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

AGUS SUNARTO

FPV 2001 5
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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

CHARACTERIZATION OF WHITE SPOT SYNDROME VIRUS (WSSV)
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By
AGUS SUNARTO

November 2001

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 limfoïd, 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 $328\pm24$ nm dan lebar $122\pm27$ nm. Nukleokapsid adalah berbentuk silinder, berukuran panjang $253\pm30$ nm dan lebar $80\pm7$ 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.
ACKNOWLEDGEMENTS

My genuine appreciation goes to my chairman, Dr. Hassan Hj. Mohd. Daud, for the support, assistance and guidance throughout the study. I am greatly indebted to Professor Dr. Mohamed Shariff Mohamed Din, for all unflinching support to complete my study, in particular for his conscientious reading of this thesis. I am also grateful to Dr. Abdul Rahman Omar for his unselfish help and comments.

I am thankful for the scholarship provided by the Agricultural Research Management Project (ARMP-II Indonesia) without which it would have been impossible for me to pursue this study. I would like to express my thanks to Dr. M. Fatuchri Sukadi, the Director of Central Research Institute for Fisheries (CRIFI), and Dr. Akhmad Rukyani, Head of Research Institute for Freshwater Fisheries (RIFF) for allowing me to pursue my post graduate study. I am also thankful to my seniors in Pathology Section of RIFF for their support.

I appreciate very much the technical assistance provided by Mr. Ho, Ms. Sulaika and Ms. Azilah for sample preparation of transmission and scanning electron microscopy. Special thanks to my Lab. mates: Mr. and Mrs. Wang, Abeer, Samson, Tan, Lee, Najiah, Fennie, Reza, Sanjoy and Dev for their invaluable assistance and friendship. I am thankful to the Indonesian Student Association for their concern and encouragement. Accomplishment of this work is not possible without true loves of my wife, Novy and cute baby, Prasasti Chika Razita.
I certify that an Examination Committee met on 21st November 2001 to conduct the final examination of Agus Sunarto on his Master of Science thesis entitled “Characterization of White Spot Syndrome Virus (WSSV) from Indonesian Shrimp Farms and Development of Polymerase Chain Reaction (PCR) Assay for Its Detection” in accordance with the Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 1 JAN 2002
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AGUS SUNARTO

Date: 21st November 2001
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LIST OF ABBREVIATIONS

AcMNPV ........... Autographica californica multiple nuclear polyhedrosis virus
ASCC ................................................................. Asian Shrimp Culture Council
BMNV ................................................................. Baculovirus midgut gland necrosis virus
bp ........................................................................ base pair
BP ........................................................................ Baculovirus penaei
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

xx
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 Penaeus monodon non-occluded baculovirus
PRDV ............................................................ Penaeid rod-shaped DNA virus
RNA .................................................................. Ribonucleic acid
RR ..................................................................... Ribonuclease reductase
RV-PJ .......................................................... Rod-shaped nuclear virus of Penaeus japonicus
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
Taq ........................................................................... Thermus aquaticus

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.