UNIVERSITI PUTRA MALAYSIA

CLONING AND EXPRESSION OF THE SPIKE PROTEIN OF NEPHROPATHOGENIC INFECTIOUS BRONCHITIS VIRUS STRAIN MH5365/95

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By

YAP MAY LING

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

September 2004

DEDICATED TO……..

My Late Father,

YAP KAM SANG

My Mother,

CHAN HA MOOI

My Brothers and Sister,

KOON HOI,

KOK HOU,

KOK WAI,

MEI YUEN.

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

CLONING AND EXPRESSION OF THE SPIKE PROTEIN OF NEPHROPATHOGENIC INFECTIOUS BRONCHITIS VIRUS STRAIN MH5365/95

By

YAP MAY LING

July 2004

Chairman: **Associate Professor Siti Suri Arshad, Ph.D.**

Faculty: **Veterinary Medicine**

Infectious bronchitis is a highly contagious respiratory and kidney disease of poultry. The etiological agent, the prototype coronavirus, infectious bronchitis virus (IBV) is a member of the family *Coronaviridae*. The objective of this research is to isolate S1 protein from nephropathogenic IBV strain MH5365/95. The spike (S1) gene encodes for the S1 surface glycoprotein which is involved in virus attachment and infectivity. In addition, the S1 possesses the main immunological determinants essential for immune response and host protection. Production and isolation of the S1 protein from the rest of viral immunogens is necessary for further subunit vaccine development and protein structure-functional studies. To achieve these, therefore, the S1 gene of the IBV strain MH5365/95 was cloned into *Escherichia coli* expression system and also into the Baculovirus Expression Vector System (BEVS). This is the first study ever conducted in Malaysia in which the viral immunogen of a local IBV strain was cloned and expressed as a recombinant protein in heterologous cell system. The 1.75 kb S1 gene

was amplified from the viral genomic RNA by RT-PCR method. It was cloned into the *E. coli* expression vector, pGEX-2T, and the baculovirus transfer vector, pAcG-2T. The recombinant clones were verified by restriction enzyme analysis, PCR and partial DNA sequencing. The recombinant S1 was expressed as glutathione S-transferase (GST) fusion protein in both the expression systems. In *E. coli* cells, the GST-S1 fusion protein was expressed at a relatively low level despite the optimization studies done. In Western blot analysis using an anti-GST polyclonal antibody, the *E. coli*-derived fusion protein was identified as a protein band having a molecular weight of approximately 90 kDa. Upon co-transfection of the recombinant baculovirus transfer vector with BaculoGold® linearized baculovirus genomic DNA in *Sf*9 insect cells, a viable recombinant baculovirus AcNPV (recS1-AcNPV) was verified by PCR and purified through plaque assay. The recS1-AcNPV recombinant baculovirus failed to produce any occlusion bodies in *Sf*9 cells consequent upon the replacement of baculoviral polyhedrin gene by S1 gene. In western blot analysis, the *Sf*9-derived GST-S1 fusion protein was identified as a protein band with molecular weight of approximately 105 kDa, reacting with anti-GST polyclonal antibody. The size of the S1 moiety was estimated to be approximately 79 kDa. This result showed that level of glycosylation in the *Sf*9-derived S1 protein was not equal to that of the authentic S1 glycoprotein. Moreover, the same protein was unable to react with the polyclonal antiserum raised against IBV MH5365/95. Thus, further analysis on the glycosylation pattern, conformation and antigenicity of the S1 protein is necessary.

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

PENGKLONAN DAN PENGEKSPRESAN PROTEIN PEPAKU VIRUS BRONKITIS BERJANGKIT STRAIN MH5365/95 NEFROPATOGENIK

Oleh

YAP MAY LING

Julai 2004

Pengerusi: **Profesor Madya Siti Suri Arshad, Ph.D,**

Fakulti: **Perubatan Veterinar**

Bronkitis berjangkit merupakan penyakit pernafasan dan penyakit ginjal paling menular dalam ayam itik. Agen etiologinya, koronavirus prototip, iaitu virus bronkitis berjangkit (IBV) merupakan ahli famili *Coronaviridae*. Objektif penyelidikan ini adalah untuk mengasingkan protein S1 daripada virus bronkitis berjangkit strain MH5365/95 nefropatogenik. Gen pepaku (S1) mengekod untuk glikoprotein permukaan S1 yang terlibat dalam pelekapan dan kejangkitan virus. Sambil itu, ia juga mempunyai penentu imunologi utama untuk gerak balas imun dan perlindungan perumah. Penghasilan dan pemencilan protein S1 daripada imunogen virus lain adalah perlu untuk perkembangan vaksin subunit dan kajian struktur-fungsi protein seterusnya. Untuk mencapai matlamat ini, maka gen S1 bagi strain IBV MH5365/95 telah diklonkan ke dalam sistem pengekspresan *Escherichia coli* dan juga ke dalam Sistem Vektor Pengekspresan Bakulovirus (BEVS). Ini merupakan pengajian terulung yang dijalankan di Malaysia di mana imunogen virus strain IBV tempatan diklon dan diekspreskan sebagai protein rekombinan di dalam sistem sel heterologos. Gen S1 bersaiz 1.75 kb ini telah

