



UNIVERSITI PUTRA MALAYSIA

***IDENTIFICATION OF PROTEINS AND GENES AS POTENTIAL
BIOMARKERS IN BROWN-MARbled GROUper (Epinephelus
fuscoguttatus Forsskål) RESISTANCE TO Vibrio sp.***

LOW CHEN FEI

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By
LOW CHEN FEI

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

November 2014

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

IDENTIFICATION OF PROTEINS AND GENES AS POTENTIAL BIOMARKERS IN BROWN-MARBLED GROUPEL (*Epinephelus fuscoguttatus* Forsskål) RESISTANCE TO *Vibrio* sp.

By

LOW CHEN FEI

November 2014

Chair: Mariana Nor Shamsudin, PhD

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The gram-negative marine bacterium, *Vibrio* sp. has frequently been identified as the causative pathogen responsible for the infectious disease vibriosis in the marine aquaculture industry. This disease is one of the major challenges facing brown-marbled grouper aquaculture, causing fish farmers globally to suffer substantial economic losses. In this study, several experiments were conducted using a range of methodologies to identify proteins and genes that are immune response-related upon *Vibrio* infection in brown-marbled grouper. Serum proteome profiles from two-dimensional gel electrophoresis were compared between infected grouper and control grouper after 4 hours of pathogen challenge. Differentially expressed proteins were then identified by MALDI TOF. The serum proteins that were highly expressed during early *Vibrio* infection of grouper were putative apolipoprotein A-I, natural killer cell enhancement factor and lysozyme g. The transcriptome of brown-marbled grouper spleen was studied by RNA-sequencing using Next Generation Sequencing technology. Gene expression in grouper spleen was compared between the infected grouper and control grouper. A total of 4162 unigenes were up-regulated in infected grouper, and 4988 unigenes were down-regulated. Gene ontology classification showed 338 differentially expressed genes were involved in immune system processes. Cell killing and antioxidant activity class have highest percentage of differentially expressed unigene of 34.48% and 37.74% respectively. Up-regulated unigenes in the cell killing class included transporter-associated with antigen processing 2, and cytotoxic and regulatory T cell protein. KEGG pathway annotation identified eight immune-related pathways and also seven non-immune response-related pathways that were significantly enriched in differentially expressed genes. Among the most abundantly up-regulated unigenes, four unigenes were found to be novel and were not annotated in any of the database. These novel unigenes warrant further identification and characterization of its function in immune response of grouper against pathogens. Lastly, brown marbled grouper fingerlings observed for seven days after experimental infection with *Vibrio*

parahaemolyticus determined grouper susceptible to infection, with these fish having skin lesions ≥ 5 mm. Grouper that were resistant to infection had no observable skin lesion. Skin lesion specimens observed under the scanning electron microscope revealed disintegration of skin around the lesion, and presence of bacterial cells under high magnification of 6000X. Serum proteome profiles were compared between the resistant and susceptible grouper by two-dimensional gel electrophoresis. Putative parvalbumin beta-2 subunit I, alpha-2-macroglobulin, natectin and immunoglobulin light chain were identified to be differentially expressed in resistant grouper. In summary, resistance of grouper to bacterial infection involved complex mechanisms consisting of different pathways with distinct methods of activation and regulation. Genes and proteins altered in these pathways are potential markers to identify *Vibrio* resistant grouper as well as targets for immunomodulation and disease prevention through vaccination. It could therefore be concluded that putative parvalbumin beta-2 subunit I, alpha-2-macroglobulin, natectin and immunoglobulin light chain are among the important proteins participating critically in disease resistance mechanism in grouper, which are over-expressed to function collectively, thus contributing to the resistance of grouper to *V. parahaemolyticus* infection.

