UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF RECOMBINANT OUTER MEMBRANE PROTEINS VACCINE AND ITS IMMUNOPROTECTIVE ABILITY AGAINST Vibrio alginolyticus IN HYBRID GROUPER (Epinephelus fuscoguttatus Forsskal x Epinephelus lanceolatus Bloch)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

DEVELOPMENT OF RECOMBINANT OUTER MEMBRANE PROTEINS VACCINE AND ITS IMMUNOPROTECTIVE ABILITY AGAINST *Vibrio alginolyticus* IN HYBRID GROUPER (*Epinephelus fuscoguttatus* Forsskal *x Epinephelus lanceolatus* Bloch)

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August 2016

Chairman : Ina Salwany Md. Yasin, PhD
Faculty : Agriculture

The productions of grouper may become restricted due to many constraints, including diseases. The most significant bacterial disease attacking groupers is vibriosis and its major causative agent is *Vibrio alginolyticus.* This study was conducted to develop recombinant cells vaccine containing antigenic OmpK and OmpW genes from *V. alginolyticus* by gene cloning and protein expression, and to evaluate the immune response and protection efficacy of hybrid grouper following exposure to virulent *V. alginolyticus*. The selected *V. alginolyticus* strain VA2 was first identified to confirm it as *V. alginolyticus* by phenotypic and genotypic characterization. In addition, the antigenicity analysis of the outer membrane proteins (OMPs) of *V. alginolyticus* strain VA2 was carried out. The OMPs were isolated and purified, followed by protein profiling using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and antigenicity analysis was done using Western blotting. The SDS-PAGE profiling revealed a mixture of major and minor protein bands. The 23 and 31 kDa protein bands, postulated to be the OmpW and OmpK proteins, respectively, were antigenic as shown by Western blot. The vaccine was constructed by purifying PCR products of OmpK and OmpW genes from *V. alginolyticus*, followed by transformation into cloning and expression hosts, *Escherichia coli* TOP10 and BL21(DE3), respectively. Sequencing analysis revealed that the full length of target genes OmpK and OmpW of *V. alginolyticus* strain VA2 were 846 and 642 bp, respectively. The sequences of the target genes were highly similar to published sequences of *Vibrio* species in GenBank. The multiple sequence alignment showed that their amino acid sequences were highly conserved

between different *Vibrio* species. High numbers of antigenic sites were also predicted in the OmpK and OmpW protein sequences. Pilot expression was done for recombinant OmpK and OmpW to determine the optimum expression temperature and time, which were revealed to be 30°C for 10 h. The expressed target proteins were then detected by His-tag monoclonal antibody using SDS-PAGE and Western blotting, in which the presence of fusion proteins at 48.3 kDa and 40.3 kDa for OmpK and OmpW respectively, were confirmed. For vaccination, formalin killed whole-cell of *V. alginolyticus* strain VA2 was prepared at concentration of 1.59×10^7 and 8.3×10^7 CFU/ml for OmpK and OmpW respectively. Six groups of 150 hybrid groupers each, at approximately 30 g was acclimatized beforehand for two weeks. These six groups were for treatments of rOmpK, rOmpW, rOmpK+rOmpW, *Eschericia coli* only, PBS only and control without manipulation. Vaccination was done by intraperitoneal injection on day 0. Booster dose was given on day 14. On day 28, all fish were challenged with virulent strain of *V. alginolyticus* at concentration of 1×10^9 CFU/mL by intraperitoneal (IP) injection. Blood serum and gut samples were collected biweekly until week 10. The survival percentages were highest for OmpK+OmpW and OmpK vaccinated groups at 100%, followed by OmpW vaccinated group at 67%, PBS group at 12%, and *E. coli* group at 0%. Enzyme-linked immunosorbent assay (ELISA) analysis showed significant ($p<0.05$) antibody difference between vaccinated and non-vaccinated groups from week 2 to week 10. After booster dose, the antibody level of bivalent vaccine OmpK+OmpW was significantly higher ($p<0.05$) than other groups. However, it was not significantly different ($p>0.05$) than monovalent vaccine OmpK after challenge at week 4. Gut histology showed presence of gut-associated lymphoid tissue (GALT) in vaccinated groups only. This showed that while bivalent vaccine OmpK+OmpW is effective, monovalent vaccine OmpK alone is also strong enough to give protection to groupers against high concentration of virulent *V. alginolyticus*.

