



UNIVERSITI PUTRA MALAYSIA

***PROFILING IMMUNE MARKERS FOR IDENTIFICATION OF
LEUKOCYTES IN BROWN-MARbled GROUper
Epinephelus fuscoguttatus FORSSKAL***

CHONG CHOU MIN

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By

CHONG CHOU MIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of
Philosophy**

August 2014

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***Specially dedicated to my beloved parents and both of my beloved late
grandfathers***



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

**PROFILING IMMUNE MARKERS FOR IDENTIFICATION OF
LEUKOCYTES IN BROWN-MARbled GROUPER
Epinephelus fuscoguttatus (FORSSKAL, 1775)**

By

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August 2014

Chair : Maha Abdullah, PhD
Faculty : Institute of Bioscience

The high market value of grouper has encouraged a greater impetus for its development in aquaculture. Rapid expansion of mariculture of grouper however has been hampered by serious outbreaks of infectious diseases in the industry which poses a pressing need for disease prevention programs and supportive input from immunological studies on this premium fish. However, fundamental difficulties such as rapid blood clotting during blood collection, unsuitability of standard reagents for isolating leukocytes and paucity in monoclonal antibody (MAb) markers for identifying leukocyte subsets has hindered the progress of immunological studies in this field. The general aim of this study was to develop methodologies to isolate and identify leukocyte subpopulations in grouper. Various methodologies were used. Experimentation with different combinations of anticoagulants identified a new preparation which effectively prevented the remarkably rapid blood clotting capabilities of grouper (*Epinephelus fuscoguttatus*). This anticoagulant also prevented the blood coagulation in other tropical fish. Isopycnic density gradient showed that all of the grouper peripheral leukocytes possess heterogeneous densities and were hardly able to be purified using this method. Cytological assessment via microscopic observations of Romanowsky stained grouper blood cells revealed the five major cell components present, namely lymphocyte, thrombocyte, erythrocyte, monocyte, and granulocyte. Cytochemical stainings were also useful to characterise these cells. Putt's Eosin staining was found to differentiate grouper leukocytes into five clusters in flow cytometry and each was shown to correlate significantly with the aforementioned main cell types. Distinct flow cytometric light scattering characteristics of these subsets with eosin mirrored the biophysics of the cells in microscopic studies. The utilisation of flow cytometric eosin profile in cell subset identification is novel and applicable on other fish species. The profile was used to indicate granulocyte: lymphocyte ratio, whereby the index reflects inflammation status in fish groups treated with *Vibrio parahaemolyticus* relative to the control group. A panel of MAbs was developed against various blood cells of *E. fuscoguttatus* using the hybridoma fusion technique. Four (EF118, EF124, EF353 and EF512) were further characterised. With a combination of

immunochemistry assessments, flow cytometric analyses, and mitogen-induced leukocyte proliferation assays. EF118 was identified as a general marker for grouper lymphocytes, EF512 was identified a subset of lymphocyte, EF124 appeared to be specific for monocytes/macrophages, while EF353 stained mature blood cells (haematocytes). These markers were also used to profile changes in cellular compositions in grouper blood and spleen of controls and 4 hours acute infection with *V. parahaemolyticus*. Results showed modulation in percentage of EF118 and EF124 positive cells in these tissue/organ. Immunopanning with the EF118 monoclonal antibody was also tested to isolate specific cells from peripheral blood of grouper and successfully purified more than 95% of lymphocyte. With the successfully establishment of the methodologies and reagents in isolation and characterisation of leukocytes subsets in grouper blood and lymphoid organs, in addition to the development of MAB and non-MAB markers, immune responses of the grouper could be monitored against various environmental or biotic stressors and immunoprophylactic treatments that will lead to improvement of programs in aquaculture of brown-marbled grouper.

