UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF Vibrio harveyi PROTEASE DELETION MUTANT AS LIVE ATTENUATED VACCINE AGAINST VIBRIOSIS IN Epinephelus fuscoguttatus (FORSSKAL, 1775)

ASLIZAH BINTI MOHD ARIS

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By

ASLIZAH BINTI MOHD ARIS

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February 2018

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

Groupers aquaculture industries have faced a high risk of bacterial diseases such as Vibriosis. Various types of vaccine have been studied including attenuated vaccine. However, the potential of live attenuated vaccine is still understudied. This study was conducted to develop a live attenuated protease derivative from pathogenic *Vibrio harveyi* strain *Vh1* as a live vaccine candidate using site-directed mutagenesis (SDM) and allelic exchange replacement techniques. In SDM, an overlapping PCR was performed to generate a deletion in a specific amino acid residue that represents a catalytic site of the protease gene. Seven mutant variants consisted of single deletion catalytic sites (DelD, DelH, and DelS), double deletion catalytic sites (DelDDelH, DelDDelS and DelHDelS) and triple mutation catalytic sites (DelDDelHDelS) were successfully developed. Evaluation of protease activity showed DelDDelHDelS recombinant recorded highest relative protease activity. A mutagenesis plasmid was further constructed using pRE112 as suicide plasmid, prior to the transformation into *E. coli* SM10*λpir*. The resulting mutagenesis plasmid (pRE112-DelDDelHDelS) was integrated into the chromosome of *V. harveyi* strain *Vh1* by employing a combined mixed broth-membrane filter technique for conjugation and the allelic replacement technique. Subsequently, the attenuated strain was designated as *V. harveyi* attenuated strain *MVh-vhs* and was further used as live vaccine candidate throughout this study. *In vivo* study was performed in *Epinephelus fuscoguttatus* to evaluate the safety and pathogenicity level of the attenuated strain. Fish challenged with the parental strain showed obvious clinical signs of Vibriosis such as hemorrhages on the ventral abdominal pelvic area, external lesion and white depigmentation in skin. The median lethal dosage (LD_{50}) in fish challenged with the parental strain was found at 10^6 CFU/fish. Histopathological analysis showed the presence of immunological response activity in both fish treatment. In contrast, 100% survival with no indication of vibriosis was detected in fish challenged with the attenuated strain. Vaccination with the attenuated strain in juvenile *E. fuscoguttatus* was performed using a single dose IP administration at 10^5 CFU/fish. The bacterial challenge was done after four weeks post vaccination with the pathogenic *V. harveyi* strain *Vh1* at dosage concentration of 10^8 CFU/fish following IP administration. Fish vaccinated with

the attenuated strain showed 52% relative percentage survival (RPS). *De novo* transcriptomic analysis revealed that the vaccinated *E. fuscoguttatus* with the attenuated strain conferred both innate and adaptive immunity. The innate adaptive involved in regulation of the autophagosome pathway and coagulation and complement cascade pathway. Adaptive immunity relies on the regulation of antigen processing and presentation pathway. In addition, *V. harveyi* attenuated strain *MVh-vhs* possessed an unmarked gene deletion and the attenuation properties were found stable after 15 *in vitro* passages. As a conclusion, the *V. harveyi* attenuated strain *MVh-vhs* has significant potential to be applied as a live vaccine candidate against vibriosis in *E. fuscogutattus*.

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

PEMBANGUNAN MUTAN *Vibrio harveyi* LESAPAN PROTEASE SEBAGAI VAKSIN TERATENUASI HIDUP MELAWAN VIBRIOSIS DALAM *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

