EFFICIENCY OF INJECTABLE FORMALIN-INACTIVATED WHOLE-CELL BACTERINS OF *Vibrio harveyi* VACCINE IN PREVENTING VIBRIOSIS IN MARINE RED HYBRID TILAPIA (*Oreochromis* spp.)

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 Doctor of Philosophy

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Vibriosis is a serious illness caused by a group of bacteria called *Vibrio* in aquaculture industries while vaccination is an alternative method in protecting fish from diseases. However, the evaluation and testing of developed vaccines against vibriosis in real hosts are expensive and tedious to conduct especially on the laboratory scale. In addition, the use of marine tilapia in studying vibriosis was very limited and little attention was given. Thus, studying the ability and susceptibility of marine red hybrid tilapia against vibriosis and the efficacy of developed formalin-inactivated whole-cell bacterins of *Vibrio harveyi* (FK*Vh*) vaccine was important. This research's aims to develop vibriosis in marine red hybrid tilapia and to evaluate the efficacy of FK*Vh* vaccine itself.

A disease development study was conducted with 90 marine red hybrid tilapias and divided into 15 fishes per group with triplicate. Group A was infected with 2.3 x 10^9 CFU/mL of virulent *V. harveyi* inoculum and Group B was injected with phosphate buffered saline (PBS) and act as a negative control. Various organs such as the liver, brain, spleen and kidney were collected and subjected to immunoperoxidase (IP) staining for histopathology. Following infection with *V. harveyi*, infected fish showed some clinical signs such as inappetence and the presence of mucus in water was seen. Meanwhile, uninfected fish do not show any clinical signs. Group A showed 80% mortality after infection and group B showed 0% mortality. The distribution and intensity of brown colour using IP staining were higher in infected group A compared to uninfected group B. For the study of the immune responses, 200 marine red hybrid tilapias were used and divided equally into 2 groups with 30 fishes per group with triplicate. Group 1 was vaccinated with 100 µL of the FK*Vh* vaccine containing 10^6 cells/fish on week 0 and a booster dose was administered on week 2. Group 2 was injected with 100 µL of PBS on weeks 0 and 2. Samples of skin mucus, serum, and gut lavage were collected and subjected to ELISA and lysozyme assay. The initial

assessment at one week post-vaccination showed that injectable FK*Vh* vaccine immunized fish significantly ($P < 0.05$) stimulated the IgM level of all samples compared to unvaccinated fish. The lysozyme activity was also significantly ($P < 0.05$) higher in the vaccinated group compared to the unvaccinated group and increased drastically after the vaccine was given on week 0, indicating vaccination possibly induce lysozyme activity in samples to some extent.

A study of mucosal immunity in both SALT and GALT was done using 200 fishes and divided into 2 groups. Group 1 was vaccinated while group 2 was the unvaccinated group. The results showed the size and numbers of GALT and SALT in the vaccinated group were significantly ($P < 0.05$) higher compared to the unvaccinated group. Size, density and number of lymphocytes of GALT and SALT kept higher by weeks. Moreover, the GALT and SALT's size and numbers of lymphocytic cells in this study were positively correlated with the elicitation of antibody (IgM) level in gut lavage in immunological assessment in the previous chapter. In the protective efficacy study of FK*Vh* vaccine, the unvaccinated group showed high cumulative mortality (80%) compared to the vaccinated group which was only (13%). The histopathology analysis examined the presence of meningoencephalitis in the brain concurrently with haemorrhages. Kidney, spleen, gill and liver also showed severe haemorrhagic, congestion, eosinophilic granular inflammatory cells infiltration, necrosis, vacuolation, haemosiderin deposition, melanomacrophages aggregations, hypercellularity and fusion of secondary lamella.

Combining our results demonstrate that FK*Vh* vaccine could elicit significant innate and adaptive immunological responses against *V. harveyi* in marine red hybrid tilapia. This vaccine is highly effective to control vibriosis in marine red hybrid tilapia as tilapia is well-known as a freshwater fish. Besides, marine tilapia also can be the best candidate for the fish model in studying marine bacterial diseases. Therefore, this vaccine can effectively protect marine tilapia against vibriosis and also could offer a promising strategy for an effective therapeutic measure in the aquaculture industry.