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

**PENCIPTAAN DAN PENCIRIAN PENANDA IMUN BAGI PENGENALAN
LEUKOSIT DALAM IKAN KERAPU HARIMAU *Epinephelus
fuscoguttatus* (FORSSKAL, 1775)**

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Harga pasaran bagi ikan kerapu yang tinggi telah mendorong perkembangan akulkultur ikan tersebut di seluruh dunia. Namun begitu, pembangunan marikultur kerapu telah dilarang oleh penyebaran pelbagai penyakit berjangkit yang serius dalam industri tersebut. Senario ini merangsang dan mendorong pengajian imunologi dan pencegahan penyakit bagi ikan kerapu. Tetapi, maklumat mengenai sistem imun dalam ikan tropika yang sedia ada adalah terhad. Pembekuan darah yang cepat semasa proses pengumpulan darah dan kesukaran pengasingan leukosit adalah antara masalah yang dihadapi dalam pengajian imun ikan tropika. Kekurangan antibodi monoklonal untuk membezakan jenis leukosit adalah faktor lain yang menghalang kajian imunologi dalam bidang ini. Tujuan umum kajian ini adalah untuk mencipta kaedah berkesan bagi mengasingkan dan mengenal pasti jenis leukosit ikan kerapu harimau. Pengujikajian pelbagai kombinasi antikoagulan telah mendorong penciptaan antikoagulan yang baru dan berkesan dalam pencegahan darah ikan kerapu harimau (*Epinephelus fuscoguttatus*) daripada membeku. Anticoagulan tersebut menyelesaikan isu pembekuan darah yang cepat yang dihadapi oleh ikan tropika. Pengemparan kadar zon yang berasaskan isopiknik cerun ketumpatan menunjukkan bahawa semua jenis leukosit yang terdapat dalam darah periferia kerapu memiliki ketumpatan yang bertindih dan sama. Ini menyebabkan sel-sel tersebut tidak dapat dipisahkan kepada subset individunya dengan menggunakan pendekatan kaedah pengemparan kadar zon semata-mata. Pemeriksaan sitologi menunjukkan kehadiran lima jenis sel utama dalam darah periferia ikan kerapu, iaitu limfosit, trombosit, eritrosit, monosit dan granulosit. Teknik pewarnaan enzimatik telah digunakan untuk menggambarkan sifat sitokimia sel-sel ini. Putt's eosin didapati berjaya membezakan leukosit kerapu kepada dalam lima kelompok melalui analisis sitometri aliran. Setiap kelompok yang diperhati dalam simetri aliran didapati berkait rapat secara ketara dengan kelima-lima leukosit dalam darah. Prinsip asas kaedah baru ini adalah berdasarkan perbezaan afiniti terhadap eosin Y bagi sel-sel yang keterterapan sel membrannya telah diubahsuaikan oleh pengawet. Gambar rajah serakan cahaya sitometri aliran yang berbeza

mengambarkan ciri-ciri biofizik sebenar sel-sel leukosit tersebut. Penggunaan eosin profil dalam sitometri adalah inovasi yang baru untuk mengenal pasti subset sel dan kaedah ini didapati boleh diaplikasikan oleh ikan tropika yang lain. Profil eosin ini telah digunakan untuk menunjuk nisbah limfosit kepada granulosit dalam darah ikan. Nisbah ini adalah indeks yang menunjukkan status keradangan bagi sesuatu vertebrat. Pembezaan nisbah ini telah dikesan dalam kumpulan ikan yang telah dijangkiti dengan *Vibrio parahaemolyticus* berbanding dengan kumpulan kawalan. Empat antibodi monoklonal telah dihasilkan dengan teknik fusi hibridoma. Melalui penilaian imunokimia, analisis sitometri aliran, dan ujian proliferasi limfosit, keempat-empat antibodi ini telah dikenal pasti sebagai penanda sel ikan kerapu. Mereka masing-masing merupakan penanda sel kepada limfosit, limfosit subset, monosit/ makrofaj, atau sel-sel darah matang. Penanda-penanda ini telah digunakan untuk mengaji ketukaran komposisi sel dalam darah dan limpa di kalangan kerapu yang disuntik *V. parahaemolyticus* berbanding dengan kumpulan kawalan. Aplikasi teknik panning dengan menggunakan piring petri yang dilapisi penanda limfosit (antibodi monoklonal EF118) telah berjaya mengasingkan limfosit dengan perolehan sel dan ketulenan pengasingan yang lebih tinggi berbanding dengan teknik pendekatan kaedah pengemparan kadar zon. Penciptaan antikoagulan dan penanda-penanda sel yang baru ini dijangka akan memberi rangsangan positif kepada pengajian imunologi ikan kerapu. Pelbagai teknik, pendekatan dan rekabentuk eksperimen baru dapat dicapai dengan mengaplikasikan perciptaan ini.