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PEMBANGUNAN VAKSIN PROTEIN MEMBRAN LUAR REKOMBINAN DAN KEBOLEHANNYA MEMBERI PERLINDUNGAN IMUN TERHADAP *Vibrio alginolyticus* KEPADA IKAN KERAPU HIBRID (*Epinephelus fuscoguttatus* Forsskal x *Epinephelus lanceolatus* Bloch)

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Pengeluaran ikan kerapu boleh menjadi terhad disebabkan pelbagai kekangan, termasuk penyakit. Penyakit bakteria yang paling serius menyerang kerapu adalah vibriosis dan agen penyebab utamanya adalah *Vibrio alginolyticus.* Kajian ini dijalankan untuk menghasilkan vaksin sel rekombinan yang mengandungi antigen gen OmpK dan OmpW dari *V. alginolyticus* dengan cara pengklonan dan ekspresi protein, serta untuk menilai tindak balas imun ikan kerapu hibrid yang didedahkan kepada *V. alginolyticus* yang virulen. *Vibrio alginolyticus* strain VA2 yang dipilih telah dikenal pasti terlebih dahulu sebagai *V. alginolyticus* oleh pencirian fenotip dan genotip. Tambahan pula, analisis keantigenan protein membran luar (OMPs) *V. alginolyticus* strain VA2 telah dilakukan. Protein membran luar (OMPs) tersebut diasingkan dan ditulenkan, diikuti pemprofilan protein oleh elektroforesis gel poliakrilamida sodium dodesil sulfat (SDS-PAGE) dan analisis keantigenan oleh pemblotan Western. Pemprofilan SDS-PAGE menunjukkan campuran jalur protein utama dan minor. Jalur protein yang bersaiz 23 dan 31 kDa yang diandaikan adalah protein OmpW dan OmpK, masing-masing, adalah bersifat antigen menurut pemblotan Western. Vaksin ini dihasilkan dengan menulenkan produk PCR gen-gen OmpK dan OmpW dari *V. alginolyticus*, diikuti dengan transformasi ke dalam perumah pengklonan dan pengekspresi, masing-masing *Escherichia coli* TOP10 dan BL21(DE3). Analisis penjujukan menunjukkan saiz keseluruhan gen OmpK dan OmpW *V. alginolyticus* strain VA2 adalah masing-masing 846 dan 642 bp. Jujukan gen tersebut mempunyai persamaan yang sangat tinggi dengan

jujukan spesies *Vibrio* dalam GenBank. Penjajaran berbagai turutan menunjukkan jujukan asid amino mereka dikekalkan dalam kalangan spesies *Vibrio*. Jumlah tempat antigenik yang tinggi juga diramalkan dalam jujukan protein OmpK dan OmpW. Ekspresi ujian telah dilakukan bagi OmpK dan OmpW untuk menentukan suhu dan jangka masa ekpresi yang optimum, iaitu 30°C selama 10 jam. Protein sasaran yang diekspres kemudiannya dikesan oleh antibodi monoklonal His-tag menggunakan SDS-PAGE dan pemblotan Western, yang mana gabungan protein OmpK dan OmpW bersaiz 48.3 kDa dan 40.3 kDa, masing-masing, telah dikenal pasti. Bagi pemvaksinan, sel keseluruhan *V. alginolyticus* strain VA2 yang dimatikan dengan menggunakan formalin telah disediakan pada kepekatan 1.59×10^7 dan 8.3×10^7 CFU/ml untuk OmpK dan OmpW masing-masing. Enam kumpulan ikan kerapu hibrid, setiap kumpulan sebanyak 150 ekor, dengan berat setiapnya kira-kira 30 g diaklimatisasikan terlebih dahulu selama dua minggu. Enam kumpulan ini adalah untuk rawatan rOmpK, rOmpW, rOmpK+rOmpW, *Eschericia coli* sahaja, larutan garam bertimbal fosfat (PBS) sahaja dan kawalan tanpa manipulasi. Pemvaksinan dilakukan dengan suntikan intraperitoneum pada hari 0. Dos peningkatan diberikan pada hari 14. Pada hari 28, semua ikan dicabar dengan *V. alginolyticus* yang virulen pada kepekatan 1×10^9 CFU/mL dengan suntikan IP. Serum darah dan sampel usus diambil setiap dua minggu sehingga minggu 10. Peratusan kemandirian adalah tertinggi untuk kumpulan-kumpulan vaksinasi OmpK+OmpW dan OmpK pada 100%, diikuti kumpulan vaksinasi OmpW pada 67%, kumpulan PBS pada 12% dan kumpulan *E. coli* pada 0%. Analisis asai imunoserap terangkai enzim (ELISA) menunjukkan perbezaan ketara ($p<0.05$) antibodi antara kumpulan vaksinasi dan tiada vaksinasi dari minggu kedua hingga minggu 10. Selepas dos peningkatan, tahap antibodi vaksin bivalen OmpK+OmpW meningkat dengan ketara ($p<0.05$) berbanding kumpulan-kumpulan lain. Bagaimanapun, tiada perbezaan ketara ($p>0.05$) antaranya dengan vaksin monovalen OmpK selepas dicabar pada minggu 4. Histologi usus menunjukkan kehadiran sel limfoid berkaitan usus (GALT) dalam kumpulan vaksinasi sahaja. Ini menunjukkan vaksin bivalen OmpK+OmpW adalah efektif, sementara vaksin monovalen OmpK sahaja sudah cukup kuat untuk memberi perlindungan kepada kerapu terhadap *V. alginolyticus* virulen kadar tinggi.