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

KEBERKESANAN VAKSIN FORMALIN-TIDAK AKTIF SELURUH-SEL BAKTERIA *Vibrio harveyi* SECARA SUNTIKAN DALAM MENGAWAL PENYAKIT VIBRIOSIS TERHADAP IKAN TILAPIA MERAH HIBRID MARIN (*Oreochromis* spp.)

Oleh

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Vibriosis adalah penyakit serius yang disebabkan oleh sekumpulan bakteria yang digelar *Vibrio* dalam industri akuakultur sementara vaksinasi adalah langkah alternatif yang dapat mencegah ikan dari penyakit. Walaubagaimanapun, evaluasi dan ujian terhadap vaksin yang dicipta dalam perumah yang sebenar adalah sangat mahal dan sukar untuk dilaksanakan terutamanya dalam skala makmal. Tambahan pula, penggunaan tilapia marin dalam kajian terhadap penyakit vibriosis adalah sangat terhad dan sedikit perhatian diberikan. Jadi, kajian tentang kebolehan dan keupayaan marin tilapia merah hibrid terhadap penyakit vibriosis dan efikasi vaksin FK*Vh* sangat penting. Tujuan kajian ini adalah untuk menjangkitkan vibriosis terhadap ikan tilapia merah marin dan menilai efikasi vaksin FK*Vh* terhadapa penyakit tersebut.

Kajian penyakit dijalankan dengan menggunakan 90 ekor ikan yang dibahagikan kepada 2 kumpulan secara sama rata di mana 15 ekor dalam setiap kumpulan secara peniga. Kumpulan A dijangkitkan dengan 2.3 x 10^9 CFU/mL *V. harveyi* virulen manakala kumpulan B disuntik dengan hanya menggunakan PBS sebagai kawalan negatif. Beberapa organ seperti hati, otak, hempedu dan buah pinggang dikumpulkan dan dilakukan warnaan imunoperoksida (IP) untuk melihat perubahan histopatologi. Sejurus jangkitan *V. harveyi* diberikan, ikan yang dijangkiti dilihat hilang selera makan dan mukus dilihat di permukaan air. Sementara itu, ikan yang tidak dijangkiti tidak menunjukkan sebarang perubahan klinikal. Kumpulan A menunjukan 80% mortaliti selepas jangkitan dan kumpulan B menunjukan 0% mortaliti. Perkadaran taburan bakteria dan intensiti warna coklat yang ditunjukan dari pewarnaan IP adalah tinggi dalam kumpulan A yang dijangkitkan berbanding kumpulan B yang tidak dijangkitkan. Untuk kajian tindak balas imunologikal, 200 ikan tilapia merah hibrid marin telah digunakan dan dibahagikan kepada 2 kumpulan dengan 30 ekor ikan setiap kumpulan secara

peniga. Kumpulan 1 telah divaksinkan secara intraperitoneum dengan 100 µL FK*Vh* yang mengandungi 10^6 sel/ikan pada minggu 0. Dos penggalak diberikan pada minggu ke 2. Kumpulan 2 diberi suntikan 100 µL PBS pada minggu 0 dan minggu 2. Sampel mukus kulit, serum dan cecair usus untuk ELISA dan aktiviti lisozim. Taksiran awalan dilakukan pada minggu pertama pasca-vaksinasi menunjukkan FK*Vh* vaksin berjaya menstimulasi antibody IgM secara signifikan ($P < 0.05$) berbanding ikan yang tidak divaksinasi. Aktiviti lisozim dalam kumpulan yang divaksinasi secara singifikannya ($P < 0.05$) meningkat secara mendadak pada tahap permulaan dalam kajian ini selepas vaksinasi diberikan pada minggu 0, menunjukan bahawa vaksinasi merencatkan aktiviti lisozim sampel dalam keadaan tertentu.

