



UNIVERSITI PUTRA MALAYSIA

**CHARACTERIZATION OF WHITE SPOT SYNDROME VIRUS (WSSV)
FROM INDONESIAN SHRIMP FARMS AND DEVELOPMENT OF
POLYMERASE CHAIN REACTION (PCR) ASSAY
FOR ITS DETECTION**

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By

AGUS SUNARTO

**Thesis Submitted in Fulfilment of the Requirement for the Degree of
Master of Science in the Faculty of Veterinary Medicine
Universiti Putra Malaysia**

November 2001



In the name of Allah, the most merciful and the most beneficent

**I dedicate this work to my late father,
with all the blessings**



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science

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Chairman : Hassan Hj. Mohd. Daud, Ph.D.

Faculty : Veterinary Medicine

A study was carried out to clarify the viral white spot disease in Indonesian shrimp farms and to develop a polymerase chain reaction (PCR) assay for its detection. Giant tiger shrimp (*Penaeus monodon* Fabricius) were collected from Indonesian shrimp farms that had a history of high mortality. The identification of shrimp infected with white spot was based on the clinical signs, particularly on the appearance of white spots on the cephalothorax and body shell. The shrimp was either preserved in Davidson's fixative, 4% glutaraldehyde or 70% ethanol and subsequently were used for histopathological study, ultrastructural analysis and DNA extraction, respectively.

Clinical history of the diseased shrimp included reduced feed intake before dying which surged rapidly up to 100% within a week. The disease



occurred in shrimp of all ages, regardless of stocking density and culture system. The pathognomonic clinical sign of white spots on the carapace developed from a tiny spot to 3 mm in diameter to a hibiscus-like shape.

Histopathological examination of the diseased shrimp revealed generalised tissue damage and cellular changes in subcuticular epidermis, gill, stomach, hematopoietic tissue, lymphoid organ, hepatopancreas, heart, nervous tissue and muscle. Marked eosinophilic to basophilic intranuclear Cowdry A-type inclusion bodies were observed in infected cells. Transmission electron microscopy observation of diseased shrimp confirmed the features of Cowdry A-type intranuclear inclusion body as seen under light microscope and the presence of virus particles in the intranuclear inclusion bodies in hypertrophied nuclei.

The virus was a non-occluded, ovoid, trilaminar enveloped and measured 328 ± 24 nm and 122 ± 27 nm in length and width, respectively. The nucleocapsid was cylindrical, measured 253 ± 30 in length and 80 ± 7 nm in width with unique appearance of 14 to 17 striated structures. The core of the nucleocapsid was highly electron-densed and separated from the envelope by an electron-lucent layer. The virus morphogenesis took place in the nucleus with membranous labyrinth as its support system. The virus had four structural proteins namely 19, 23, 27 and 75 kDa in size.

Nested PCR assays developed using primers designed from WSSV-DNA sequence available in GenBank[®] (Thai and Korean isolates) and from published



primers (Taiwanese and Japanese isolates) proved to be specific and sensitive for the detection of WSSV from Indonesian shrimp farms. However, the primer pairs constructed from highly conserved region of ribonuclease reductase gene from Thai isolate was the most sensitive PCR assay against WSSV.

Based on the gross signs, histopathological changes, ultrastructural observation and PCR results, it was confirmed that white spot disease occurred in Indonesian shrimp farms due to viral agent. Based on the viral ultrastructure, morphogenetic pathway and the genomic homology sequence, the virus was similar with WSSV previously reported in other Asian countries.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**PENCIRIAN SINDROM BINTIK PUTIH (SBP) DARI KOLAM UDANG
INDONESIA DAN PEMBANGUNAN KAEDAH REAKSI BERANTAI
POLIMERASE (RBP) UNTUK PENGESANANNYA**

Oleh

AGUS SUNARTO

November 2001

Pengerusi : Hassan Hj. Mohd. Daud, Ph.D.

