UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF INTESTINAL IMMUNITY DEVELOPMENT IN JUVENILE TIGER GROUPER, Epinephelus fuscoguttatus (Forsskål, 1775) FOLLOWING ORAL EXPOSURE TO BIOENCAPSULATED VIBRIOSIS VACCINE

MOHD FIRDAUS BIN NAWI

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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 Doctor of Philosophy

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Chairman : Professor Mohd Zamri Saad, PhD
Faculty : Veterinary Medicine

Vibriosis outbreaks have been reported in cultured groupers in several countries including Malaysia. Several species of *Vibrio* were isolated from the infected fish such as *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus* and *V. carchariae*. In Malaysia, the major causative agent of vibriosis is *V. alginolyticus* and the disease can affect all growth stages of groupers, although juvenile stage is more susceptible. Recent study indicated the intestinal tract is the major portal of entry of *V. alginolyticus* rather than the gill or skin.

Study was conducted to investigate the intestinal immunomorphology development as well as the mucosal immunity of normal juvenile tiger groupers, *Epinephelus fuscoguttatus* and followed by a study on the immune response following oral adminstrations of bioencapsulated vibriosis vaccine. At the start of the experiment, sixty juvenile tiger groupers obtained from Fisheries Research Institute (FRI) Tg. Demong were euthanized by an overdose of Ethyl 3-aminobenzoate methanesulfonate before the entire intestine was removed and was separated into the anterior, mid and posterior portions. The samples were fixed in 10% buffered formaldehyde for at least 24 h and subjected to histological examinations. The results revealed that the intestinal mucosal immunity in juvenile tiger grouper existed as early as 30 days old, and every region of the intestine has different role either in food absorption or in immunity. However, it was clear that there was a gradual change in function of the intestinal regions from major nutrient absorption function in the anterior intestine to intermediate absorption and immunity functions in the mid intestine to the mainly immunity function in the posterior intestine.

Following the immunomorphology study on normal juvenile tiger groupers, the humoral components of the mucosal immunity of normal juvenile tiger groupers were accessed to complete the investigation. Similar number of juvenile tiger

grouper as previous experiment were used. Body mucus samples were obtained by gentle scraping of the body surface with sterile plastic scraper involving an area of approximately 2cm in length. After that, the fish were slaughtered and intestinal sample were obtained. Then, all the samples were subjected to lysozyme assay, alkaline phosphatase assay and enzyme-linked immunosorbent assay (ELISA). This assessment study indicated that lysozyme, alkaline phosphatase and natural IgM antibody could be detected as early as in 30 days old and increased parallel with the increasing age of the fish. These findings also demonstrated that every immune substance has its own localize area as manifested by high activities or concentrations. Highest activity of lysozyme was detected in the intestine, especially the posterior and mid regions, while highest level of alkaline phosphatase was detected in the anterior intestine. Highest concentration of natural IgM was detected in the posterior intestine followed by the body mucus. Since all of the important immune substances existed as early as 30 days old juvenile age, it was suggested that vaccination program could be started at that particular age or earlier.

Based on the results of the assessment study, it was decided that administration of the vaccine to the juvenile tiger groupers could be done as early as 15 days old. Bioencapsulated vibriosis vaccine were fed to the juvenile tiger grouper twice; first administration that using bioencapsulated rotifer *(Brachionus plicatilis)* at 15 days old and followed by booster at 30 days old using the bioencapsulated *Artemia*. The bioencapsulated vibriosis vaccine was prepared by soaking the rotifer and artemia in sterile sea water containing 3.4×10^9 CFU/mL formalin-killed *V. alginolyticus* for 2 hours. This vaccination study revealed that oral administrations of bioencapsulated vaccine to 15-day old tiger grouper were able to stimulate short-term innate immunity that lasted 15 days but was able to provide humoral protection for 60 days in the body mucus and more than 105 days in the intestines. Therefore, oral administrations of bioencapsulated vibriosis vaccine into 15 days old tiger groupers followed by booster at 30 days old were able to protect the juvenile tiger groupers for a period of 105 days until they reach 120 days old.

The last investigation was conducted to determine the intestinal immunomorphology responses following oral administrations of bioencapsulated vibriosis vaccine. This findings revealed that the oral administrations of bioencapsulated vibriosis vaccine into 15 days old and followed by booster dose at 30 days old juvenile tiger groupers are able to trigger the proliferation and movement of important immune related cells such as goblet cells, lymphoid cells and plasma cells for a period of 3 months, between 30 and 120 days old. The study also demonstrated that the vaccination did not caused changes in the morphology of the intestine as observed in the length and numbers of villi. Thus, it is suggested that oral vaccination of juvenile tiger groupers with bioencapsulated vibriosis vaccine could be given as early as 15 days old and followed by booster at 30 days old to provide protection for the entire hatchery stage.