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I certify that a Thesis Examination Committee has met on 26 August 2016 to conduct the final examination of Nehlah binti Rosli on her thesis entitled "Development of Recombinant Outer Membrane Proteins Vaccine and its Immunoprotective Ability Against *Vibrio alginolyticus* in Hybrid Grouper (*Epinephelus fuscoguttatus* Forsskal *x Epinephelus lanceolatus* Bloch)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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

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LIST OF ABBREVIATIONS/NOTATIONS/GLOSSARY OF TERMS

%	Percentage
λ	lambda
µg	microgram
µl	microliter
µM	micromolar
ANOVA	Analysis of variance
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CFU	colony forming units
DAB	3,3'-Diaminobenzidine
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
E. fuscoguttatus	*Epinephelus fuscoguttatus*
E. lanceolatus	*Epinephelus lanceolatus*
EFA	essential fatty acid
FKC	formalin-killed cells
ELISA	Enzyme-Linked Immunosorbent Assay

GALT	Gut-Associated Lymphoid Tissue
H_2SO_4	sulphuric acid
H&E	haematoxylin and eosin
Ig	immunoglobulin
IHN	infectious haematopoietic necrosis
IP	intraperitoneal
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITS	Internal Transcribed Spacer
kb	kilobase pair
kDa	kilodalton
LB	Luria Bertani
LIC	ligation-independent cloning
M	molar
MCS	multiple cloning site
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
mM	millimolar
MS-222	tricaine methanesulfonate solution
NaCl	sodium chloride
ng	nanogram
nm	nanometer
OD	optical density
OMP	Outer membrane protein

PBS	phosphate buffer saline
PCR	polymerase chain reaction
pmol	picomole
ppt	parts per thousand
RBC	red blood cells
RE	restriction enzyme
RNA	ribonucleic acid
rpm	revolutions per minute
RPS	relative percentage survival
rRNA	ribosomal ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal broth with Catabolite repression
sp.	species
TBE	tris-boric EDTA
TBS	tris-buffer saline
TCBS	thiosulfate-citrate-bile salts-sucrose
TMB	3,3′,5,5′-tetramethylbenzidine
TSA	tryptic soy agar
TSB	tryptic soy broth
U	unit
V	voltage
VHS	viral haemorrhagic septicaemia
V. alginolyticus	*Vibrio alginolyticus*

VNN	viral nervous necrosis
v/v	volume per volume
w/v	weight per volume
x g	multiples of gravitational acceleration

CHAPTER 1

INTRODUCTION

Vibriosis is a disease caused by bacteria belonging to the genus *Vibrio,* and has been recognized as one of the most severe and destructive diseases in the aquaculture industry, causing high mortality in marine fish (Cai *et al.*, 2013a). *Vibrio* species is a rod-shaped, gram-negative bacterium from the family Vibrionaceae with a broad host range (Cai *et al.*, 2013a). Studies had shown the virulence of *V. alginolyticus*, which is considered the main bacterial pathogen causing outbreaks to humans as well as aquatic animals such as shrimp, shellfish and marine fish (Cai *et al.*, 2013a; Zorrilla *et al.*, 2003; Lee *et al.*, 1996; Lightner, 1993). Vibriosis caused by *V. alginolyticus* is able to affect all fish growth stages which often leads to severe mortality. Due to various diseases including vibriosis, survival of cage-cultured groupers in Malaysia are up to 30-40% only (Liao and Leaño, 2008). *Vibrio alginolyticus* itself causes more than 50% mass mortality of worldwide fishery culture (Salosso and Jasmanindar, 2014), consequently leading to serious economic damage (Martins *et al.*, 2010).

