UNIVERSITI PUTRA MALAYSIA

ANTIGENIC ANALYSIS OF OUTER MEMBRANE PROTEIN OF Vibrio SPECIES AND DEVELOPMENT OF VERSATILE RECOMBINANT vhDnaJ VACCINE AGAINST VIBRIOSIS

FATHIN AMIRAH MURSIDI

FP 2018 98

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By

FATHIN AMIRAH MURSIDI

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

February 2018

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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February 2018

Chairman : Associate Professor Ina Salwany Md Yasin, PhD
Faculty : Agriculture

Vibriosis is one of the most catastrophic bacterial disease caused by infection of *Vibrio* spp. which frequently attacks marine cultures in all life stages. Recently, vibriosis was reported to cause vast mortality of tiger grouper cultured in deep sea cages in Langkawi, Malaysia. This disease frequently occurs during dry months when the water temperature is elevated. Although vibriosis is controlled through vaccination, the existence of different strains and antigenic diversities of *Vibrio* species and their serotypes have led to slow progress of vaccine development. Therefore, the development of a versatile vaccine that can fight against multiple *Vibrio* by eliciting protection against homologous and heterologous strains is urgently needed both for hindering vibriosis infections and to avoid the exploitation of antibiotics in aquaculture industry. Hence, the aim of this research was to search for the most antigenic OMPs protein among *Vibrio* sp. by analysing the protein's ability to elicit homologous and cross antigenicities and to develop a potentially versatile recombinant *Vibrio* vaccine. The safety of the developed vaccine was also assessed in gnotobiotic *Artemia* species. OMPs of *Vibrio harveyi* strain VH1, *V. alginolyticus* strain VA2, *V. parahemolyticus* strain VPK1 and *Photobacterium damselae* strain PDS1 isolated from diseased groupers were extracted and characterized by SDS-PAGE and detection of immunogenic proteins were done by Western immunoblotting. The polyclonal antiserum of *V. harveyi* strain VH1 raised from rabbit induced strong antigenic responses on homologous OMPs antigens of *V. harveyi* strain VH1 and cross reacted against heterologous OMPs antigens of *V. parahemolyticus* strain VPK1, *V. alginolyticus* strain VA2 and *P. damselae* strain PDS1 at molecular weight of 32 kDa. Therefore, further studies were conducted on the antigenically heterologous 32 kDa OMP of *V. harveyi* strain VH1 as a potential vaccine candidate. The 32 kDa protein band was a molecular chaperone DnaJ, designated as vhDnaJ after submitted for N-terminal amino acid sequencing analysis.

The vhDnaJ gene was amplified and cloned in pET-32 Ek/LIC vector and expressed in host BL21 (DE3) *Escherichia coli*. Bioinformatics analysis indicated that the target gene was highly conserved among *Vibrio* sp. and highly antigenic by comprising 40 antigenic sites. Successful recombinant vhDnaJ protein expression expressed under 30°C for 10 hour was detected at 49 kDa band by SDS-PAGE and Western immunoblotting by using Anti-HisTag monoclonal antibodies. The bioencapsulation of the inactivated recombinant vhDnaJ cells vaccine into *Artemia* sp. demonstrated that the species could survive up to ±83.3% after 36 h post-encapsulation, signifying the vaccine was safe and might be beneficial to the host. In conclusion, the cumulative evidences of the 32 kDa OMP of *V. harveyi* strain VH1 by being the most antigenic against homologous and heterologous isolates and highly conserved among the tested *Vibrio* strains in *in-vitro* antigenicity and bioinformatics study could be a promising versatile vaccine antigen against multiple *Vibrio* sp. in grouper culture. Moreover, the successful expression of the protein of interest and verified safety of the developed recombinant vhDnaJ vaccine in *Artemia* sp. open the way for future preparation of crude recombinant vaccine as well as to assess its efficacy in marine fish.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS KEANTIGENAN PROTIN MEMBRANE LUAR DARI *Vibrio* SPESIES DAN PEMBANGUNAN REKOMBINAN VAKSIN vhDnaJ YANG VERSATIL MELAWAN VIBRIOSIS

