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SAFETY AND EFFICACY OF LIVE ATTENUATED SERINE PROTEASE *Vibrio harveyi* (MVh_vhs) VACCINE AGAINST VIBRIOSIS IN ASIAN SEABASS [*Lates calcarifer* (Bloch, 1790)]

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment 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 fulfilment of the requirement for the degree of Master of Science

SAFETY AND EFFICACY OF LIVE ATTENUATED SERINE PROTEASE Vibrio harveyi (MVh_vhs) VACCINE AGAINST VIBRIOSIS IN ASIAN SEABASS [Lates calcarifer (Bloch, 1790)]

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

Vibrio species is bacteria that cause natural outbreak of Vibriosis in marine aquaculture and cause the serious economic loss indirectly particularly in the early development of farmed fishes' species. Live attenuated serine protease *V. harveyi* (MVh_vhs) was developed previously as vaccine candidate for farmed fishes against *V. harveyi*. In the first study, the gnotobiotic *Artemia franciscana* was used to evaluate the safety and efficacy of the newly developed live attenuated vaccine. The high concentration of MVh_vhs at concentration of 10^9 CFU/mL is safe and had improved the *Artemia* larvae survival. It is also indicated that 10^9 CFU/mL of MVh-vhs with 24 hours incubated *Artemia* larvae contributed higher survival at 36 h against multiple *Vibrio* challenge. Thus, we concluded that the incubation time affected bacterial concentration uptake by *Artemia* larvae and affect the effectiveness of *Artemia* bioencapsulation for targeted hosts. Skin abrasion often occurs in farmed fish following labour handling, injury by farm facilities, cannibalism and ectoparasites. In the second study, the Asian seabass (*Lates calcarifer)* fingerling was chosen as model to describe the effect of skin abrasion on the infectivity of *V. harveyi* on Asian seabass fingerling and compared to bacterial load and fish survival following immersion challenge before vaccination. No mortality was observed in control and non-skin abraded. However, fish of skin abraded group that were exposed to 10^7 and 10^8 CFU/mL of live *V. harveyi* showed 100% mortality by 96 h and 120 h, respectively. Infected fish of skin abraded group were classified into three stages: mild, moderate and severe infection. High bacterial loads were recorded for the severely infected dead fish, compared to low loads in moderately infected fish. Significantly higher bacterial load was recorded in the intestine, liver and gills of the severely infected dead fish.

The findings revealed that skin injury causes Asian seabass fingerling to be more susceptible to *V. harveyi* infection. The study showed the lethal dose (LD_{50}) of live *V. harveyi* on skin abraded Asian seabass fingerling for *Vibrio* challenged was determined at $10^{6.83} \approx 10^{7}$ CFU/mL. In the third study, the bath vaccination was chosen to be applied with the formalin killed *V. harveyi* and MVh_vhs for immunization of the Asian seabss fingerling model to investigate the immune related gene expressions. The high expression of chemokine ligand 4 (CCL4) and major histocompatibility complex I (MHC I) in the fish skin indicated the MVh_vhs induced mucosal innate and adaptive immunity while high expression of CCL4 and MHC I in the fish liver indicated the MVh_vhs, induced internal innate and adaptive immunity. After immersion challenge of wildtype *V. harveyi* with skin-abrasion on the vaccinated Asian seabass fingerling model, MVh_vhs contributed high survival indicated more than 50% survival for the fingerlings compared with formalin-killed *V. harveyi* and control at 120 h. The results indicated that the MVh_vhs can be applied through low cost and feasibly of bath vaccination to protect early protection of marine farmed fishes from natural occurrence of vibriosis.