Untuk kajian imuniti mukosal SALT dan GALT dilakukan menggunakan 200 ikan yang dibahagikan kepada 2 kumpulan. Kumpulan 1 adalah kumpulan vaksinasi sementara kumpulan 2 adalah kumpulan yang tidak divaksinasi. Keputusan menunjukan saiz dan jumlah GALT dan SALT dalam kumpulan vaksinasi secara signifikannya ($P < 0.05$) lebih tinggi berbanding kumpulan tidak divaksinasi. Saiz, ketumpatan dan bilangan sel limfotik pada GALT dan SALT semakin tinggi setiap minggu. Tambahan lagi, saiz dan bilangan limfotik sel bagi GALT dan SALT menunjukan kolerasi positif dengan elisitasi paras antibodi (IgM) dalam cecair usus dalam taksiran imunologikal yang terdapat dalam bab sebelum ini. Dalam kajian perlindungan efekasi vaksin FK*Vh,* kumpulan yang tidak divaksinasi menunjukkan kumulatif kematian yang tinggi (80%) berbanding kumpulan yang divaksinasi yang hanya menunjukan 13% sahaja kematian kumulatif. Analisis patologi menunjukan banyak perubahan patologi dalam organ otak termasuk meningiensefalitis beserta pendarahan. Buah pinggang, limpa dan hati menunjukan hemoraj, kongestan (*congestion*), *eosinophilic granular inflammatory cells infiltration,* nekrosis, vakulasi, pemendapan hamosiderin, pengumpulan makrofajmelano, *hypercellularity* dan lakuran lamella kedua.

Menggabungkan dapatan dari kajian ini, jelas menunjukan bahawa FK*Vh* vaksin mampu mengelisit tindak balas imunologi inat dan adaptif, terhadap kedua imun sistemik dan mukosal terhadap *V. harveyi* pada ikan tilapia hibrid merah marin. Vaksin ini sangat efektif dalam mengawal vibriosis yang disebabkan oleh *V. harveyi* virulen terhadap ikan tilapia hibrid merah marin yang dikenali sebagai ikan air tawar. Disamping itu, tilapia marin juga adalah calon terbaik untuk menjadi model dalam kajian penyakit ikan marin. Oleh itu, vaksin ini sangat efektif dalam melindungi ikan tilapia marin terhadap vibrosis dan mampu menjadi strategi yang terjamin sebagai pendekatan terapeutik dalam industri akuakultur.

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"**Research indeed is a collaborative work. No one does it by oneself**"

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

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LIST OF ABBREVIATIONS

µl	microliter
µm	micrometee
ºC	Degree Celcius
ANOVA	Analysis varience
BHI	Brain-heart infusion
BSA	Bovine serum albumin
cfu	Colony forming unit
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
g	gram
h	hour
H&E	Haematoxylin and eosin
IACUC	Institutional Animal Care and Use Comittee
Ig	immunoglobulin
IP	immunoperoxidase
min	minute
ml	milliliter
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
rpm	Revolution per minute
SD	Standard deviation
SEM	Standard error of the mean

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The aquaculture industry has been expanding for more than two decades in Southeast Asia. The industry has been successful in reducing our dependence on the capture fisheries sector which was reduced year by year. Furthermore, the aquaculture industry also contributes a huge social and economic impact, especially by increasing the incomes of poor farmers in rural areas as well as job opportunities for the locals. The Asia-pacific region itself has contributed more than 90% of the total world aquaculture production and this figure reflects the importance of the industry in this region (FAO,2020). Due to the declining state of marine fishery resources, world aquaculture production needs to be progressively surpassed capture fisheries to fulfil the consumers' demand while sustaining the marine fish stocks. With the rapid growth of the aquaculture industry, the “farming more than catch” milestones were reached in 1997 for diadromous fishes and other species (FAO, 2020).

Globally, intensive aquaculture has been implemented for years to increase productivity. Despite the advantages of intensive aquaculture techniques, there are some possible oppositions to the industry. Intensive farming systems usually increase the stress level in fish due to high stocking density and high feeding levels, resulting in decreased water quality (Hastuti et al., 2019). The stressors increase susceptibility to infectious diseases causing substantial losses to fish farming. Therefore, the need to protect the highly intensive culture system from bacterial infectious disease has become a priority among the aquaculturists community, as the intensive culture of aquatic animals brings along more severe diseases that cause economic and welfare issues (Kole et al., 2018).

Tilapia is one of the most important freshwater fish species that are cultured intensively all over the world. Global cultured tilapia represents 6.88 million tons (FAO, 2020a). Tilapia is very popular due to its rapid growth, suitability for aquaculture, low production cost, high acceptability also in the market (Gu et al., 2017) and ease to adapt in both tropical and sub-tropical regions (Shelton, 2002). However, the production of tilapia is increasing day by day from the particular ecosystem, but this sector globally has been facing several health risks affected by emerging and re-emerging bacterial diseases, which causes severe economic losses in this industry (Adikesavalu et al., 2017).