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

**PENGENALAN PROTEIN DAN GEN YANG BERPOTENSI SEBAGAI
PENANDA BIO DALAM IKAN KERAPU (*Epinephelus fuscoguttatus*
Forsskål) RINTANG TERHADAP *Vibrio* sp.**

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Bakteria marin gram-negatif, *Vibrio* sp. sering dikenalpasti sebagai patogen yang menyebabkan penyakit berjangkit yang dikenali sebagai vibriosis dalam industri akuakultur marin. Penyakit ini adalah salah satu cabaran utama yang dihadapi dalam akuakultur ikan kerapu, menyebabkan penternak ikan di seluruh dunia mengalami kerugian ekonomi yang besar. Dalam kajian ini, beberapa reka bentuk eksperimen yang merangkumi pelbagai kaedah telah dijalankan untuk mengenal pasti protein dan gen yang berkaitan dengan gerak balas imunisasi ikan kerapu terhadap jangkitan *Vibrio*. Profil proteome serum dari elektroforesis gel dua dimensi telah dibandingkan di antara kerapu kawalan dan kerapu yang dijangkiti patogen selepas empat jam. Protein yang diekspres lain dari kawalan telah dikenalpasti melalui kaedah MALDI TOF. Protein serum yang meningkat secara mendadak semasa jangkitan awal *Vibrio* dalam ikan kerapu ialah apolipoprotein A-I, factor perangsangan sel pembunuh semula jadi dan lisozim g. Transkriptom limpa ikan kerapu dikaji melalui penjujukan RNA dengan menggunakan teknologi penjujukan "next generation". Gen dalam limpa kerapu telah dibandingkan antara kerapu kawalan dan kerapu terjangkit. Sebanyak 4162 unigen yang diekspres telah meningkat dalam kerapu terjangkit, manakala 4988 unigen yang telah menurun. Klasifikasi gen ontologi menunjukkan 338 gen yang diekspres secara berbeza adalah terlibat dalam proses sistem imun. Kelas sel pembunuh dan aktiviti anti-pengoksidaan telah menunjukkan peratusan paling tinggi dalam gen yang diekspres secara berbeza iaitu 34.48% dan 37.74%. Dalam kelas sel pembunuh, unigen yang ekspresi telah meningkat termasuk "transporter-associated with antigen processing 2" dan "cytotoxic and regulatory T cell protein". Anotasi rangkaian KEGG mengenalpasti bahawa lapan rangkaian KEGG yang berkaitan dengan keimunan, dan tujuh rangkaian KEGG yang tidak berkaitan dengan keimunan telah diperkaya secara ketara dengan gen yang diekspres secara berbeza. Antara unigen yang ekspresi telah meningkat, empat unigen dikenalpasti sebagai novel dan tidak dianotasi dalam pangkalan data. Unigen yang novel ini perlu dikaji lebih mendalam untuk

mengetahui fungsi unigen ini dalam gerak balas keimunan ikan kerapu terhadap patogen. Ikan kerapu yang dijangkiti *Vibrio parahaemolyticus* secara eksperimen dan diperhatikan selama tujuh hari menunjukkan kerapu yang mudah terdedah kepada jangkitan mempunyai luka di kulit yang berukuran $\geq 5\text{mm}$. Kerapu yang rintang jangkitan tidak mempunyai sebarang luka di bahagian kulit. Spesimen kulit yang terluka diperhatikan di bawah mikroskop imbasan elektron menunjukkan perpecahan kulit di sekitar luka, dan kehadiran sel-sel bakteria dapat diperhatikan di bawah kuasa pembesaran setinggi 6000X. Profil proteome serum telah dibandingkan antara kerapu yang rintang terhadap jangkitan dan kerapu yang mudah terdedah kepada jangkitan oleh elektroforesis gel dua dimensi. Parvalbumin beta-2 subunit I, alpha-2-macroglobulin, nattectin dan rantai ringan imunoglobulin telah dikenalpasti diekspres secara berbeza dalam kerapu yang rintang terhadap jangkitan. Secara ringkasnya, kerapu yang rintang terhadap jangkitan bakteria melibatkan mekanisme yang kompleks yang terdiri daripada pelbagai rangkaian proses biologi yang berbeza dengan kaedah yang berbeza dari segi pengaktifan dan pengaturannya. Gen dan protein yang dikawal atur secara berbeza dalam rangkaian proses biologi ini adalah penanda bio yang berpotensi untuk digunakan dalam mengenalpasti kerapu yang rintang terhadap jangkitan *Vibrio* serta digunakan dalam imunomodulasi dan terapi dalam vaksin. Kesimpulannya, Parvalbumin beta-2 subunit I, alpha-2-macroglobulin, nattectin dan rantai ringan imunoglobulin merupakan protein yang penting yang terlibat secara kritikal dalam mekanisme rintang terhadap jangkitan dalam ikan kerapu, yang mana ekspresinya telah meningkat dan berfungsi bersama justeru menyumbang kepada daya rintang terhadap jangkitan *V. parahaemolyticus* dalam ikan kerapu.