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

KESELAMATAN DAN KEBERKESANAN VAKSIN *Vibrio harveyi* PROTEASE SERIN TERATENUASI HIDUP (MVh_vhs) TERHADAP VIBRIOSIS DALAM IKAN SIAKAP [*Lates calcarifer* (Bloch, 1790)]

Oleh

CHIN YONG KIT

Jun 2019

Pengerusi : Prof. Madya. Ina Salwany Md Yasin, PhD
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Spesies *Vibrio* ialah bakteria yang menyebabkan wabak semula jadi vibriosis dalam akuakultur marin dan menyebabkan kerugian ekonomi yang secara tidak langsung, terutamanya ikan ternakan pada tumbesaran awal. *Vibrio harveyi* protease serin teratenuasi hidup (MVh_vhs) telah dihasilkan sebelum ini sebagai calon vaksin untuk ikan ternakan terhadap *V. harveyi*. Dalam eksperimen pertama, *Artemia franciscana* gnotobiotik digunakan untuk menilai keselamatan dan keberkesanan hasil baru vaksin teratenuasi hidup ini. Pengujian keselamatan bakteria menunjukkan MVh_vhs yang berkepekatan tinggi pada 10^9 CFU/mL adalah selamat dan memperbaik kemandirian larva *Artemia*. Pengujian keberkesanan bakteria juga menunjukkan larva *Artemia* yang diinkubasi 24 jam dengan MVh_vhs yang berkepekatan tinggi pada 10^9 CFU/mL menunjukkan kemandirian tinggi pada kadar 50% apabila didedahkan cabaran pelbagai jenis *Vibrio* dalam masa 36 jam. Kesimpulannya, masa inkubasi memberi kesan kepada larva *Artemia* dalam pengambilan bakteria dan Keberkesanan bioenkapsulasi *Artemia* untuk haiwan akuatik. Abrasi kulit sentiasa berlaku bagi ikan ternakan melalui pengendalian pekerja, kecederaan akibat fasiliti ladang, kanibalisme dan ektoparasit. Dalam eksperimen kedua, anak ikan siakap (*Lates calcarifer)* dipilih sebagai model untuk menerangkan kesan abrasi kulit terhadap kejangkitan *V. harveyi* bagi anak ikan siakap melalui perbandingan beban bakteria dengan kemandirian ikan tersebut selepas cabaran rendaman. Tiada kematian direkodkan pada kumpulan contoh kawalan dan kumpulan tanpa abrasi kulit selepas cabaran rendaman. Walau bagaimanapun kumpulan ikan abrasi kulit yang didedah kepada *V. harveyi* berkepekatan 10^7 and 10^8 CFU/mL menunjukkan 100 % kematian dalam masa 96 jam dan 120 jam. Ikan yang telah dijangkit dalam kumpulan abrasi kulit dibahagi kepada tiga peringkat, iaitu: jangkitan ringan, jangkitan sederhana dan jangkitan teruk. Beban bakteria tertinggi direkod daripada ikan mati akibat jangkitan teruk berbanding dengan beban bakteria jangkitan rendah dan jangkitan sederhana. Beban bakteri yang tertinggi direkod bagi usus, hati dan

insang ikan mati untuk jangkitan teruk. Penemuan ini telah menunjukkan abrasi kulit menyebabkan anak siakap lebih mudah terdedah pada jangkitan *V. harveyi*. Eksperimen ini menunjukkan dos mortaliti (LD50) *V. harveyi* bagi anak ikan siakap secara abrasi kulit untuk cabaran rendaman *V. harveyi* adalah $10^{6.83} \approx 10^7$ CFU/mL. Dalam eksperimen ketiga, vaksinasi rendaman durasi singkat telah dipilih untuk penggunaan *V. harveyi* dibunuh secara fomalin dan MVh_vhs terhadap keimunan model anak ikan siakap supaya menyiasat ekspresi gen imun. Ekspresi ligan kemokin 4 (CCL4) dan kompleks kehistoserasian utama 1 (MHC I) yang tinggi terdapat dalam kulit ikan menunjukkan MVh_vhs mengaruh keimunan inat dan keimunan pengesuaian mukosal manakala ekpresi CCL4 dan MHC I yang tinggi terdapat dalam hati ikan menunjukkan MVh_vhs mengaruhkan keimunan inat dan pengesuaian dalam. Selepas cabaran rendaman *V. harveyi* bagi model ikan divaksin yang telah diabrasi kulit, MVh_vhs menyumbangkan kemandirian tinggi yang lebih 50% berbanding dengan *V. harveyi* dibunuh secara fomalin dan contoh kawalan dalam masa 120 jam. Hasilan ini menunjukkan MVh_vhs dapat diaplikasi dengan vaksinasi rendam durasi singkat yang berkos murah dan senang supaya dapat melindungi ikan ternakan marin daripada kejadian vibriosis semula jadi.