Oleh

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Vibriosis adalah salah satu penyakit yang amat merbahaya berpunca daripada jangkitan bakteria *Vibrio* spp. yang kebiasaannya menyerang spesis marin. Baru-baru ini, vibriosis yang menyebabkan kematian sejumlah besar ikan kerapu yang dibiakkan di sangkar laut dalam telah dilaporkan di Langkawi, Malaysia. Wabak vibriosis ini kebiasaannya berlaku apabila suhu air meningkat. Walaupun vibriosis telah dikawal melalui pemvaksinan, kewujudan strain yang berbeza dan kepelbagaian antigenik dalam *Vibrio* sp. dan serotip telah menyebabkan pembangunan vaksin semakin perlahan. Justeru itu, pembangunan vaksin versatil yang boleh melawan pelbagai patogen dengan menghasilkan perlindungan antigenik menentang homolog dan heterologus strain diperlukan dengan segera untuk menghalang jangkitan vibriosis dan mengelakkan penyalahgunaan antibiotik dalam industri akuakultur. Oleh itu, tujuan penyelidikan ini adalah untuk menyelidik protin yang paling antigenik dengan menganalisis kemampuan protin membran luar (OMP) daripada *Vibrio* sp. yang berbeza untuk menghasilkan reaksi homologi dan silang dan membangunkan rekombinan vaksin yang mempunyai potensi serbaguna. Keselamatan vaksin tersebut juga telah dinilai dengan menggunakan gnotobiotik *Artemia* model. OMP daripada *Vibrio harveyi* strain strain VH1*, V. alginolyticus* strain VA2*, V. parahemolyticus* strain VPK1 dan *Photobacterium damselae* strain PDS1 yang telah dipisahkan daripada kerapu berpenyakit telah diekstrak dan dicirikan menggunakan kaedah sodium dodecyl sulfat-gel elektroforesis poliakrilamide (SDS-PAGE) dan pengesanan protin imunogenik melalui kaedah Pemblotan Western. Antiserum poliklonal daripada *V. harveyi* strain VH1 yang dibangunkan daripada arnab telah merangsang gerak balas yang kuat terhadap antigen protin *V. harveyi* strain VH1 dan reaksi silang terhadap protin antigen *V. parahemolyticus* strain VPK1*, V. alginolyticus* strain VA2 dan *P. damselae* strain PDS1 di kedudukan jalur 32 kDa. Oleh itu, kajian seterusnya telah dijalankan

dengan memilih jalur protin 32 kDa *V. harveyi* strain VH1 sebagai calon vaksin. Jaluran protin tersebut telah diproses melalui penjujukan amino asid Terminal-N dan keputusan menunjukkan protin tersebut adalah "molekul pengiring DnaJ". Gen tersebut telah diamplifikasikan dan diklonkan kedalam vector pET-32 EK/LIC sebelum dimasukkan kedalam perumah ekspresi BL21 (DE3) *Eschericia coli.* Bioinformatik analisis menunjukkan bahawa gen tersebut sangat terpelihara di kalangan *Vibrio* sp. dan sangat antigenik dengam memiliki 40 tapak antigenik. Protin yang telah berjaya diekspresikan dibawah suhu 30°C selama 10 jam dianalisa melalui "SDS-PAGE" dan Pemblotan Western menggunakan monoclonal antibodi Anti-HisTag telah menunjukkan jalur protein gabungan yang sangat menonjol pada saiz 49 kDa, yang mengandungi 32 kDa protin vhDnaJ dan protin Tag bersaiz 17 kDa. Rekombinan vhDnaJ vaksin yang tidak aktif dimasukkan ke dalam *Artemia* sp. secara bio menunjukkan spesis tersebut mampu hidup sebanyak ±83.3% selepas 36 jam, menunjukkan bahawa vaksin tersebut selamat dan mungkin memberi kesan yang baik kepada *Artemia.* Kesimpulannya, bukti-bukti yang terkumpul menunjukkan bahawa OMP *V. harveyi* strain VH1 bersaiz 32 kDa adalah paling antigenik dan paling terpelihara di antara *Vibrio* strain berdasarkan ujikaji antigenik dan bioinformatik *in-vitro* dan boleh menjadi antigen vaksin yang bagus serta berpotensi untuk melawan pelbagai *Vibrio* dalam ternakan kerapu. Tambahan pula, pada masa hadapan, ekspresi protin pilihan dan rekombinan vhDnaJ vaksin telah terbukti selamat dan telah membuka jalan untuk penghasilan vaksin rekombinan mentah dan untuk menilai keberkesanan vaksin tersebut dalam ternakan ikan marin.