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I certify that a Thesis Examination Committee has met on 4th August 2014 to conduct the final examination of Chong Chou Min on his thesis entitled "Profiling immune markers for identification of leukocytes in brown-marbled grouper *Epinephelus fuscoguttatus* Forsskal" 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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LIST OF ABBREVIATIONS

A ₄₅₀	Optical density reading measured at 450 nm wavelength
APC	Allophycocyanin
ATCC	American Type Culture Collection
BCR	B-cell receptor
BG	β-glucuronidase
BSA	Bovine serum albumin
CAF	Citrate-acetone formaldehyde
CFU	Colony-forming unit
CO ₂	Carbon dioxide
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGC	Eosinophilic granular cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FL1	First fluorescence detector in flow cytometry
G:L ratio	Granulocyte to lymphocyte ratio
GIFT	Genetically improved farmed tilapia
HAT	Hypoxanthine, Aminopterin and Thymidine
HBSS	Hank's balanced salt solution
Hp	Hours post-fertilisation
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
i.m.	Intramuscularly
i.p.	Intraperitoneally
ICE	Isotonic citrate EDTA
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IUPAC	International Union of Pure and Applied Chemistry
I-15	Leibovitz-15
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAb	Monoclonal antibody
MGG	May-Grünwald Giemsa staining
MHC	Major histocompatibility complex
MS222	Tricaine methane sulfonate
Na ₂	Sodium
NBE	α-naphthyl butyrate esterase
NCC	Non-specific cytotoxic cell

NK cells	Natural killer cell
PAMPs	Pathogen associated molecular patterns
PAS	Periodic acid Schiff
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PMMA	Polymethylmethacrylate
ppt	Parts-per-trillion
PRP/R	Pattern recognition proteins or receptors
RNA	Ribonucleic acid
SIP	Stock isotonic Percoll
<i>Sp.</i>	Species
TCR	T-cell receptor
TLR	Toll-like receptor
TRITC	Tetramethylrhodamine isothiocyanate
USA	United states of America

CHAPTER 1

INTRODUCTION

The brown-marbled grouper (*Epinephelus fuscoguttatus*) of the Serranidae family or Tiger grouper as it is known in South-East Asia is a species of warmwater marine finfish (Unsworth *et al.*, 2007). Groupers are the most rigorously exploited live fish group (Pierre *et al.*, 2008) due to the remarkable quality of its flesh and high demand in the Asian market with an estimated cost ranging from USD 15–50 kg⁻¹ with fluctuations in the price and increased demand during the festive seasons (Harikrishnan *et al.*, 2010; Othman, 2008; Pierre *et al.*, 2008; Parameswaran *et al.*, 2007; Zhou *et al.*, 2007; Antoro *et al.*, 2006). Habitat loss and commercial fishery (Cornish, 2004 ; Zatcoff *et al.*, 2004) however, have led to the development of mariculture of grouper in many countries including Malaysia, with a two-fold increase in the wholesale value of reared grouper from the year 2002 to 2004 (Othman, 2008).

Unfortunately, the mariculture industry is susceptible to diseases such as vibriosis which occurs at various stages of grouper cultivation (Sivaram *et al.*, 2004) and serious infections which are potentially capable of wiping out entire fish stocks (Oh *et al.*, 2012; Gong *et al.*, 2011; Parameswaran *et al.*, 2007; Chi *et al.*, 2003). Immunology-based strategies such as vaccination and investigations on the susceptibility of infections may improve management and increase productivity of fish culture.