Besides streptococcosis and motile aeromonad septicaemia (MAS), vibriosis is also had reported reported to occur in tilapia. This disease is widely accused of a high mortality rate among fishes in marine and aquaculture worldwide (Shruti

and Haldar, 2012). *V. harveyi, V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* were the major *Vibrio* species infecting marine and aquaculture fishes (Austin and Austin, 2012; Amalina et al., 2019). In the study done by El-Sharaby et al. (2017), 35% of positive vibriosis cases were reported in infecting tilapia in Egypt while 12.8% of cases were reported in Saudi Arabia (Anwar et al., 2010). The disease spreads rapidly among fish stocked in the same cages and causes high mortality, especially in small-sized fish stocked at high density (Julian et al., 2012). When the outbreak occurred, the mortality of the fish affected reached 50%, especially among juveniles. The clinical signs of vibriosis include red spots on the ventral and lateral areas of the fish, swollen body, dark skin lesions and ulcer that ulcer releasing blood exudate. However, in acute or severe epizootics, the infection is very rapid and most of the infected fish die without showing any clinical signs (De Souza Valente & Wan, 2021)

The most common practice in treating fish bacterial diseases involves the application of antibiotics (Darwish et al, 2002; Assane et al., 2019). Unfortunately, serious complications raised through the spread of antibiotics resistance bacteria and the accumulation of antibiotic residues in the environment cause serious problems to human and environmental health (Watts et al., 2017). Owing to the issues, the application of antibiotics is no longer encouraged. Following the ban on antibiotics in aquaculture, vaccination has been advocated as one of the options for reducing disease-related morbidity and mortality (Sudheesh et al., 2016; Zhang et al., 2018). Vaccines are more effective and less harmful to fishes and the environment compared to antibiotics (Ina-Salwany et al., 2019) because vaccine can stimulate the fish's immune system to produce antibodies that protect the fish against disease compared to antibiotics, which kill or halt infections. Vaccination often produces particular memory cells, which can trigger a fast-anamnestic response upon infection by the targeted pathogen, making it is the most effective technique for controlling infectious illnesses in animals with adaptive immunity (Yamaguchi et al., 2019). Therefore, vaccination is considered the best method and feasible way to prevent bacterial diseases that can stimulate host adaptive immune responses to fight the infection or diseases caused by a pathogen.

An effective method to counter vibriosis is by vaccination which this method can increases the resistance of the immune system to subsequent pathogen infection (Nehla et al., 2017). Inactivated vaccines that prepared in formalin solution have been widely applied in aquaculture in recent decades and reached a satisfactory level of protection against pathogens (Wei et al., 2020). Therefore, inactivating the microorganisms either by heat or chemical made the whole-cells bacterium vaccine and this type of vaccine enable to stimulate an immune response against vibriosis in tilapia.

1.2 Problem Statement

In Malaysia, *Vibrio* sp. is most commonly isolated from cultured marine fishes such as snapper (*Lutjanus* spp.), grouper (*Epinephelus* spp.) and Asian seabass (*Lates calcarifer*) meanwhile, *Streptococcus* spp. and *Aeromonas* spp. are most frequently isolated from freshwater fish such as tilapia (Siti-Zahrah et al., 2008; Noraini et al., 2013; Monir et al., 2020; Azzam-Sayuti et al., 2021). Tilapias are the fish for future aquaculture which can grow in brackish to marine water (Fouz et al, 2002; Vinh et al., 2006). Under stress conditions (including poor water quality and high density of fish), tilapia can easily be infected by several opportunistic pathogens belong to the genera *Streptococcus*, *Enterococcus*, *Aeromonas*, *Pseudomonas*, *Vibrio*, *Flexibacter* and *Edwarsiella* (Plumb, 1999). Vibriosis is a serious illness and very pathogenic to fishes including tilapias as it can cause mortality up to 100% and cause severe economic losses. In Malaysia, the rearing of brackish to marine red hybrid tilapia in farms kept on going especially in Terengganu and Melaka because of high demand from consumers. Although there was no outbreak of vibriosis in tilapia recorded in Malaysia, the study of this diseases was important as a preparation before the outbreak occurred.