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I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Chin Yong Kit on his thesis entitled “Safety and Efficacy of Live Attenuated Serine Protease Vibrio harveyi (MVh_vhs) Vaccine Against Vibriosis in Asian Seabass [Lates calcarifer (Bloch, 1790)]” 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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LIST OF ABBREVIATIONS

%	Percentage
µl	Microlitre
Mm	Micromolar
®	Registered
™	Trademark
ºc	Degree Celsius
Asp (D)	Aspartate
BLAST	Basic Local Alignment Search Tool
$CaCl_2$	Calcium chloride
CFU	Colony forming units
DNA	Deoxyribonucleic acid
ECPs	Extracellular products
H&E	haematoxylin and eosin
His (H)	Histidine
LD_{50}	Median lethal dosage
LPS	Lipopolysaccharide
M	Molar
Mg	Milligram
MgCl2	magnesium chloride
Mm	Millimolar
MS-222	Tricaine methanesulfonate solution
NaCl	Sodium chloride
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction

Ser (S)	Serine
TCBS	thiosulfate-citrate-bile salts-sucrose
TSA	tryptic soy agar
TSB	tryptic soy broth
CCL4	Chemokine ligand 4
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
ß-actin	Beta-actin

CHAPTER 1

INTRODUCTION

1.1 Background of study

Global aquaculture production increase sharply annually since year 1990 to achieve 80.03 million tonnes (USD 243.5 bilion) which is 47 % of total global fish production that achieved 171 million tonnes in year 2016 compared with no increment for wild fish capture production in these last years (FAO, 2018). Increment of global marine aquaculture production from 23.2 million metric tonnes since year 2011 to 28.7 million metric tonnes at year 2016 (FAO, 2018). Based on the statistic, aquaculture is the fastest food production sector for decades and is predicted to exceed wild fish capture production influenced by increasing of human population and economic development in the world (Liu et al., 2018). In Malaysia, freshwater aquaculture sector contributed to 163757 metric tonnes valued at RM 992 million while marine aquaculture sector contributed 139129.5 metric tonnes valued at RM 1566.8 million in year 2012 (SEAFDEC, 2015).

Asian seabass (*Lates calcarifer*), snappers (*Lutjanus* sp.) and groupers (*Epinephelus* sp.) are the major common marine finfish species found in Malaysia marine aquaculture (Halwart et al., 2007). In fact, Malaysia is one of important Asian seabass producing countries that contributed about 5000 metric tonnes from year 2008 increased to 25000 metric tonnes of Asian seabass, which 20 % of total global Asian seabass production at year 2010 (Tveteras, 2016). These facts showed that the Asian seabass is important marine fish species that commonly cultured in Malaysian marine aquaculture.

However, farmed aquatic animals are susceptible to infectious disease and transmission of pathogens due to environmental stress that caused by high density of farmed aquatic animals, clean water shortage and insufficient waste removal (Kautsky et al., 2000). The spread of infectious disease and discharge of effluents which had high level of nutrient, antibiotics or nonnative species are negative impacts of marine aquaculture industry for seawater environment (Engle and Bohorquez, 2009). Moreover, infectious disease of aquaculture usually occur in lower latitudes areas that relatively warmer climate due to favorable condition for the proliferation of infectious pathogen and have chance to spread to other upper latitudes areas that increased temperature due to climate change (Leung and Bates, 2013). Pathogenic *Vibrio* species growth rapidly in warm water temperate area and spread to other area due to climate change (Baker-Austin et al., 2016).