ACKNOWLEDGEMENTS

First and foremost, all praises be to Allah SWT for His blessing and guidance for making this project possible to be completed within the given time.

I would like to express my deepest gratitude to my research supervisor, Dr. Ina Salwany Md Yasin for her invaluable supportiveness and patient in giving full effort in guiding, teaching, enthusiastic encouragement and useful critiques to this research work. To my co-supervisors, Dr. Mohammad Noor Amal Azmai and Dr. Chong Chou Min, thank you so much for the input and recommendations you gave to improve my research studies.

To my supportive labmates, Mrs. Diyana Nadhirah Khairul Parman, Ms. Nehlah Rosli, Dr. Zarirah Zulperi, and Ms. Saleema Matusin, thank you guys for providing priceless contributions in making this project running more smoothly. An extra thanks to Prof. Dr. Mohd Zamri Saad, Mrs. Ida Muryany Md Yasin, Dr. Dzarifah Zulperi and Mrs. Aslizah Mohd Aris for your contributions along the way.

To all the staffs in Department of Aquaculture UPM, especially Mrs. Nur Shafika Maulad Abd Jalil, thank you for your assistance throughout my study here. Not to forget Mr. Jamil Samad from Faculty of Veterinary UPM, thank you for your help for letting me use your laboratory and materials.

I would like to extend my thanks to the people from National Fish Health Research Centre (NaFish) Penang and to Dr. Siti Zahrah Abdullah and Dr. Nur Nazifah Mansor for your input to improve my studies.

I would like to thanks to my greatest blessing in life, my wonderful parents Mr. Mursidi Haji Umi and Mrs. Raja Napisah Raja Embong as well as my siblings for their unequivocal personal support throughout, as always, for which my mere expression of thanks likewise does not suffice.

Last but not least, an appreciation to Fundamental Research Grant Scheme (FRGS Fund) for funding my research studies under grant number 5524625.

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment 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
µg	microgram
µl	microliter
µM	micromolar
ASW	Artificial sterile water
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
Blastp	protein-protein BLAST
bp	base pair
BSA	bovine serum albumin
CFU	colony forming units
DAB	3,3'-Diaminobenzidine
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E. fuscoguttatus	*Epinephelus fuscoguttatus*
E. lanceolatus	*Epinephelus lanceolatus*
FKC	formalin-killed cells
H_2SO_4	sulphuric acid
Ig	immunoglobulin
IHN	infectious haematopoietic necrosis
IP	intraperitoneal
IPTG	isopropyl β-D-1-thiogalactopyranoside
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
NaCl	sodium chloride
$Na_2S_2O_3$	sodium thiosulfate
NaOCl	Sodium hypochlorite
ng	nanogram
nm	nanometer
OD	Optical density
OMP	Outer membrane protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
P. damselae	*Photobacterium damselae*
pmol	picomole
RE	restriction enzyme
rpm	revolutions per minute
RPS	relative percentage survival
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
V. alginolyticus	*Vibrio alginolyticus*
V. harveyi	*Vibrio harveyi*
V. parahemolyticus	*Vibrio parahemolyticus*
v/v	volume per volume
w/v	weight per volume
x g	multiples of gravitational acceleration

CHAPTER 1

INTRODUCTION

For many decades, aquaculture industry has blooming boundlessly throughout the world primarily in Asia. Marine grouper culture which belong to family Serranidae, subfamily Epinephelinae has recognized to be among the top species in aquaculture production in most Asian countries (Harikrishnan et al., 2011). The high interest towards grouper aquacultures has encouraged Malaysian government to increase hatcheries number and has provided infrastructures for aquacultures development (Pomeroy, 2002).