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I certify that a Thesis Examination Committee has met on 17 November 2014 to conduct the final examination of Low Chen Fei on his thesis entitled "Identification of Proteins and Genes as Potential Biomarkers in Brown-Marbled Grouper (*Epinephelus fuscoguttatus*, Forsskål, 1775) Resistance To *Vibrio* sp." 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

VNN	Viral nervous necrosis
TDH	Thermostable direct haemolysin
TRH	TDH-related haemolysin
NGS	Next-Generation Sequencing
SNP	Single nucleotide polymorphism
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
FAO	Food and Agriculture Organization
RAS	Recycle aquaculture systems
SGIV	Singapore grouper iridovirus
RPS	Relative percentage survival
ROS	Reactive oxygen species
PRRs	Pattern recognition receptors
LPS	Lipopolysaccharide
PAMPs	Pathogen associated molecular patterns
CLRs	C-type lectin receptors
MHC	Major histocompatibility complex
MALT	Mucosa-associated lymphoid tissue
GALT	Gut-associated lymphoid tissue
SALT	Skin-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
2-DE	Two-dimensional gel electrophoresis
PMF	Peptide mass fingerprinting
PBS	Phosphate buffered saline
CFU	Colony forming unit
Ppt	Parts per thousand
MS-222	Tricaine mesylate
IPG	Immobilized pH gradient
FPKM	Fragments Per kb Per Million Fragments
NKEF	Natural killer cell enhancement factor
nr	Non redundant
KEGG	Kyoto Encyclopedia of Genes and Genomes
COG	Clusters of Orthologous Group
GO	Gene Ontology
SEM	Scanning electron microscope

CHAPTER ONE

INTRODUCTION

Fish is one of the major protein sources that contributes a relatively high percentage of animal protein to the human diet. However, due to rapid population growth that increases the demand for fish protein, the present fish catch is insufficient to compensate the increasingly high market demand for fish. This has promoted the expansion of the aquaculture industry. High market value marine fish is being cultured extensively in parallel with the rapid expansion of the aquaculture industry. Brown marbled grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775) is one of the high market value marine fish that is well received in the industry. However, high incidence of disease outbreaks has been reported with intensive culture, leading to huge economic losses in the industry. Viral diseases such as viral nervous necrosis (VNN) have been reported in several cultured grouper species included *E. coioides*, *E. fuscoguttatus* as well as *E. bruneus*, and the geographic distribution is worldwide including in Asia (Japan, Taiwan, Indonesia, Brunei Darussalam), North America (United States of America) and Europe (France, Italy, United Kingdom) (Nagasawa *et al.*, 2004). Meanwhile, several gram negative *Vibrio* species have been frequently reported as the causative agents of a bacterial disease known as vibriosis. Vibriosis has been reported in cultured *E. coioides*, *E. tauvina* and *E. malabaricus* in Brunei Darussalam, Malaysia, Taiwan, Indonesia and Thailand (Nagasawa *et al.*, 2004).

Aquaculture methods include extensive farming, semi-intensive farming and intensive farming. Among the different culture methods, intensive farming is the most common way that is being practiced in most aquaculture industry. The intensive culture techniques include land-based intensive flow-through farming, recirculation aquaculture systems, and cage farming. Application of intensive culture techniques which includes high stocking density of fish has led to high organic content, low aeration and increased pollution which have sustained the multiplication of pathogens including bacteria and viruses in the culturing system. The aquatic environment that contains very high concentrations of these pathogens promotes outbreak of diseases. Use of agricultural antibiotics in fish farming and immune-suppression in fish caused by water pollution are among the factors that contribute to the outbreak of diseases (Kai 1993). Vibriosis, a common infectious disease frequently reported in the aquaculture industry is caused by strains of Gram-negative bacteria, collectively called *Vibrio*. *Vibrio* is widely distributed in the marine environment. Among the *Vibrio* species, *Vibrio parahaemolyticus* and *Vibrio cholera* are important pathogenic agents for aquatic animals, and *Vibrio alginolyticus* is also frequently identified as a disease-causing pathogen. Rapid expansion of the aquaculture industry and the increase in the intensity of mariculture has expanded the list of *Vibrio* species causing fish diseases. The outbreaks of vibriosis in aquaculture have led to huge economic losses

globally. Thus, strategies to improve culture conditions such as selective breeding are proposed to produce new strains of fish that are highly resistant to disease pathogens, inclusive of *Vibrio*.