Oleh

ASLIZAH BINTI MOHD ARIS

Februari 2018

Pengerusi: Prof. Madya Ina Salwany Md Yasin, PhD
Fakulti: Institut Biosains

Industri akuakultur kerapu telah menghadapi risiko tinggi penyakit berasaskan bakteria seperti Vibriosis. Pelbagai jenis vaksin telah dikaji termasuk vaksin teratenuasi. Walau bagaimanapun, potensi vaksin teratenuasi masih belum dikaji secara menyeluruh. Kajian ini dijalankan untuk membangunkan terbitan protease yang teratenuasi dari patogen *V. harveyi* sten *Vh1* sebagai calon vaksin hidup dengan menggunakan kaedah mutagenesis tapak terarah (SDM) dan teknik penggantian alel. Dalam SDM, PCR bertindih dilakukan untuk menjana lesapan dalam sisa asid amino tertentu yang mewakili tapak pemangkin bagi gen protease. Tujuh varian mutan terdiri daripada lesapan pemangkin tapak tunggal (DelD, DelH dan DelS), lesapan pemangkin dua tapak (DelDDelH, DelDDelS dan DelHDelS) dan lesapan kesemua tapak pemangkin (DelDDelHDelS) telah berjaya dibangunkan. Aktiviti protease menunjukkan rekombinan DelDDelHDelS mencatatkan aktiviti relatif protease yang tertinggi. Selanjutnya, plasmid mutagenesis dibina menggunakan pRE112 sebagai plasmid perantara, sebelum transformasi ke dalam *E. coli* SM10λ*pir*. Plasmid mutagenesis (pRE112-DelDDelHDelS) telah diintegrasi ke dalam kromosom *V. harveyi* strain *Vh1* dengan menggunakan kaedah gabungan media-membran penapis melalui teknik konjugasi dan penggantian alel. Strain attenuasi telah direka bentuk sebagai *V. harveyi* teratenuasi strain *MVh-vhs* dan seterusnya digunakan sebagai calon vaksin hidup sepanjang kajian ini. Kajian *in vivo* telah dilakukan terhadap *E. fuscoguttatus* untuk menilai tahap keselamatan dan kepatogenan bagi strain teratenuasi. Ikan yang diuji dengan strain pathogen asal menunjukkan tanda-tanda klinikal yang jelas untuk Vibriosis seperti pendarahan di kawasan abdomen pelvis, luka luaran dan depigmentasi putih pada kulit. Dos maut median (LD_{50}) ikan yang diuji dengan strain asal pathogen adalah 10^6 CFU/ikan. Analisis histopatologi menunjukkan kehadiran bagi aktiviti tindak balas imunologi dalam kedua-dua rawatan ikan. Sebaliknya, 100% kemandirian ditunjukkan dalam ikan yang diuji dengan strain teratenuasi serta ianya tidak menunjukkan kesan vibriosis. Vaksinasi dengan strain teratenuasi dalam juvana *E. fuscoguttatus* dilakukan dengan menggunakan suntikan IP dos tunggal pada 10^5

CFU/ikan. Cabaran bakteria dilakukan selepas empat minggu suntikan vaksinasi dengan *V. harveyi* strain *Vh1* pada kepekatan dos 10^8 CFU/fish dengan menggunakan suntikan IP. Ikan yang disuntik dengan strain teratenuasi menunjukkan 52% peratusan relatif masih hidup (RPS). Analisis transkriptomik *de novo* menunjukkan vaksinasi *E. fuscoguttatus* dengan strain teratenuasi menghasilkan immuniti semulajadi dan adaptif. Hasil dapatan kajian menunjukkan pengawalaturan immuniti semula jadi melibatkan laluan autophagosome serta laluan pembekuan dan lata komplemen. Pengawalaturan dalam kekebalan adaptif bergantung kepada laluan pemprosesan antigen dan persembahan. Di samping itu, *V. harveyi* teratenuasi strain *MVh-vhs* mempunyai gen penglesapan tidak bertanda yang stabil dari segi atenuasi selepas 15 siri inokulasi secara *in vitro*. Kesimpulannya, *V. harveyi* teratenuasi strain *MVh-vhs* mempunyai potensi yang besar untuk digunakan sebagai calon vaksin sebenar melawan vibriosis pada *E. fuscogutattus*.

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I certify that a Thesis Examination Committee has met on 6 February 2018 to conduct the final examination of Aslizah binti Mohd Aris on her thesis entitled "Development of *Vibrio harveyi* Protease Deletion Mutant as Live Attenuated Vaccine Against Vibriosis in *Epinephelus fuscoguttatus* (Forsskal, 1775)" 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Annie Christianus, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Zunita binti Zakaria, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Abdul Rahman bin Omar, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Internal Examiner)

K. Pani Prasad, PhD
Professor
Central Institute of Fisheries Education
India
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 March 2018

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:

Ina Salwany Binti Md Yasin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Mohd Zamri Bin Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Hassan Bin Hj Mohd Daud, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
ROBIAH BINTI YUNUS, PhD
Professor and Dean
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Universiti Putra Malaysia

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This is to confirm that:

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Signature: _____

Name of Chairman of Supervisory Committee: Assoc Prof Dr. Ina Salwany Binti Md Yasin

Signature: _____

Name of Member of Supervisory Committee: Prof Dr. Mohd Zamri Bin Saad

Signature: _____

Name of Member of Supervisory Committee: Assoc. Prof Dr. Hassan Bin Hj Mohd Daud

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λ	lambda
∞	infinite
%	percentage
µl	microlitre
µM	micromolar
®	Registered
™	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*
E. fuscoguttatus	*Epinephelus fuscoguttatus*
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
LD_{50}	Median lethal dosage
LPS	Lipopolysaccharide
M	molar