diamplifikasi daripada RNA genom virus melalui kaedah RT-PCR. Gen ini diklonkan ke dalam vektor pengekspresan *E. coli*, pGEX-2T, dan vektor pemindahan bakulovirus, pAcG-2T. Klon rekombinan ini telah disah betul melalui analisis enzim penyekat, PCR dan penjujukan DNA separa. S1 rekombinan telah diekspres sebagai protein lakuran glutation S-transferase (GST) dalam kedua-dua sistem pengekspresan tersebut. Dalam sel *E. coli*, protein lakuran GST-S1 telah diekspres pada aras agak rendah walaupun kajian pengoptimuman telah dijalankan. Dalam analisis pemblotan Western mengguna antibodi poliklon anti-GST, protein lakuran terbitan *E. coli* ini telah dikenal pasti sebagai jalur protein yang berat molekulnya lebih kurang 90 kDa. Selepas ko-pentransjangkitan vektor pemindahan bakulovirus rekombinan dengan DNA genom bakulovirus linear BaculoGold® ke dalam sel serangga *Sf9*, satu bakulovirus rekombinan AcNPV (recS1-AcNPV) telah disah betul melalui PCR dan ditulenkan melalui assai plak. Bakulovirus rekombinan recS1-AcNPV gagal untuk menghasilkan jasad oklusi dalam sel *Sf9* kerana telah berlaku penggantian gen polihedrin bakulovirus dengan gen S1. Dalam analisis pemblotan Western, protein lakuran GST-S1 terbitan *Sf9* telah dikenal pasti sebagai jalur protein berat molekul lebih kurang 105 kDa yang bertindak balas dengan antibodi poliklon anti-GST. Saiz moieti S1 ini dianggarkan lebih kurang 79 kDa. Hasil kajian ini menunjukkan yang aras pengglikosilatan dalam protein S1 terbitan *Sf9* tidak sama dengan glikoprotein S1 asli. Kajian menunjukkan bahawa protein yang sama tidak mampu untuk bertindak balas dengan antiserum poliklon yang dihasilkan terhadap IBV MH5365/95. Dengan demikian, analisis lanjutan terhadap pola pengglikosilatan, konformasi dan keantigenan protein S1 adalah perlu.

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I certify that an Examination Committee met on **23th July 2004** to conduct the final examination of **Yap May Ling** on her **Master of Science** thesis entitled "Cloning and Expression of the Spike Protein of Nephropathogenic Infectious Bronchitis Virus Strain MH5365/95" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows :

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Date :

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.

YAP MAY LING

Date:

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~	approximately
AcNPV	*Autographa californica* nuclear polyhedrosis virus
AcMNPV	*Autographa californica* multiple nucleocapsid per virus
AF	allantoic fluid
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BEVS	Baculovirus Expression Vector System
bp	base pair
BSA	bovine serum albumin
BV	budded virus
^{o}C	degrees centrigrade
CDNB	1-chloro-2,4-dinitrobenzene
cm^2	centimeter square
CPE	cytopathic effect
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpi	day-post-infection
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ECV	extracellular virus
FBS	fetal bovine serum
GST	glutathione S-transferase
GV	granulosis virus
h	hour
HA	haemagglutination
HI	haemagglutination-inhibition
hpi	hour-post-infection
IB	infectious bronchitis
IBV	infectious bronchitis virus
IgG (H+L)	immunoglobulin G (high and low)
IPTG	isopropyl 1-thio-β-D-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria Bertani
M	membrane
M	molar
µg	microgram
µl	microliter
µm	micrometer
mA	milliampere
MAb	monoclonal antibody

MCS	multiple cloning site
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
m.o.i.	multiplicity of infection
MOPS	3-*N*-morpholino propanesulfonic acid
mRNA	messenger ribonucleic acid
MTA	methylamine tungstate
MW	molecular weight
N	nucleocapsid
NBT	nitro blue tetrazolium
nm	nanometer
NOV	nonoccluded virus
NPV	nuclear polyhedrosis virus
NTE	natrium chloride-tris-EDTA buffer
OB	occlusion body(ies)
OD	optical density
ORF	open reading frame
ori	origin of replication
OV	occluded virus
%	percentage
PAb	polyclonal antibody
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unit
polh	polyhedrin
PVDF	polyvinyl difluoride
RBS	ribosome binding site
RE	restriction enzyme
RNA	ribonucleic acid
rpm	round per minute
rS1-AcG	recombinant plasmid with S1 gene, derived from pAcG-2T vector
rS1-GEX	recombinant plasmid with S1 gene, derived from pGEX-2T vector
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
S	spike
S1	spike subunit 1
S2	spike subunit 2
SDGC	sucrose density gradient centrifugation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
*Sf*9	*Spodoptera frugiperda* cell line
TAE	tris-acetate-EDTA buffer

TE	tris-EDTA buffer
TEM	transmission electron microscopy
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethylene diamine
U	unit
UV	ultraviolet
V	volt
VN	virus-neutralization
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Infectious Bronchitis

The poultry industry constitutes a significant sector in world agriculture. In United States, more than 8 billion birds are produced yearly with a value exceeding $20 billion (Sharma, 1999). In Malaysia, 644.56 thousand million tan of poultry meat and 6,534 million of chicken or duck eggs were consumed in year 2001 (Department of Veterinary Services Malaysia, 2002). The industry relies on intensive farming to supply meat and eggs at relatively low cost.

Infectious bronchitis virus (IBV), a member of family *Coronaviridae* and prototype of coronavirus, is the etiological agent of infectious bronchitis (IB), a highly contagious respiratory, kidney and urogenital tract disease in chickens. IB is prevalent in all countries with intensive poultry industries, with the incidence of infection approaching 100% in most locations (Ignjatovic and Sapats, 2000). It has a significant economic impact; in broilers, production losses are due to poor weight gains, condemnation at processing and mortality, whilst in laying birds, losses are due to suboptimal egg production and downgrading of eggs.