UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A COMPETITIVE CHAIN REACTION ASSAY FOR QUANTITATIVE ANALYSIS OF WHITE SPOT SYNDROME VIRUS GENE TRANSCRIPTION AND VIRAL REPLICATION IN SHRIMPS

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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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Despites much research on infectivity and diagnostics of white spot syndrome virus (WSSV), little is known about the viral replication kinetics and quantitative gene expressions. Therefore, a time course quantitative study was carried out using competitive polymerase chain reaction (cPCR) to measure viral growth in grow-out *Penaeus monodon* experimentally infected via feeding of WSSV infected tissue. The current tissue tropism studies demonstrated that gills have higher viral load followed by integument and abdominal muscle. Gills and integument were infected as early as 14 hour post infection (hr p.i.) compared to 24 hr p.i. for abdominal muscle. Gills are therefore recommended for extraction of DNA in routine PCR screening of WSSV. A classification of infection level was proposed to categorise infection into light (0 to 24 hr p.i.), moderate (24 to 48 hr p.i.) and moribund (48 to 120 hr p.i.) stage according to viral loads detected in

gills, which were 0 to 1×10^3, 1×10^3 to 1×10^7 and 1×10^7 to 1×10^9 copies per mg tissue respectively for the three infection stages. As the viral load was low at light infection, but increased exponentially at moderate infection and maintained at high level at moribund infection, such pattern of growth in viral loads is comparable to the eclipse, logarithmic and plateau phase of viral growth curve. White spots and reddish discoloration on the exoskeleton were apparent in moderate and moribund infection stage, but terminal clinical signs such as abnormal swimming behaviour and heavy mortality could only be observed in the later.

Previous studies on WSSV early genes expression were often qualitative rather than quantitative. By using competitive reverse transcriptase PCR (cRT-PCR), early gene ribonucleotide reductase large subunit (RR1) and thymidine kinase-thymidylate kinase (TK-TMK) mRNA expressions were non detectable at light infection stage (12 hr p.i.), but abundant at moderate (24 hr p.i.) and moribund (60 hr p.i. and above) infection stages. Geomeans of RR1 expression in whole heart samples were 9.69×10^4 and 2.36×10^7 copies at moderate and moribund infection stage respectively. Thus, both genes are probably vital in establishing WSSV infection, and their expressions are useful as marker in anti-viral studies of WSSV.

Shrimp immunity was emphasised under the European Commission's Shrimp Immunity and Disease Control (SI & DC) project. At present, prophenoloxidase (proPO) activating system and penaeidins, the predominant antimicrobial peptides, are well studied in bacterial and fungal infection, but not in viral infection. The mRNA expression of proPO was detected low and infrequent throughout infection with two-step PCR in heart and lymphoid organ. Penaeidin expression was however abundant with geomean of 4.35×10^4 copies in light infection (12 hr p.i.) but downregulated to 8.94×10^3 copies at moderate infection (24 hr p.i.) and non-detectable at moribund stage in whole heart samples. The lack of penaeidin and proPO mRNA upregulation suggests that they have little if any importance in the response to viral infection.

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

PEMBANGUNAN SATU ESEI REAKSI POLYMERAS BERANTAI KOMPETITIF UNTUK ANALISIS KUANTITATIF TRANSKRIPSI GENE DAN REPLIKASI VIRUS PENYAKIT SINDROM BINTIK PUTIH DALAM UDANG

Oleh

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Walaupun banyak kajian telah dibuat dalam jangkitan and diagnostik virus penyakit bintik putih (VPBP), kinetik replikasi virus dan expressi gen kuantitatif masih kurang diketahui. Oleh itu, kajian kuantitatif berpandukan masa telah dijalankan dengan menggunakan reaksi polymeras berantai kompetitif (cPCR) untuk menentukan pertumbuhan virus dalam udang *Penaeus monodon* yang dijangkiti VPBP dalam eksperimen dengan memberi makan tisu terjangkit VPBP. Kajian tropisma tisu ini menunjukkan bahawa insang mengandungi beban virus yang tertinggi diikuti oleh kulit dan otot abdomen. Insang dan kulit dijangkiti seawal 14 jam pasca infeksi (j p.i.) dibandingkan dengan 24 j p.i. untuk otot abdomen. Oleh sebab itu, adalah dicadangkan supaya insang digunakan untuk pengambilan DNA untuk pengujian PCR rutin untuk VPBP. Satu klasifikasi aras jangkitan diperkenalkan untuk mengkategori jangkitan kepada peringkat ringan (0