The pathogenicity of *V. parahaemolyticus* has been reported to be due to its ability to produce haemolysins, a chemical substance that exhibit beta-haemolysis on high-salt blood agar (Noriea *et al.*, 2010). Haemolysins cause haemorrhagic septicaemia in marine fish, leading to mortality in cultured fish populations (Wong & Leong 1990). Previous studies showed that the majority of genes contributing to the virulence of *V. parahaemolyticus* include thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) and thermolabile haemolysin (Iida *et al.*, 1998; McCarthy *et al.*, 1999). The manifestation of *V. parahaemolyticus* infection in fish appears as tail rot, red spots on the head, swollen and necrotic intestine (Alcaide *et al.*, 1999; Shruti & Soumya 2012) as well as lesions on the body (Wong & Leong 1990). Other commonly observed signs included anaemia, ascetic fluid, petechial haemorrhages on the muscle wall and accumulation of liquid in the air bladder (Shruti & Soumya 2012). The early stage of *V. parahaemolyticus* infection is commonly marked by the appearance of red spots on the skin surface, which develop in size until a circular to oval-shaped, deep haemorrhagic ulcer is noticeable, exposing the skeletal musculature (Sankar *et al.*, 2012). The colonization and interaction of bacteria with the fish host is a complex process, and variation in disease severity is expected. These well-defined signs allow distinction of disease-susceptible from disease-resistant variants and are important in the subsequent study of the mechanisms of infection in fish.

A robust immune response in fish is the main mechanism of defense to resist infections. Mediators of the innate and adaptive immune responses consists of a large number of proteins, which function in a variety of mechanisms to restrict the invasion, and prevention of systemic spread of the pathogen that could progress into lethal infection (Wu *et al.*, 2004; Lauren & Hao 2007). The effectiveness of fish immune response against invasion by a pathogen determines the infection outcome. Recent progress has indicated the potential in development of marker assisted selection strategies in fish breeding schemes. Many of these include mediators of immune response. These studies have also revealed the complexities in pathways and vast number of molecules involved. Therefore, further investigation using genome-wide techniques may be helpful to determine the mechanisms of bacterial infection in fish due to specific resistance mechanisms that are peculiar to the fish species.

De novo transcriptome sequencing by Next-Generation Sequencing (NGS) technology is a suitable platform to rapidly identify differentially expressed genes on a large scale and also facilitates functional studies. Transcriptome sequencing provides information of global gene expression profiles, in

addition to the discovery of novel genes and single nucleotide polymorphisms (SNPs), as well as the assembly of full-length genes (Huang *et al.*, 2011; Vera *et al.*, 2008; Emrich *et al.*, 2007). To date, the genome sequence of *Epinephelus fuscoguttatus* is still unavailable. The application of NGS in the study of brown marbled grouper infected with *Vibrio* may identify markers associated with the immune system and this would be useful for further studies in identification of disease resistant variants. On the other hand, study of grouper immune response upon bacterial infection through a proteomic approach identifies the proteins of interest and their regulation. Expression of proteins is regulated according to physiological needs, and their concentration may not be represented at mRNA level. Not all expressed genes are translated into proteins, but depend on the rate of translation and also the rate of mRNA degradation. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) provides higher gel resolution and is thus, suitable for comparative analysis of total proteome profile, integrated with spectrometric and bioinformatics approaches.

Hypothesis

Experimental infection of brown marbled grouper with *Vibrio* will identify resistant grouper expressing differential proteins compared to grouper that are susceptible to *Vibrio* infection. These differentially expressed proteins can be identified through comparative two-dimensional gel electrophoresis. In terms of genes expression, transcriptome sequencing of grouper spleen will identify differentially expressed genes in response to *Vibrio* challenge.

Specific objectives

- ~ To isolate and identify differentially expressed proteins in serum proteome profiles of infected grouper and *Vibrio* resistant grouper.
- ~ To identify differentially expressed genes in spleen transcriptome profiles of *Vibrio*-infected grouper.
- ~ To identify potential biomarkers of disease resistance in grouper.

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