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

PENILAIAN PERKEMBANGAN IMUNITI USUS KERAPU HARIMAU JUVANA, *Epinephelus fuscoguttatus* (Forsskål, 1775) BERIKUTAN PENDEDAHAN ORAL TERHADAP KEPADA VAKSIN VIBRIOSIS.

Oleh

MOHD FIRDAUS BIN NAWI

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Wabak Vibriosis telah dilaporkan dalam ternakan kerapu harimau di beberapa buah negara termasuk Malaysia. Sejumlah spesis *Vibrio* telah diisolasi daripada ikan yang terkena jangkitan antaranya *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus d*an *V. carchariae*. Di Malaysia, agen penyebab utama jangkitan vibriosis adalah *V. alginolyticus* dan penyakit ini boleh menjangkiti semua peringkat perkembangan ikan kerapu, namun peringkat juvana adalah paling mudah dijangkiti. Hasil kajian terbaru menunjukkan usus adalah sumber kemasukan utama berbanding insang atau kulit.

Kajian telah dijalankan untuk mengkaji pembangunan immunomorfologi usus serta keimunan mukosa kerapu harimau juvana normal, *Epinephelus fuscoguttatus* dan diikuti dengan kajian mengenai tindak balas yang berlaku selepas pemberian vaksin bioenkapsulasi vibriosis secara oral. Pada permulaan eksperimen, enam puluh ekor kerapu harimau juvana yang diperoleh dari Fisheries Research Institute (FRI) Tg. Demong dimatikan dengan merendam dalam air mengandungi dos berlebihan Ethyl methanesulfonate 3-aminobenzoate dan kemudiannya kesemua usus dikeluarkan dan dipisahkan kepada bahagian hadapan, pertengahan dan belakang. Sampel-sampel usus terbabit diawet dalam larutan 10% formalin untuk sekurang-kurangnya 24 jam dan diproses mengikut teknik histologi. Hasil kajian menunjukkan keimunan mukosa didalam usus kerapu harimau juvana telah wujud seawal umur 30 hari, dan setiap bahagian usus mempunyai peranan yang berbeza-beza sama ada untuk pemprosesan makanan atau imuniti. Walau bagaimanapun, ia adalah jelas bahawa terdapat perubahan secara beransur-ansur dalam fungsi bahagian usus, dari fungsi penyerapan nutrien utama dalam usus hadapan, fungsi separa penyerapan dan keimunan dalam usus pertengahan kepada fungsi keimunan sepenuhnya dalam usus belakang.

Selepas kajian immunomorfologi pada kerapu harimau juvana normal, kajian seterusnya adalah penilaian komponen humoral keimunan mukosa dalam kerapu harimau juvana normal dilakukan untuk melengkapkan keseluruhan kajian penilaian. Sejumlah kuantiti kerapu harimau juvana sama seperti dalam eksperimen terdahulu telah digunakan. Sampel lendir badan diambil dengan cara mengikis lembut permukaan badan ikan seluas 4cm^2 dengan menggunakan pengikis plastik yang steril. Selepas itu, ikan-ikan tersebut disembelih dan sampel usus diambil. Kemudian, semua sampel dianalisa menggunakan teknik esei lysozyme, esei alkali phosphatase dan esei enzim-berkaitan imunoserapan (ELISA). Kajian penilaian ini menunjukkan bahawa lysozyme, alkaline phosphatase dan antibodi IgM semula jadi boleh dikesan seawal usia 30 hari dan meningkat seiring dengan pertambahan usia ikan. Penemuan ini juga menunjukkan bahawa setiap sebatian imun mempunyai kawasan tersendiri seperti yang dimanifestasikan oleh aktiviti atau kepekatan yang tinggi. Aktiviti lysozyme paling tinggi dikesan di dalam usus, terutamanya di kawasan usus belakang dan pertengahan, manakala tahap tertinggi alkaline phosphatase dikesan dalam usus hadapan. Kepekatan tertinggi IgM semulajadi pula dikesan dalam kawasan usus belakang dan diikuti dengan mukus badan. Oleh kerana semua bahan-bahan imun yang penting telah wujud us seawal usia 30 hari, ia telah dicadangkan bahawa program vaksinasi boleh bermula pada usia tersebut atau lebih awal.

Berdasarkan dapatan daripada kajian penilaian, pemberian vaksin kepada kerapu harimau juvana boleh dilakukan seawal usia 15 hari. Vaksin bioenkapsulasi vibriosis telah diberi makan kepada ikan kerapu harimau juvana sebanyak dua kali; pertama kali menggunakan rotifer (*Brachionus plicatilis*) yang telah dibioenkapsulasi pada usia 15 hari dan diikuti oleh dos tambahan pada usia 30 hari menggunakan *Artemia* yang telah dibioenkapsulasi. Vaksin bioenkapsulasi vibriosis disediakan dengan cara merendam rotifer dan *Artemia* selama 2 jam dalam air laut steril mengandungi 3.4 x 10^9 CFU / mL *V. alginolyticus* yang dimatikan dengan formalin.