1.2 Problem statement

Vibriosis is the disease that are caused by pathogenic *Vibrio* spp. which gave serious impact for global marine aquaculture toward increase mortality of farmed fishes and had potential zoonotic to human (Haenen et al., 2014). Aquaculture farms in Malaysia and other tropical neighboring countries always suffered vibriosis outbreaks particularly in the important food fish species that affected aquaculture production and Malaysia economy. Among the fish species are Asian Seabass (*Lates calcarifer)*, brown marble Grouper (*Epinephelus fuscoguttatus*), Orange-spotted Grouper (*Epinephelus coioides*), Red Snapper (*Lutjanus* sp.) and Hybrid Grouper (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus)*(Albert and Ransangan, 2013). Antibacterial drugs were widely used as veterinary medicine for aquaculture treatments to control infectious bacterial disease included vibriosis to prevent any loss of aquaculture activities (Sekkin et al., 2011). *Vibrio harveyi*, *V. alginolyticus* and *V. parahaemolyticus* are well common causative agent of Vibriosis for farmed marine animals (Kalatzis et al., 2018).

However, antibiotics was restricted for treatment of fish bacterial disease because production of antibiotic-resistant bacteria upon long-term using of antibiotics (Martinez-Diaz and Hipolito-Morales, 2013). The uses of improved husbandry/management practices, movement restrictions, genetically resistant stock, dietary supplements, nonspecific immunostimulants, vaccines and therapeutics, water disinfection biological control, antimicrobial compounds, water disinfection, and control of movement are the best recommendations to prevent infectious diseases of fish (Assefa and Abunna, 2018). Vaccine is one of alternatives that have been proposed to overcome disease-caused mortality and morbidity after restriction of antibiotic for aquaculture (Cheng et al., 2014). Vaccination depends on host adaptive immune mechanisms to acquire antigens in vaccine for enhancing longer period of specific immunity against the particular pathogen (Rowley and Pope, 2012).

Several factors that needed to be considered before fish vaccination included targeted fish species, status of fish immune system, fish production cycle and life history of aquaculture system (Assefa and Abunna 2018). Formalin killed vaccine was created against disease of farmed aquatic animals but formalin-killed vaccine did not provided acceptable protection for targeted host (Liu et al., 2015b). Moreover, protective effects of vaccination for marine fish fingerling stage was poorly understood and was not well studied. Injection vaccination and handling stress are the problems for fish fingerling or larvae before they are developed into large enough for the vaccination (Sommerset et al., 2005). Therefore, further studies are needed to investigate pathogenesis of *Vibrio* species, vaccine efficacy and delivery particular for marine fishes at fingerling stage.

1.3 Justification of study

Live attenuated vaccine is pathogenic or beneficial microorganism that are engineered into genetical attenuated forms to deliver heterologous antigens for stimulating host immunity against pathogens which included the donor gene from it was derived (Lin et al., 2015). In fact, live attenuated vaccine can stimulate strong cellular and antibody response with long life immunity by only one or two doses but have risk to revert their pathogenicity which cause disease (NIAID, 2017). In our laboratory, newly developed vaccine known as live attenuated *V. harveyi* (MVh_vhs) is genetic modified strains of *V. harveyi* with deletion of catalytic triad sites which are Aspartate (D153), Histidine (H123) and Serine (S228) in serine protease (vhs) gene (Mohd-Aris et al., 2018). The serine protease gene was chosen for attenuation because serine protease is one of the extracellular products of *V. harveyi* that contributed pathogenic properties (Austin and Zhang, 2006).

Therefore, in the present study, the live attenuated *V. harveyi* vaccine candidate was tested using brine shrimps as a model to determine bacterial virulence of the *Vibrio* spp. strains and the safety of live attenuated *V. harveyi* (MVh_vhs) through their survival. Brine shrimp, *Artemia salina* have features that live in high salinity of water environment and short life-span with good resilience which is suitable as good host model organisms to study virulence of marine pathogenic bacteria (Lee et al., 2014).