Fakulti : Perubatan Veterinar

Satu kajian telah dijalankan untuk menjelaskan penyakit sindrom bintik putih di kolam udang Indonesia dan pembangunan kaedah reaksi berantai polimerase (RBP) untuk pengesanannya. Udang harimau (*Penaeus monodon* Fabricius) telah dikumpulkan dari kolam udang Indonesia di Indonesia yang mempunyai masalah kematian yang tinggi. Udang berpenyakit bintik putih ditentukan berdasarkan tanda-tanda klinikal, terutamanya dengan kehadiran bintik putih di cangkerang sefalotorak dan badan. Udang samada di simpan di dalam pengawet Davidson, 4% glutaraldehyde atau 70% etanol, dan selanjutnya digunakan untuk kajian histopatologi, ultrastruktur, analisis struktural protein dan ekstraksi DNA.



Sejarah klinikal dan tanda-tanda kasar penyakit termasuklah selera makan berkurangan sebelum kematian mencapai 100% dalam masa satu minggu. Penyakit terjadi pada udang di semua peringkat usia tanpa bergantung kepada kadar pelepasan dan sistem kultur. Tanda klinikal khas penyakit ini iaitu bintik putih di bawah cangkerang. Bintik putih bermula sebagai titik kecil dan berkembang sehingga diameternya mencapai 3 mm dengan bentuk seakan-akan bunga raya.

Kajian histopatologi terhadap udang yang dijangkiti menunjukkan kerosakan tisu dan perubahan pada sel subkutikular epidermis, insang, perut, tisu hematopoietik, organ limfoid, hepatopankreas, jantung, tisu syaraf dan otot. Badan inklusi eosinofilik atau basofilik jenis Cowdry-A dapat diperhatikan dengan nyata di dalam nukleus sel. Pemerhatian dengan mikroskop transmisi elektron pada kepingan ultratipis telah memastikan badan inklusi jenis Cowdry-A yang dilihat dengan mikroskop cahaya dan kehadiran butiran virus di dalam badan inklusi tersebut.

Virus adalah tidak terkatup, bujur, bersarung tiga lapis dan berukuran panjang 328 ± 24 nm dan lebar 122 ± 27 nm. Nukleokapsid adalah berbentuk silinder, berukuran panjang 253 ± 30 nm dan lebar 80 ± 7 nm dengan 14-16 struktur unik berlapis. Teras nukleokapsid adalah padat elektron dan dipisahkan daripada sarung oleh satu lapisan yang tidak padat elektron. Proses pembentukan virus berlaku di dalam nucleus dengan membranous labirin membran sebagai sistem

sokongan. Virus mempunyai empat protein struktur dengan berat molekul 19, 23, 27 dan 75 kDa.

RBP tersarang dibuat dengan menggunakan primer yang dicipta daripada jujukan DNA WSSV yang ada di GenBank® (jujukan Thailand dan Korea) dan daripada primer terbitan Taiwan dan Jepun yang mana terbukti spesifik dan sensitif terhadap pengesanan WSSV daripada kolam udang di Indonesia. Walau bagaimanapun, pasangan primer daripada Thailand yang dibina daripada gen ribonuklease reduktase yang mempunyai kawasan terpelihara yang tinggi telah memberikan sensitiviti yang tertinggi terhadap WSSV.

Berdasarkan tanda-tanda klinikal, perubahan histopatologi dan keputusan RBP, ianya telah dipastikan bahawa penyakit bintik putik yang terjadi di kolam udang Indonesia adalah disebabkan oleh virus. Di dalam hal struktur ultra virus, tapak luhuran morfogenetik dan kesamaan jujukan genomik, virus tersebut adalah serupa dengan WSSV yang telah dilaporkan di negara-negara Asia yang lain, sebelum ini.