Dapatan dari kajian vaksinasi menunjukkan bahawa pemberian vaksin yang telah dibioenkapsulasi kepada kerapu harimau yang berusia 15 hari dapat merangsang imuniti semula jadi jangka pendek yang bertahan selama 15 hari tetapi dapat memberi perlindungan humoral selama 60 hari dalam mukus badan dan lebih daripada 105 hari dalam usus. Oleh itu, pemberian vaksin vibriosis yang telah dibioenkapsulasi secara oral kepada kerapu harimau berusia 15 hari diikuti dos tambahan pada usia 30 hari dapat melindungi kerapu harimau juvana untuk tempoh 105 hari sehingga mereka mencapai usia 120 hari.

Kajian terakhir yang telah dijalankan adalah untuk menentukan tindakbalas immunomorfologi usus selepas pemberian vaksin vibriosis yang dibio-enkapsulasi secara oral. Dapatan kajian ini menunjukkan bahawa pemberian vaksin vibriosis yang dibio-enkapsulasi kepada kerapu harimau juvana berusia 15 hari dan diikuti oleh dos tambahan pada umur 30 hari dapat merangsang proses pembahagian dan

pergerakan sel-sel penting berkaitan keimunan seperti sel-sel goblet, sel-sel limfoid dan sel-sel plasma untuk tempoh 3 bulan, iaitu antara umur 30 dan 120 hari. Kajian ini juga menunjukkan bahawa pemberian vaksin ini tidak menyebabkan sebarang perubahan dalam morfologi usus sebagaimana yang boleh dilihat pada panjang dan bilangan villi yang tidak berbeza antara ikan yang divaksin dan tidak divakin. Oleh itu, adalah dicadangkan bahawa pemvaksinan oral kerapu harimau juvana dengan vaksin vibriosis yang dibio-enkapsulasi boleh diberikan seawal umur 15 hari dan diikuti oleh dos tambahan pada usia 30 hari untuk menyediakan perlindungan yang diperlukan bagi keseluruhan peringkat hatcheri.

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I certify that a Thesis Examination Committee has met on 17 May 2017 to conduct the final examination of Mohd Firdaus bin Nawi on his thesis entitled "Assessment of Intestinal Immunity Development in Juvenile Tiger Grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775) Following Oral Exposure to Bioencapsulated Vibriosis Vaccine" 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.

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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:

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Name of Member of Supervisory Committee : Prof. Dr. Md Zuki bin Abu Bakar

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LIST OF ABBREVIATIONS

BSA	Bovine Serum albumin
CFU	Colony Forming Units (bacteria)
cm	Centimeter
ELISA	Enzyme-Linked Immunosorbent Assay
E. fuscoguttatus	*Epinephelus fuscoguttatus*
FKB	Formalin-Killed Bacteria
GALT	Gut-associated Lymphoid Tissue
h	Hour
IgM	Immunoglobulin M
M	Molar
PBS	Phosphate-Buffered Saline
rpm	Revolutions per minute
RM	Malaysian Ringgit
OD	Optical Density
V. alginolyticus	*Vibrio alginolyticus*
µm	Micrometer
USD	United States Dollar

CHAPTER 1

INTRODUCTION

Aquaculture industry has been expanding for more than two decades in Southeast Asia. The industry has been successful in reducing our dependence on capture fisheries sector that was reduced year by years. Furthermore, aquaculture industry also contributes huge social economic impact, especially via increasing the incomes of poor farmers in the 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 important of the industry in this region (Nagasawa, 2004).

Groupers are economically important species in mariculture industry due to their high economic value and are a major component of the coastal fisheries where they are abundantly cultured in Southeast Asia (Yu-Hsuan *et al*., 2010). In Asia, grouper culture industry was started in 1980s (Pierre *et al*., 2007). The groupers that belong to the subfamily Epinephelinae and Serranidae are broadly distributed in the tropical and subtropical areas. According to FishBase (www.fishbase.org) data, there are up to twenty-one species of groupers that are cultured in Asia. The most popular cultured species are tiger grouper (*Epinephelus fuscoguttatus*), orange-spotted grouper (*E. coioides*), malabar grouper (*E. malabaricus*), greasy-grouper (*E. tauvina*), duskytail grouper (*E. bleekeri*), giant grouper (*E. lanceolatus*), humpback grouper (*Cromileptes altivelis*), spotted coralgrouper (*P. maculatus*), potato grouper (*E. tukula*), leopard coralgrouper (*Plectropomus leopardus*), yellow grouper (*E. awoara*), longtooth grouper (*E. bruneus*), Hong Kong grouper (*E. akaara*), convict grouper (E. septemfasciatus), camouflage grouper (*E. polyphekadion*) and palemargin grouper (*E. bontoides*). Several culturing systems have been used to culture grouper such as floating net cages, earthen pond and cage culture that are commonly practiced in Southeast Asia including Malaysia (Nagasawa, 2004). In Malaysia, the most cultured species are tiger grouper, giant grouper, potato grouper and hybrid grouper. The cultured groupers are normally for domestic consumption and export.