Nevertheless, intensive farming of groupers cultivated in net cages in the brackish water plus in limited area have made them to become very susceptible to disease infection, especially by bacterial *Vibrio* species causing strenuous mortality that totally disrupting both economic and social development as well as food safety worldwide (Chatterjee and Haldar, 2012; Li et al., 2015). Vibriosis, caused by infection of *Vibrio* sp. has been extensively reported as a major threatening and most catastrophic diseases to grouper culture involving grouper fry, fingerlings, juveniles, adults and brood stocks (Sarjito et al., 2009; Ilmiah et al., 2013; Novriadi and Haw, 2014). *Vibrio* species like *Vibrio harveyi, V. alginolyticus, V. parahemolyticus, V. vulnificus, V. anguillarum,* and *Photobacterium damselae* have long been recognized as the main pathogens in *Epinephelus* sp. and other marine species causing severe gastroenteritis syndrome and haemorrhagic septicaemia (Ningqiu et al., 2008; Li et al., 2010; Hu et al., 2012; Huang et al., 2012; Peng et al., 2016; Pang et al., 2016).

A case study in Malaysia reported only 30-40% cage-cultured groupers could survive due to various diseases including vibriosis (Liao and Leano, 2008). Besides that, Abdullah et al., (2017) reported cage-cultured groupers and snappers in fish farm in Langkawi Island in Kedah, Malaysia were majorly infected with vibriosis along with viruses for up to 77.78% and 76.05%, respectively. Abdullah et al. (2015) also reported three main *Vibrio* sp. (*V. vulnificus, V. alginolyticus,* and *P. damselae*) were isolated from diseased tiger grouper after vibiosis outbreak in deep sea cage in Langkawi with prevalence rates from 50% to 90%. Another case of vibriosis outbreaks reported in Malaysia discovered *V. harveyi* as the main culprit followed with *V. parahemolyticus, V. alginolyticus, V. ponticus, V. fluvialis* and many other *Vibrio* sp. infecting Asian seabass (*Lates calcarifer*), orange-spotted grouper *(Epinephelus coioides*), red snapper (*Lutjanus* sp.), brown marble grouper (*Epinephelus fuscoguttatus*), and hybrid grouper (*E. fuscoguttatus x E. lanceolatus*) (Albert and Ransangan, 2013). This disease also commonly reported infecting grouper (*Epinephelus* sp.) (Huang et al., 2012), European sea bass (*Dicentrarchus labrax*) (Bellos et al., 2015), red sea bream (*Pagrus major*) (El-Galil and Mohamed, 2012), and catfish (*Siluriformes*) (Geng et al., 2014). Despite of this problem, government of Malaysia has boost the research and development (R&D) on the frequently isolated *Vibrio* sp. to further study on their virulence properties and

immunogenic potential for preventative measures against infection in mariculture species including grouper to maintain healthy broodstock and disease-free seed and fingerling (Pomeroy, 2002).

Preventions of vibriosis are mainly dependent on antibiotics which are still effective, however the usage are strictly not recommended since the extensive use of the antimicrobial drugs have led to the emergence of drug resistance strains and pose risk to horizontally transfer the resistance gene to fish and human's pathogen (Ningqiu et al., 2008, Maiti et al., 2012; Li et al., 2014). Moreover, application of antibiotics also caused problems of food safety and environmental pollution as well (Evelyn, 1996). Bacterial resistance to common antibiotics has reached frightening levels in multiple countries around the world making the antibiotics treatment to common diseases is no longer effective (WHO, 2014). Many studies have reported some *Vibrio* sp. such as *V. harveyi, V. vulnificus* and *V. parahemolyticus* exhibit multiple antibiotic resistance in aquaculture productions (Elmahdi et al., 2016). Thus, new strategies to avoid the massive misuse of antibiotics to control infection in aquaculture are urgently needed. Vaccination is an alternative prophylactic measure with a safer strategy to control diseases by increasing antibody titre, boost immunological memory, and enhance rate of survival of infected fish (Defoirdt et al., 2007). Fish vaccination is now approved as a standard protocol in modern aquaculture (Huang et al., 2012).