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LIST OF ABBREVIATIONS

AcMNPV	<i>Autographica californica</i> multiple nuclear polyhedrosis virus
ASCC	Asian Shrimp Culture Council
BMNV	Baculovirus midgut gland necrosis virus
bp	base pair
BP	<i>Baculovirus penaei</i>
CBV	Chinese baculovirus
CL	Crystalline lattice
CTAB	n-Cetyl n,n,n-trimethyl ammonium bromide
DGF	Directorate General of Fisheries
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
EEDS	Explosive epidemic disease of shrimps
ELISA	Enzyme-linked immunosorbent assay
Et-Br	Ethidium bromide
ha	hectares
H&E	Hematoxylin and eosin
HE	Haemocytic enteritis
HHNBV	Hypodermal and haematopoietic necrosis baculovirus
ICTV	International Committee on Taxonomy of Viruses



IB	Inclusion body
IFAT	Indirect fluorescent antibody technique
IHHNV	Infectious haematopoietic hypodermal necrosis virus
HPV	Hepatopancreatic parvo virus
kbp	kilo base pair
LOP	Lymphoid organ pathology
MBV	Monodon baculovirus
MF	Membranous fibrillar
ML	Membranous labyrinth
nPCR	nested Polymerase chain reaction
PAV	Penaeid acute viremia
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PL	Post larvae
PmNOBIII	Third <i>Penaeus monodon</i> non-occluded baculovirus
PRDV	Penaeid rod-shaped DNA virus
RNA	Ribonucleic acid
RR	Ribonuclease reductase
RV-PJ	Rod-shaped nuclear virus of <i>Penaeus japonicus</i>
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SEMBV	Systemic ectodermal and mesodermal baculovirus
SHN	Septic hepatopancreas necrosis
ssDNA	Single-stranded deoxyribonucleic acid



<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-Boric acid-EDTA
TCBV	Type C baculovirus
TE	Tris-EDTA
TEM	Transmission electron microscopy
T_m	Melting temperature
T_{opt}	Optimum annealing temperature
TSV	Taura syndrome virus
WSBV	White spot syndrome baculovirus
WSS	White spot syndrome
WSSV	White spot syndrome virus
YHV	Yellow head virus



CHAPTER I

GENERAL INTRODUCTION

The Background

Shrimp farming of Penaeidae family has become a major world industry. Currently, over 40 countries reported some level of shrimp aquaculture, but production is clearly dominated by China, Thailand, Ecuador and Indonesia. Culture of black tiger shrimp (*Penaeus monodon* Fabricius) is the most important aquaculture industry in Indonesia. It is notable that Indonesia has a large potential area of approximately 4 million ha of mangrove tidal swamps for shrimp culture, plus generations of experience in shrimp pond aquaculture. The government has given a high priority to shrimp aquaculture. The industry is expected to contribute US\$6.78 billion or 70% of Indonesia's fisheries production by 2003.

Since the government launched the programme on shrimp pond intensification, which is referred as 'program intensifikasi tambak' in the Indonesian language, in 1984, shrimp pond culture is expanding rapidly. This programme has been successful in increasing shrimp production from 15,400 metric tonnes in 1986 up to 140,131 metric tonnes in 1991. Concomitant with the growth of the shrimp culture industry was the recognition of the ever-increasing importance of disease; especially those caused by infectious agents. Bacteria and

virus have been identified as the main causative agents of diseases of cultured shrimp in Indonesia. In addition, fungus and protozoa are also frequently reported. Bacteria are a major problem in hatcheries, while viral diseases cause massive mortality in pond-reared shrimp.

Since the middle of 1994, a disease that causes cumulative mortality of up to 100% was reported in numerous shrimp farms in northern coast of East (Anon, 1994), Central and West Java, Indonesia (DGF, 1995; Sunarto, 1995). The new disease, in which the pathognomonic characteristic sign was the presence of white spots on the cuticle was referred to as white spot syndrome ('penyakit bercak putih' in the Indonesian language), was the most threatening disease that had ever occurred in Indonesian shrimp farms. The two earlier viral diseases of shrimp, i.e. monodon baculovirus (MBV) and yellow head virus (YHV) were less pathogenic than the newly emerged white spot syndrome virus (WSSV), the causative agent of white spot syndrome (WSS).

The economic impact of white spot syndrome in Indonesian shrimp industry is difficult to determine. It is estimated that in 1999 only 20% of shrimp ponds were in operation. Many of the ponds remained unoperated (Rukyani, 1999), with some being converted to milkfish ponds. This phenomenon may be associated with environment deterioration and disease outbreaks, particularly the white spot syndrome.