MCS	multiple cloning site
mg	milligram
MgCl2	magnesium chloride
mM	millimolar
MS-222	tricaine methanesulfonate solution
NaCl	sodium chloride
NGS	Next Generation Sequencing
OE-PCR	Overlapping Extension PCR
OMPs	Outer membrane proteins
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate uffered saline
PCR	Polymerase chain reaction
RE	restriction enzyme
RNA	ribonucleic acid
RPM	revolutions per minute
RPS	relative percentage survival
rRNA	ribosomal ribonucleic acid
SDM	Site directed mutagenesis
Ser (S)	Serine
sp	species
TCBS	thiosulfate-citrate-bile salts-sucrose
TSA	tryptic soy agar
TSB	tryptic soy broth
V	voltage
V. harveyi	*Vibrio harveyi*
v/v	volume per volume
wpi	Week post infection
wpv	Week post vaccination
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Aquaculture industry is an important sector in agriculture. It rapidly gains global interest as it plays important roles in food security. It also contributes toward economic development and social stability worldwide (IFPRI, 2015; WorldFish, 2011; World Bank, 2007). In order to cater for the high demand, intensification and commercialization become the main focus. However, outbreaks of fish diseases appear to increase due to these practices (Bondad-Reantaso *et al.*, 2005). Vibriosis is a disease of importance among aquatic animals (Wei and Wee, 2014). In Malaysia, the first documented disease outbreak was in 1989 that involved cage-cultured grouper, snapper and seabass (Shariff, 1995). These outbreaks led a total loss of USD7.4M loss, between years 1990 to 1995 in aquaculture facilities in Malaysia (Shariff, 1995). In addition, early mortality syndrome (EMS) was reported in 2010 in Malaysia that was responsible for the collapse of shrimp farming. *Vibrio parahaemolyticus* is believed to be the cause of EMS in shrimp farming. EMS was reported to be occured in Malaysia, as well as in China, Vietnam and Thailand (Wei and Wee, 2014). A recent report revealed that *V. parahaemolyticus*, *V. alginolyticus* followed by *Vibrio harveyi*, *Vibrio owensii* and *Vibrio campbelli* were the most common species infecting aquatic animals (Nor-Amalina *et al*., 2017). The symptom of vibriosis includes lethargy, septicemia, necrotizing enteritis, vasculitis, inappetite, haemorrhages, internal organ liquefaction, skin lesion, eye blindness (Austin and Austin, 2007).

The common practice in treating a fish bacterial disease involves the application of antibiotics. They are effective to cure bacterial diseases (Grisez and Tan, 2005). Unfortunately, serious complications developed through the spread of antibiotic resistance bacteria and the accumulation of antibiotic residues in the environment and food products, creating serious problems to human and environmental health (Defoirdt *et al*., 2011; Plant and LaPatra, 2011; Defoirdt *et al*., 2007; Grisez and Tan, 2005; Vadstein, 1997). Owing to the issues, the application of antibiotics is no longer encouraged. Therefore, vaccination is now considered as the best approach to prevent a specific disease outbreak. A number of vaccines have been developed to combat Vibriosis in aquatic organisms. For example, Vibrogen 2, AquaVac®, Lipogen Forte and Forte VI which is based on the killed attenuated vaccine. The majority of commercialized vaccines using killed attenuated *Vibrio anguillarum*, formulated with only O1, or a mixture of serotypes O1 and O2a. Some of developed vaccines had applied different types of polyvalent oil-adjuvanted vaccines, including a different combination of *Vibrio anguillarum* with other pathogens, such as *Vibrio ordalii*, *Vibrio salmonicida* and *Aeromonas salmonicida*. These killed vaccines are commonly used for salmonids by the intraperitoneal route. However, there is lack information and research documentation to study its protective efficacy in different hosts other than salmonids and seabass.