Outbreaks of vibriosis have been frequently reported in grouper (Huang *et al.*, 2012), sea bream (El-Galil and Mohamed, 2012), sea bass (Bellos *et al.*, 2015) and catfish (Geng *et al.*, 2014). In 2010, two major vibriosis outbreaks on April and October caused high mortality of cultured marine fish species including Asian seabass (*Lates calcarifer*), brown-marbled grouper (*E. fuscoguttatus*), red snapper (*Lutjanus* sp.), orange-spotted grouper (*E. coioides*) and hybrid grouper (*E. fuscoguttatus x E. lanceolatus*) in an aquaculture facility in Sabah, Malaysia (Albert and Ransangan, 2013).

In a study by Kua *et al.* (2011), abnormal mortality of 4-5 abalone/day instead of 1-3 abalone/month were reported for abalone cultured in Penang, Malaysia, in which it was confirmed that the abalones were infected by *Pasteurella* sp. and *V. alginolyticus*. In Karimunjawa islands, North Java Sea, Indonesia, the causative agents of vibriosis associated with groupers were investigated and the result showed that they include *V. alginolyticus* (Sarjito *et al.*, 2009).

Vibriosis normally occurs when the fish is exposed to the pathogen in the presence of stress factors (El-Galil and Mohamed, 2012; Martins *et al.*, 2010). Vibriosis had been known to cause septicemia, identified by the presence of fin haemorrhages, loss of appetite, skin lesions (Martins *et al.*, 2010), skin

darkening, pale gills caused by anemia, enlarged spleen, internal ascetic fluid (Hendrikson and Zenoble, 1983), and fins and mouth erythema (Reed and Francis-Floyd, 2009). Good husbandry is crucial in preventing spread of diseases. Antibiotics and chemotherapeutic agents are commonly used as strategies to control vibriosis (Sonia and Lipton, 2012). Fish farmers prefer antibiotics as opposed to vaccines due to the former being widely available and the latter being scarce. However, antibiotic treatment has become less effective with the gradual spread of drug resistance (Cai *et al.*, 2013a). Another effective alternative would be vaccination, which has been shown to be able to increase the antibody titre, induce immune memory and significantly increase the survival of infected fish (Defoirdt *et al.*, 2007). Grisez and Tan (2005) observed that in Asia except for Japan, the use of vaccines for controlling fish diseases is not common, even though fish production is large in Asia. Vaccines should be used as an alternative strategy to control vibriosis due to its ability to induce production of antibodies against pathogens in case of infection.

Currently, recombinant vaccine is a promising strategy to combat diseases in fish by using immunogenic protein expressed by specific antigens. Recombinant cells produce the protein of interest in large amount, which increase the antibody reaction and enhance protection. One of the gram-negative bacteria surface structure known as outer membrane have a role in interaction between bacterium and host, in which the outer membrane proteins (OMPs) could serve as potential candidates for vaccine development (Jiang *et al.*, 2003).

As the fish immune system is very specific, a classical vibriosis vaccine that had been developed, with *V. anguillarum* as the antigen, will not provide specific protection against other *Vibrios* spp. (Toranzo *et al.*, 1996). Thus, it is crucial to develop a safe and effective broad-range vaccine for fish against *V. alginolyticus* and possibly other *Vibrio* species as well. For this purpose, two interesting OMP genes, OmpK and OmpW were evaluated in our study.

In developing recombinant bivalent vaccine against *V. alginolyticus* in hybrid grouper, the objectives were:

1. To characterize *Vibrio alginolyticus* isolated from diseased grouper and analyze the antigenicity of its outer membrane proteins (OMPs).
2. To construct recombinant cell vaccines of *V. alginolyticus* antigenic outer membrane proteins OmpK and OmpW genes for the production of potential recombinant vaccines.

3. To evaluate the effectiveness of monovalent vaccines (OmpK and OmpW) versus bivalent vaccine (OmpK+OmpW) in protecting hybrid grouper against *Vibrio alginolyticus* infection.

Hypotheses of this study were:

1. The outer membrane proteins (OMPs) of *V. alginolyticus* consist of major and minor proteins, which can be detected by polyclonal rabbit antiserum.
2. For the vaccine construction, the recombinant cells carrying the genes encoding OmpK and OmpW proteins of *V. alginolyticus* can be cloned and expressed in the *Escherichia coli* prokaryotic expression system.
3. Recombinant bivalent vaccine (OmpK+OmpW) should confer better protection against *V. alginolyticus* infection in hybrid grouper, compared to monovalent vaccines (OmpK and OmpW).

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LIST OF PUBLICATIONS

Nehlah, R., Ina-Salwany, M. Y., & Zulperi, Z. (2016). Antigenicity Analysis and Molecular Characterization of Two Outer Membrane Proteins of *Vibrio alginolyticus* Strain VA2 as Vaccine Candidates in Tiger Grouper Culture. *Journal of Biological Sciences*, *16*(1), 1.

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