Although vibriosis has been administered by vaccination, however, the existence of different strains and serotypes of *Vibrio* sp. have contributed major challenges in vaccine development. Moreover, the existence of antigenic diversity of *Vibrio* strains and their serotypes have made the vaccines unable to elicit protection against multiple vibrios resulting in slow progress of vaccine development against vibriosis (Li et al., 2014). Furthermore, according to Li et al. (2010), there are no commercial vibrio vaccines that are versatile available at the moment. Until this time, studies on vaccines that could provide cross-protections have been widely reported, however most investigations were mainly focused on cross protection against homologous strains or serotype (Mutharia et al., 1993; Sabri et al., 2000; Sun et al., 2012). The studies on cross protective ability against heterogenous serotypes and species on *Vibrio* sp. are still scarce. Therefore, more efforts are needed to develop a powerful and versatile subunit vaccine that could provide cross protection against multiple *Vibrio* strains and serotypes to combat vibriosis in fish.

Outer membrane protein (OMP), a unique composition of Gram-negative bacteria is ideally located on the cell surface of bacteria and highly immunogenic. These characteristics have made this protein to be extensively used in all range of studies of antimicrobial drugs and vaccination. OMP has been revealed to provide homologous and heterologous protection and could act as polyvalent immunogens against diverse Gram-negative bacteria (Xu et al., 2005; Li et al., 2009). For this reason, more researches have been focused on determination of immunogenic characteristics of OMPs. Moreover, interests have been growing to develop a recombinant vaccine expressing immunogenic outer membrane protein (OMPs) that elicit homologous

and cross antigenicity since this type of vaccine has been proven to induce protective efficacy against multiple bacterial species and serotypes. Recombinant cells allow production of protein in large amount which further increase antibody reaction, thus intensify protection. Moreover, in recent years, the development of versatile recombinant vaccines were concentrated on conserved OMPs antigen that exist in *Vibrio* pathogens and their serotypes since such protein could provoke highly effective immune response and able to defend against different pathogens (Li et al., 2010; Lun et al., 2014; Zha et al., 2016). Li et al. (2010) had reported recombinant ompK was a versatile vaccine candidate by providing cross protection to heterogeneous virulent *V. harveyi, V. alginolyticus,* and *V. parahemolyticus* in orange spotted grouper (*Epinephelus coioides*). Another versatile vibriosis vaccine was reported by Lun et al. (2014) where immunization of zebrafish (*Danio reiro*) using recombinant LamB, a family of OMPs that was conserved antigen among various *Vibrio* sp. showed significant protection against vibriosis which was found in *V. parahemolyticus* RIMD2210633. Therefore, this study was aimed to develop a versatile recombinant vaccine against vibriosis through the search of the most antigenic protein from different strains of *Vibrio* sp. isolated from diseased marine fish by analysing the ability of their OMPs in eliciting homologous and cross antigenicity and to develop versatile recombinant *Vibrio* vaccine as well as to assess the safety of the developed vaccine in gnotobiotic *Artemia* sp. before it can be applied into final host (fish). The objectives of this study were:

1. To characterize the outer membrane protein (OMPs) profiles of *Vibrio harveyi* strain VH1, *V. parahemolyticus* strain VPK1*, V. alginolyticus* strain VA2*,* and *Photobacterium damselae* strain PDS1 isolated from diseased grouper (*Epinephelus fuscoguttatus*) and to determine homologous and cross antigenicity of the OMPs against homologous and heterologous antisera.
2. To sequence the most antigenic protein by N-terminal amino acid sequencing and to construct a recombinant cell vaccine containing the antigenic outer membrane protein vhDnaJ gene of *V. harveyi* strain VH1 in prokaryotic expression system for production of potential recombinant vaccine.
3. To evaluate the safety of formalin-killed recombinant vhDnaJ vaccine based on survivability of gnotobiotic *Artemia* model.

Hypothesis:

H_0: Outer membrane protein of *V. harveyi* strain VH1 could not elicit homologous and heterologous antigenicity against polyclonal rabbit antisera of different *Vibrio* strains and formalin-killed recombinant vhDnaJ cells vaccine could not increase the survivability of gnotobiotic *Artemia* nauplii.

H_A: Outer membrane protein of *V. harveyi* strain VH1 could elicit homologous and heterologous antigenicity against polyclonal rabbit antisera of different *Vibrio* strains and formalin-killed recombinant vhDnaJ cells vaccine could increase the survivability of gnotobiotic *Artemia* nauplii.

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