Many commercial vaccines have been developed to combat the bacterial disease in tilapia (Pridgeon & Klesius, 2011b; Shoemaker et al., 2011 and Shoemaker et al., 2012) but only one vaccine was available against *Vibrio vulnificus* (Shoemaker et al., 2011) until now in seabass. Monovalent vaccines have high protective properties against single target bacteria through injection or immersion immunization. There was little study and attention was given to the marine tilapia regarding the effectivity and ability against bacterial marine disease especially vibriosis after vaccination was given. However, considering the ability and characteristics that have in tilapia, developing formalin-inactivated whole-cell bacterins of *V. harveyi* vaccine to prevent vibrio diseases is a must. Thus, this research provides a framework for a better understanding of the ability of vibrio diseases infected in marine red hybrid tilapia and the effectiveness of this vaccine in marine red hybrid tilapia that are known as freshwater fish.

1.3 Significant of the study

The effort of developing a vaccine for injection administration against vibriosis in an unusual host is novel and is ongoing. To date from our knowledge, there is no available vaccine against vibriosis specific to marine red hybrid tilapia and there is no research report that has been claimed to develop formalin-inactivated whole-cell bacterins of *V. harveyi* vaccine which can give protection against vibriosis especially in marine red hybrid tilapia in Malaysia. Thus, this study was very important to observe the capability of marine red hybrid tilapia against this disease and know the efficacy of *the* Vibrio vaccine when using marine tilapia as an animal host. Besides chosen types of hosts, vaccine strategy also includes the decision on which diseases to be vaccinated against, as well as the types 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 compared to oral and immersion vaccination (Komar et al., 2004). Besides, injection is the first best preferable route in studying vaccination because the amount of dosage and the concentration of vaccine that entered in the host was known. Prime vaccination practice at the preliminary stage is necessary for developing the ideal vaccine. To fill in the research gap, the main purpose of this study is to assess the efficacy of injectable formalin-inactivated whole-cell bacterins of *V. harveyi* vaccine against vibriosis in marine red hybrid tilapia. Knowing the disease prevalence and development of this vaccine can help farmers, researchers and policy makers to develop control measures for Vibrios in the tilapia industry. Thus, the findings from this study can contribute to the fish health management for sustainable aquaculture.

1.4 Objectives of the study

Tilapia was mostly in freshwater and also can be found in brackish and marine water. The mechanisms and distribution of *V. harveyi* in marine red hybrid tilapia have not been studied and the immunoprotection of FK*Vh* vaccine has not been tested in marine tilapia. Therefore, the general objectives of this study are to assess the efficacy of FK*Vh* vaccine against vibriosis which was caused by *V. harveyi* in marine red hybrid tilapia.

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

1. to determine the distribution of *V. harveyi* in organs after intraperitoneal (ip) infection.
2. to analyse the innate and adaptive immune response of marine red hybrid tilapia (*Oreochromis* spp.) following vaccination.
3. to assess mucosal immunity of skin-associated lymphoid tissue (SALT) and gut-associated lymphoid tissue (GALT) of marine red hybrid tilapia (*Oreochromis* spp.).
4. to evaluate the protective efficacy of injectable FK*Vh* vaccine in protecting marine red hybrid tilapia against vibriosis.

1.5 Hypotheses of the study

Hypothesis 1:

H_0: Distribution of *V. harveyi* and pathological changes of organs of marine red hybrid tilapia (*Oreochromis* spp.) following exposure to live *V. harveyi* are not comparable.

H_1: Distribution of *V. harveyi* and pathological changes of organs of marine red hybrid tilapia (*Oreochromis* spp.) following exposure to live *V. harveyi* are comparable.

Hypothesis 2:

H_0: FK*Vh* vaccine cannot give protection to marine red hybrid tilapia (*Oreochromis* spp.) after ip infection.

H_1: FK*Vh* vaccine can give protection to marine red hybrid tilapia (*Oreochromis* spp.) after ip infection.

Hypothesis 3:

H_0: FK*Vh* vaccine unable to enhance the innate and adaptive immune response of tilapia.

H_1: FK*Vh* vaccine able to enhance the innate and adaptive immune response of tilapia.

Hypothesis 4:

H_0: Vaccination of FK*Vh* vaccine unable to develop skin-associated lymphoid tissue (SALT) and gut-associated lymphoid tissue (GALT).

H_1: Vaccination of FK*Vh* vaccine of able to develop skin-associated lymphoid tissue (SALT) and gut-associated lymphoid tissue (GALT).

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