The fish fingerling stage of Asian seabass (*Lates calcarifer)* is chosen for experiment upon fish farmers prefer hatchery cultured marine fish fingerling as seed stock for grow-out aquaculture to produce health marketable sized marine fishes because high survival and fast growth of the fingerling (Francis and Davies, 2014). Furthermore, vibriosis also cause high mortality for early development of fish because disease outbreak in fish farm (Silva et al., 2014). However, effective vaccine development and delivery are still not available. In this study, skin-abraded Asian seabass fingerling as model with immersion challenge of *Vibrio* spp. can be used to evaluate effectiveness of formalin killed *V. harveyi* and live attenuated *V. harveyi* (MVh_vhs) accurately in this experiment for future use in real situation of aquaculture.

Vibrio species were opportunistic pathogens that cause primary disease for mechanical skin damage of marine fishes in a disease outbreak (Benediktsdottir et al., 2008). The reason is standard experimental bacterial challenge method that undergoes injecting fish (subcutaneously, intraperitoneally or intramuscularly) with known bacterial concentration for finfish does not mimic natural infection which affect evaluation of potential prevention strategies against the related bacterial diseases because bypassed the fish first line defense consisting external non-specific barrier (Long et al., 2014; Henriksen et al., 2013). Moreover, the genetic and behavioral factor as well as environmental factors trigger the intracohort cannibalistic behavior for early stages of Asian seabass included fingerling and juvenile stage which lead to chasing, attacking and biting

on each other in a population lead to open wound of the fishes (Qin et al., 2004; Liu et al., 2017).

Bath administration known as bath vaccination is immersion delivery of vaccine through bathing fish into a vaccine solution with a time period to allow antigen uptake across mucosal surface for stimulating both mucosal and systemic immune response (Sudheesh and Cain, 2017). Bath or immersion vaccination are suitable methods to administrate vaccine into small size of fishes (Embregts and Forlenza, 2016) because large amount of small fishes can be vaccinated quickly and cheaply to acquire immune protection against infectious disease (Villumsen et al., 2014). In this case, previous research shown that higher relative percentage survival (RPS) of rainbow trout (*Onchorhynchus mykiss*) fry that bath vaccinated of live attenuated *Flavobacterium psychrophilum* vaccine with a booster compared with the vaccinated fry without booster after pathogenic *F. psychrophilum* challenge (Sudhessh and Cain, 2016). Gene expression used as identified immune markers in determining vaccine protective immunity served as guides to optimize vaccine antigens, formulations, antigen dose and immunization regimes for producing effective fish vaccination (Munang'andu and Evensen, 2019). Therefore, effectiveness of formalin killed *V. harveyi* and live attenuated *V. harveyi* (MVh_vhs) for vaccinated Asian seabass fingerling respectively can be evaluated accurately through survival in challenge test and gene expression result in this experiment.

1.4. Objectives

1. To determine the safety and efficacy of live attenuated *V. harveyi* on model of gnotobiotic *A. salina* metanauplii culture.

2. To determine the effects of skin abrasion on infectivity of *V. harveyi* in Asian seabass fingerling (*L. calcarifer).*

3. To examine immune protective effects of live attenuated *V. harveyi* vaccine on Asian seabass (*L. calcarifer)* fingerlings after *V. harveyi* challenge.

Hypothesis:

H_0: The live attenuated *V. harveyi* (MVh_vhs) is not safe, not effective and not protective for *L. calcarifer* fingerlings against vibriosis.

H_1: The live attenuated *V. harveyi* (MVh_vhs) is safe, effective and protective for *L. calcarifer* fingerlings against vibriosis.

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LIST OF PUBLICATIONS

Published paper:

Chin, Y.K., Al-saari, N., Mohd-Aris, A., Zamri-Saad, M. and Ina-Salwany, M.Y. 2019. Administration of live-attenuated vaccine of *V. harveyi* to improve survival of gnotobiotic brine shrimp (*Artemia salina*) model against multiple *Vibrio* infection. *International Journal of Biosciences*. 14(4). DOI: 10.12692/ijb/14.4.1-14.

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