 2:

$H_0$: Vaccination of juvenile Asian seabass with developed recombinant cell vaccines *rOmpK* and *rDnaJ* could not induce immune response after prime immunisation and results in high mortalities after challenged with pathogenic *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* strain.

$H_1$: Vaccination of juvenile Asian seabass with newly developed recombinant cell vaccines *rOmpK* and *rDnaJ* are safe, could induce good immune response prime immunisation and results in mortalities decrement after challenged with pathogenic *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* strain.

Hypothesis 3:

$H_0$: Vaccination of juvenile Asian seabass with developed recombinant cell vaccines *rOmpK* are not safe, could not induce immune response at certain dosage after prime immunisation and results in high mortalities after challenged with pathogenic *V. harveyi* strain.

$H_1$: Vaccination of juvenile Asian seabass with developed recombinant cell vaccines *rOmpK* are safe, could induce good immune response at certain dosage after prime immunization immunisation and results in mortalities decrement after challenged with pathogenic *V. harveyi* strain.

Hypothesis 4:

$H_0$: The de novo transcriptomic analysis cannot regulate the differentially-expressed genes, and specific immune response in the related pathway in post vaccinated head kidney samples of Asian seabass.

$H_1$: The de novo transcriptomic analysis successfully regulates the differentially-expressed genes, and specific immune response in the related pathway in post vaccinated head kidney samples of Asian seabass.

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