The main hurdles to sustainable aquaculture in many regions are management and control of infectious diseases (Rodger, 2016). A major problem to the grouper culture production is heavy mortality due to diseases following infections by agents such as bacteria, viruses, fungi and parasites, or non-infectious agents such as malnutrition and climate changes. One of the important diseases in grouper culture industry is vibriosis, caused by *Vibrio* sp. Vibriosis has been reported in cultured groupers in several countries including Malaysia, Indonesia, Brunei, Philippines, Thailand, Singapore, Taiwan, Israel and Kuwait (Colorni *et al*. 1981; Tendencia and Lavilla-Pitogo, 2004). Several species of Vibrio were isolated from the infected fish such as *Vibrio parahaemolyticus, V. alginolyticus, V. vulnificus* and *V. carchariae*. In Malaysia, the major causative agent of vibriosis is *Vibrio alginolyticus* and the

disease can affect all stages of groupers, although juvenile stage is more susceptible (Nik-Haiha, 2012). Following infection by *V. alginolyticus,* the fish become sluggish and show gross lesions such as darkened skin, exophthalmia, corneal opaqueness, loosened and sloughing off of scales while ulcers develop on skin (Austin *et al*., 1993). Wounds caused by physical injury or parasites and ingestions of contaminated food are the entry points of *V. alginolyticus* into fish body system, but recent study by Chen *et al*. (2008) found that the intestinal tract is the major portal of entry rather than gills or skin.

Hence, enhancement of fish intestinal immunity is vital to prevent the infection as well as increase the survivality of fish. Thus, oral vaccination of juvenile tiger groupers with bioencapsulated vibriosis vaccine was considered as the best solution. Oral vaccination with antigen being incorporated into feed is potentially an appropriate method for mass vaccination of fishes especially at juvenile stages where handling stress may lead to mortality (Le Breton, 2009). Bioencapsulation of antigen into live feed such as rotifer (*Brachionus plicatilis*) and *Artemia* (*Artemia salina*) is considered one of the efficient methods of oral vaccination. Several studies have proven the effectiveness of live feeds as vaccine carriers in stimulating protective immune responses in the host (Embregts and Forlenza, 2016). Other than vaccine, immunostimulant and probiotics are also bioencapsulated into live feeds and fed to the fish larvae to enhance their intestinal immunity (Genc *et al*., 2007; Heidarieh *et al*., 2012).

Immunomorphology is the study of the relationship between form and function of immunological parameters (Rakhmanov and Zhakov, 1980). The immunomorphology word is combination of two different words, which is "immuno" that indicates immune, immunology or immunology, and "morphology" that means the branch of biology that deals with the form of living organisms, and with relationships between their structures (Abrosimov *et al*., 2016).. Intestinal immunomorphology study means the study of cells and tissues of intestine and theirs relationship with the immune functions. The intestinal immunomorphology study in normal and vaccinated juvenile tiger grouper is very crucial since it no other such study was conducted before. Other than that, it can provide better understanding about the relationship between the intestinal cell and tissues with the local immune system especially after the administration of oral vaccination.

Thus, the objectives of this study are:

1. to assess the intestinal immunomorphology of normal juvenile tiger groupers, *Epinephelus fuscoguttatus*

2. to assess the humoral components of mucosal immunity of normal juvenile tiger groupers, *Epinephelus fuscoguttatus*

3. to determine the humoral immune responses of juvenile tiger groupers, *Epinephelus fuscoguttatus* following oral administrations of bioencapsulated live feed vibriosis vaccine.

4. to determine the intestinal immunomorphology responses of juvenile tiger groupers, *Epinephelus fuscoguttatus* following oral administrations of bioencapsulated live feed vibriosis vaccine

Hypotheses of this study were:

1. the immunomorphology of normal juvenile tiger groupers, *Epinephelus fuscoguttatus* could be assessed.

2. the humoral components of mucosal immunity of normal juvenile tiger groupers, *Epinephelus fuscoguttatus* could be assessed.

3. the humoral immune responses of juvenile tiger groupers, *Epinephelus fuscoguttatus* could be stimulated by admimistration of bioencapsulated live feed vibriosis vaccine.

4. the intestinal immunomorphology responses of juvenile tiger groupers, *Epinephelus fuscoguttatus* could be stimulated by admimistration of bioencapsulated live feed vibriosis vaccine.

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