Alternatively, a live attenuated vaccine can be used to prevent fish diseases. Basically, live attenuated viral vaccines can be developed by repeated *in vitro* passages until the virulent strain becomes avirulent (Hastein *et al*, 2005; Sommerset *et al*, 2005; Li *et al*., 2015a; Griffiths *et al*., 1998). Another possible strategy using a well-defined deletion, insertion or addition on targeted genes that control the virulence properties of the virulent strain can also attenuate the strain (Choi *et al*., 2016; Altinok *et al*., 2015; Chu *et al.,* 2015; Gao *et al*., 2014; Loessner *et al*, 2012; Xiao *et al*., 2011; Shoemaker and Klesius, 2009). This live attenuated vaccine contains the necessary antigenic determinants to induce strong and prolonged protective immunity and is able to evoke immune response at lower dosage concentration (Li *et al*., 2015b; Wang *et al*., 2014b; Ma *et al*., 2008; Ebensen *et al.,* 2004). The most research studied on the construction of live attenuated vaccine only dealing with attenuation that led to decrease the survivability and persistence of the pathogenic strain in the hosts. For example, Ma *et al*. (2008) successfully constructed a live attenuated strain MVAV6204 derivative *V. anguillarum* strain MVM425. The construction of strain MVAV6204 contains a deletion of aromatic amino acid, the folic acid synthesis gene and siderophore anguibactin. Thus, the synthesis of aromatic amino acid, folic acid and/or the virulence factor, and the siderophore anguibactin is repressed. Therefore, the ability to synthesize important amino acids and to scavenge for the iron depletion from hosts is reduced or even completely eliminated.

To our knowledge, there is no report has been claimed to construct the live attenuated strain which employed the attenuation strategies on the virulence-associated proteolytic properties. As described by Zhang *et al*. (2008), the serine endoprotease activity is responsible for the proteolytic activity of *V. harveyi*. Mutation of the serine endoprotease motif might result in a loss of proteolytic or mucinase activity. As a result, the cytotoxicity or damage effect on target cells can also be halted. In order to fill in the research gap, the main purpose of this study is to develop a live attenuated strain, a derivative *V. harveyi* from a local strain of pathogenic *V. harveyi*. Besides, the *V. harveyi* is considered as an opportunistic pathogen (Cheng *et al*., 2010; Kraxberger-Beatty *et al*., 1990) and proved to have a cross immunoprotection against other types of *Vibrio* species (Fathin-Amirah *et al*., 2015; Hu *et al*., 2012). These features definitely give an advantage to *V. harveyi* to be developed as a live vaccine candidate. The resulted attenuated strain is targeted as a live vaccine candidate against vibriosis in *Epinephelus fuscoguttatus*.

There are four objectives of this study, which are stated as below:

1) To identify and characterize the virulence-associated serine protease gene from *V. harveyi* (*Vh1* strain) that was previously isolated from diseased *E. fuscoguttatus*.
2) To construct the attenuated protease derivative strain of *V. harveyi* by employing the site directed mutagenesis, conjugation and allelic exchange replacement.
3) To determine the pathogenicity and safety levels of the attenuated strain as compared to the field *Vh1* strain in *E. fuscoguttatus* and the genetic stability of the attenuated strain.
4) To evaluate the protective ability of the newly develop attenuated strain as live vaccine using intraperitoneal vaccination regimen in *E. fuscoguttatus*.

The hypotheses of this study are as below:

Hypothesis 1:

H_0: The serine protease *vhs* gene is not associated with virulence properties of *V. harveyi* strain *Vh1*.

H_1: The serine protease *vhs* gene is associated with virulence properties of *V. harveyi* strain *Vh1*.

Hypothesis 2:

H_0: Construction of *V. harveyi* attenuated strain is not possible to be achieved by site directed mutagenesis, conjugation and allelic exchange.

H_1: Construction of *V. harveyi* attenuated strain is possible to be achieved by site directed mutagenesis, conjugation and allelic exchange.

Hypothesis 3:

H_0: The constructed *V. harveyi* attenuated strain possessed a similar pathogenicity effects which demonstrated by the *Vh1* strain in *E. fuscoguttatus*.

H_1: The constructed *V. harveyi* attenuated strain not possessed any pathogenicity effects which demonstrated by the *Vh1* strain in *E. fuscoguttatus*.

Hypothesis 4:

H_0: Vaccination of juvenile *E. fuscoguttatus* with newly developed *V. harveyi* attenuated strain could not induce immune response and results in high mortalities after challenged with pathogenic *V. harveyi* strain *Vh1*.

H_1: Vaccination of juvenile *E. fuscoguttatus* with newly developed *V. harveyi* attenuated strain could induced good immune response and results in mortalities decrement after challenged with pathogenic *V. harveyi* strain *Vh1*.

In general, the specific features of live *V. harveyi* attenuated strain *MVh-vhs* is claimed to be:

1) Novel live vaccine candidate with the unmarked gene deletion.
2) Safe and able to generate immunoprotective ability to *E. fuscoguttatus.*
3) Potentially to be commercialized in future.

It is hoped that this study might open a new direction to a possible alternative way to sustain the aquaculture sectors.

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