to 24 j p.i.), sederhana (24 to 48 j p.i.) dan berat (48 to 120 j p.i.) berpandukan beban virus yang dikesan dalam insang, yang masing-masing adalah 0 ke 1×10^3, 1×10^3 ke 1×10^7 dan 1×10^7 ke 1×10^9 salinan untuk ketiga-tiga peringkat jangkitan. Oleh kerana beban virus didapati rendah pada jangkitan ringan, tetapi bertambah secara eksponen pada jangkitan sederhana dan kekal pada paras tinggi semasa jangkitan berat, corak pertumbuhan beban virus boleh dibandingkan dengan fasa gerhana, logarithma and datar dalam keluk pertumbuhan virus. Bintik putih dan perubahan warna kemerahan pada rangka luar akan ketara dalam aras jangkitan sederhana dan berat, tetapi tanda-tanda klinikal terminal dan kematian serius cuma diperhatikan dalam yang terkemudian.

Kajian sebelum ini dalam ekspresi gen-gen awal VPBP lazimnya adalah secara kualitatif dan bukan kuantitatif. Dengan menggunakan transkriptas terbalik PCR kompetitif (cRT-PCR), ekpresi mRNA gen-gen awal virus seperti subunit besar reductase ribonucleotid (RR1) and kinase thimidin-kinase thimidilat (TK-TMK) tidak dapat dikesan dalam aras jangkitan ringan (12 j p.i.), tetapi tinggi pada aras jangkitan sederhana (24 j p.i.) dan berat (60 j p.i. dan ke atas). Min geometri ekspresi RR1 dalam sampel seluruh jantung adalah 9.69×10^4 dan 2.36×10^7 salinan masing-masing dalam aras jangkitan sederhana dan berat. Oleh itu, kedua-dua gen ini kemungkinan penting dalam pembentukan jangkitan VPBP dan ekspresi gen-gen berkenaan boleh diguna sebagai penanda dalam kajian anti-virus VPBP.

Kajian imuniti udang telah diutamakan di bawah projek Imuniti Udang Dan Kawalan Penyakit (SI & DC) Komisyen Eropah. Pasa masa kini, sistem pengaktifan prophenoloxidas (proPO) and penaeidin, sekumpulan peptid antimikrob dominan, telah dikaji secara terperinci dalam jangkitan bakteria dan fungi, tetapi bukan dalam jangkitan virus. Ekspresi mRNA proPO didapati rendah dan tidak kerap sepanjang jangkitan dengan pemeriksaan PCR dua langkah dalam jantung dan organ limfoid. Walaubagaimanapun, ekspresi penaeidin didapati tinggi dalam kedua-dua organ itu dengan min geometri setinggi 4.35×10^4 salinan pada aras jangkitan ringan (12 j p.i.) tetapi pengawalan menurun kepada 8.94×10^3 salinan pada aras jangkitan sederhana (24 j p.i.) dan tidak dapat dikesan pada aras jangkitan berat. Kekurangan peningkatan pengawalan ekspresi mRNA penaeidin dan proPO and penaeidin mencadangkan bahawa kedua-duanya adalah kurang penting dalam tindakbalas terhadap jangkitan WSSV.