Insufficient knowledge is currently available on tropical warmwater fish compared to coldwater fish species (Bowater *et al.*, 2012; Lin *et al.*, 2005). One of the main differences between cold and warm water fish is the amount of time it takes for blood clotting to occur (Kawatsu, 1986; Feeney *et al.*, 1972), which is more rapid in fish living in warmer temperature (Huss, 1993).

The rapid blood clotting mechanism in warm marine finfish is an evolutionary feature that prevents infections of open wounds which are more susceptible to pathogenic infections in warmer water systems. Apart from pathogenic infections, bleeding poses a lethal threat to fish as they possess a blood volume of relatively a fifth to a quarter of that of mammalian blood (Wolf, 1959), and the bleeding could also potentially attract aquatic predators with strong olfactory capabilities. Although the effective blood clotting has been beneficial for the warm water fish, it has hampered blood collection essential for downstream uses needed by fish farmers and researchers, such as sex determination assay, serological and health assessment, disease identification, assessment of vaccine, biomarker production as well as various endocrinology tests. Close to 60% of the world's aquaculture production is contributed by warm subtropical and tropical countries in Asia (De Silva and Soto, 2009). Thus, the development of an effective anticoagulant will significantly contribute to fundamental researches in disease prevention and management.

Like mammals, fish have various types of white blood cells, or leukocytes, which constitute a sophisticated defense mechanism called cellular immunity. These leukocytes encompass lymphocytes, monocytes, thrombocytes and granulocytes. The separation of blood cells into their respective cell lineages is often the first and crucial step for many immunological studies. Isolation of leukocytes into their respective cell lineages have been confined to the usage of buoyant centrifugation methods, for example, density gradient centrifugation as well as sedimentation (Rowley, 1990).

With the monoclonal antibody approach, great strides have been made in the identification, isolation, and characterisation of leukocyte subpopulations of some fish species. The availability of basic reagents and methodologies encourages the progress of immunological based studies including understanding mechanisms of immunity to infections and importance of abiotic/biotic stressors that may have immunomodulatory effect on boosting or suppressing immune response. Furthermore, MAbs enable comprehend evaluation of immune status in fish. Through this marker-assisted selective breeding approach, broodstock of strong immunity could be identified. With available supportive evidence for vaccine identification and production, along with selective breeding, establishment of culture programs for rearing of healthy and disease resistant fish can be conducted.

Despite immense applications of monoclonal antibodies, developed monoclonal antibodies tend to be limited to several cultured fish species, such as salmon, rainbow trout, carp, and catfish, due to complications and difficulties in MAb production. Since bony fish are the vertebrate group with the most abundant species and possess a wider diversity among the species of fish (Shigdar *et al.*, 2009), the applicability of these MAbs are limited to their corresponding species and there is no cross-reactivity with other fish species.

The lack of lineage specific antibodies has hindered progress in the study of the immune cells in many other fish species of high commercial values, including brown-marbled grouper. Without specific MAbs to recognise cell lineages, the classifications of leukocytes in these fish are based mainly on morphology, ultrastructure, and differential cytochemical staining (Katzenback and Belosevic, 2009; Shigdar *et al.*, 2009; Slierendrecht *et al.*, 1995).

In light of the paucity of MAbs, basic methods to study the immunology of grouper are limited. Neither cytochemical assessment of grouper leukocytes, nor investigation of the effectiveness of the density centrifugation approach on grouper leukocyte subtypes have been described in scientific literatures as of yet. No studies to date have reported on the flow cytometric profile of grouper or its related species.

Thus, the current project was carried out to answer and accentuate the aforementioned issues on brown-marbled grouper with the following objectives:

- i. To develop an effective anticoagulant suitable for the rapid blood clotting nature of grouper blood
- ii. To isolate various leukocyte subpopulations through physical-chemical methods.
- iii. To characterise cytochemical staining and flow cytometric profiling of grouper leukocytes
- iv. To establish monoclonal antibodies against the grouper's leukocyte subpopulations.
- v. To demonstrate the applicability of the developed monoclonal antibodies on immunological study of grouper.

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