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LIST OF ABBREVIATIONS

AcNPV	*Autographa californica* nucleopolyhedrovirus
AMV	avian myeloblastosis virus
BSA	bovine serum albumin
BGBP	beta glucan binding protein
BMNV	baculoviral midgut necrosis virus
BP	*baculovirus penaei*
bp	base pair
CBV	Chinese baculovirus
cDNA	complementary deoxyribonucleic acid
cPCR	competitive PCR
CT	competitive template
CV	coefficient of variation
DNA	deoxyribonucleic Acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
DIG	digoxigenin
EST	expressed sequence tag
ELISA	enzyme-linked immunosorbent assay
EF-1α	eukaryotic elongation factor
FAO	Food and Agriculture Organisation of the United Nations
g	gram
g	gravitational force
GADPH	glyceraldehyde 3-phosphate dehydrogenase
geomean	geometric mean
HHNBV	hematopoietic necrosis baculovirus
HPV	hepatopancreatic parvovirus
H & E	haematoxylin and eosin
HzV-1	*Heliothis zea* virus 1
hr	hour
hr p.i.	hour post infection
ICTV	International Committee on Taxonomy of Viruses
IE-2	immediate early gene 2
IHHNV	infectious hypodermal and haematopoietic necrosis virus
IPTG	isopropyl-β-D-thiogalactoside
kD	kilo Dalton
LEF-2	late expression factor 2
L-DOPA	3-4 dihydroxyphenyl L alanine
LO	lymphoid organ
mg	milligram
min	minute
mL	millilitre
mM	millimolar

MBV	*Penaeus monodon*-type baculovirus
M-MLV	Moloney murine leukemia virus
mRNA	messenger ribonucleic acid
MW	molecular weight
µL	microlitre
µM	micromolar
NBT-BCIP	5-bromo-4-chloro-3-indoyl phosphate-nitroblue tetrazolium
NT	native template
ng	nanogram
OIE	Office International des Épizooties
oligo(dT)	Oligodeoxythymidine
ORF	open reading frame
OrV	*Oryctes rhinoceros* virus
OD	optical density
PCR	polymerase chain reaction
pfu	*Pyrococcus furiosus*
pmol	picomole
PRDV	penaeid rod-shaped DNA virus
proPO	propenoloxidase
REO	reo-like viruses
RFLP	restriction fragment length polymorphism
RT	reverse transcription
RR1	ribonucleotide reductase large subunit
rRNA	ribosomal ribonucleic acid
RV-PJ	rod-shaped nuclear virus of *Penaeus japonicus*
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
SAPMP	streptavidin-paramagnetic particles
SEMBV	systemic ectodermal and mesodermal baculovirus
SDS	sodium dodecyl sulphate
SOI	severity of infection
SSC	standard sodium citrate
TE buffer	Tris-EDTA buffer
$TCID_{50}$	tissue culture infective dose
TK-TMK	thymidine kinase - thymidylate kinase
Tm	Melting temperature
UV	ultra violet
vWF	von Willebrand factor
WSBV	white spot syndrome baculovirus
WSS	white spot syndrome
WSSV	white spot syndrome virus
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

1. GENERAL INTRODUCTION

Currently, penaeid shrimp farming is probably the most lucrative aquaculture venture. According to FAO Fisheries Department, frozen shrimps and prawns are the largest fishery commodity produced and traded internationally with a total export value of 7.5 billion US$ in 2001 (Table 1.1). Giant tiger prawn (*Penaeus monodon*), the major cultured shrimp species, ranked 19th in aquaculture production quantity in 2001, but the high value of this commodity at 4.7 billion US$ has rendered it No. 1 in terms of value (Table 1.2 & 1.3). Malaysia was the 11th top shrimp producer in the world with a production of 27,014 metric tonnes in 2001. Similar to many other countries in Asia, the major culture species in Malaysia is *P. monodon*, which is also the most cultured species worldwide contributing 48% to total shrimp aquaculture production in 2001 (Table 1.4). Shrimp culture still relies heavily on wild brooders as source of post larvae and therefore constantly exposed to the risk of disease introduction. To prevent this, good disease screening facilities using sensitive molecular biology based detection techniques have to be implemented. Western Hemisphere on the other hand, has the option of culturing domesticated specific pathogen free (SPF) shrimp species which is free from major shrimp pathogens. But culturing of such