UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF RECOMBINANT ENVELOPE PROTEINS OF NEWCASTLE DISEASE VIRUS IN *ESCHERICHIA COLI* AND ANALYSIS OF THEIR IMMUNOLOGICAL PROPERTIES

WONG SING KING

FBSB 2005 2

PRODUCTION OF RECOMBINANT ENVELOPE PROTEINS OF NEWCASTLE DISEASE VIRUS IN *ESCHERICHIA COLI* AND ANALYSIS OF THEIR IMMUNOLOGICAL PROPERTIES

WONG SING KING

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

2005

PRODUCTION OF RECOMBINANT ENVELOPE PROTEINS OF NEWCASTLE DISEASE VIRUS IN *ESCHERICHIA COLI* AND ANALYSIS OF THEIR IMMUNOLOGICAL PROPERTIES

By

WONG SING KING

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

December 2004

*The fear of the **LORD** is the beginning of wisdom,*

*and knowledge of the **Holy One** is understanding.*

~ Proverbs

To my dearest father and mother
for their infinite love, care and support.
I owe them everything I have today.

To my beloved brothers and sister.

Also to my relatives and friends.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

PRODUCTION OF RECOMBINANT ENVELOPE PROTEINS OF NEWCASTLE DISEASE VIRUS IN *ESCHERICHIA COLI* AND ANALYSIS OF THEIR IMMUNOLOGICAL PROPERTIES

By

WONG SING KING

December 2004

Chairman : Professor Datin Khatijah Mohd. Yusoff, PhD

Faculty : Biotechnology and Biomolecular Sciences

Newcastle disease virus (NDV) is the causative agent of the Newcastle disease (ND) that remains as a major threat to the world poultry industry. The virus belongs to the family *Paramyxoviridae* and genus *Avulavirus*, infects more than 236 avian species, and causes up to 100% morbidity and mortality in susceptible birds. The viral envelope proteins, haemagglutinin-neuraminidase (HN) and fusion (F) proteins have been shown to play key roles in triggering the host immune responses. In order to study the immunological properties of the recombinant HN and F proteins, the HN and F genes of the Malaysian viscerotropic-velogenic NDV strain AF2240 were obtained through reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the *Pichia pastoris*, *Saccharomyces cerevisiae* and *Escherichia coli* expression vectors.

A number of eight recombinant plasmids were constructed, namely pPICZαA/HN and pPICZαA/F (*P. pastoris* system), pYES2α/HN and pYES2α/F (*S. cerevisiae* system), and pRSETA/HN, pRSETB/F, pET-43.1a/HN and pET-43.1a/F (*E. coli* system). The

recombinant plasmids were used to transform respective host cells, which were then induced for the production of the recombinant HN and F proteins. However, there was no protein expression observed in the recombinant *P. pastoris* and *S. cerevisiae* cells. Whereas, the bacterial hosts were found expressing the recombinant HN and F proteins (from the pRSETA/HN and pRSETB/F plasmids respectively), and the NusA fusion proteins, NusA-HN and NusA-F (from the pET-43.1a/HN and pET-43.1a/F plasmids respectively). The recombinant HN and F proteins were produced as insoluble inclusion bodies (IB) while the NusA-HN and NusA-F proteins were expressed in soluble form in *E. coli*.

The recombinant proteins were purified and used to immunise specific pathogen-free (SPF) chickens. ELISA results revealed that the insoluble and urea-solubilised inclusion bodies of the recombinant HN and F proteins, and the soluble NusA-HN and NusA-F proteins stimulated the production of antibodies that detect NDV. Among these antigens, the urea-solubilised HN IB appeared to induce the highest antibody titers. However, the chicken antibodies failed to neutralise the viral activities as shown in the tests such as haemagglutination inhibition (HI), neuraminidase inhibition (NI) and haemolysis inhibition (HLI). This explains the susceptibility of the immunised flocks to NDV infection upon the viral challenge. Despite of the presence of antibodies to NDV, none of the immunised chicken was protected against the viral challenge. Immunoblotting analysis on the interactions between the antigens and antibodies revealed that the anti-F antibodies did not bind to the denatured viral F glycoprotein, neither the anti-NDV serum detect the recombinant F protein. However, the anti-HN antibodies showed positive signals when used to probe the denatured viral HN glycoprotein, and in return,

the anti-NDV serum detected the recombinant HN protein. This finding indicates the potential application of the *E. coli* produced HN protein as antigen for the detection of NDV antibody.

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

PENGHASILAN PROTEIN SELAPUT REKOMBINAN VIRUS PENYAKIT NEWCASTLE DALAM *ESCHERICHIA COLI* DAN ANALISIS CIRI-CIRI IMUNOLOGINYA

Oleh

WONG SING KING

Disember 2004

Pengerusi : Profesor Datin Khatijah Mohd. Yusoff, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Virus penyakit Newcastle (NDV) merupakan agen penyebab bagi penyakit Newcastle (ND) yang masih menjadi ancaman utama kepada industri ayam sedunia. Virus ini berada dalam famili *Paramyxoviridae* dan genus *Avulavirus*. Ia menjangkiti lebih daripada 236 spesis burung dan boleh menyebabkan morbiditi dan mortaliti setinggi 100% dalam burung-burung yang terjangkit. Protein selaput virus, iaitu protein hemaglutinin-neuraminidase (HN) and protein pertaupan (F) telah ditunjukkan memainkan peranan yang penting dalam merangsangkan tindakbalas keimunan. Untuk mengkaji sifat-sifat imunologik protein rekombinan HN dan F, maka gen-gen HN and F bagi strain AF2240 NDV Malaysia yang viscerotropik-velogenik diperolehi menerusi transkripsi terbalik-tindakbalas rantaian polimerase (RT-PCR) dan seterusnya diklon ke dalam vektor pengekspresan *Pichia pastoris*, *Saccharomyces cerevisiae* and *Escherichia coli*.

Sejumlah lapan plasmid rekombinan telah dibina, iaitu pPICZαA/HN dan pPICZαA/F (sistem *P. pastoris*), pYES2α/HN dan pYES2α/F (sistem *S. cerevisiae*), serta pRSETA/HN, pRSETB/F, pET-43.1a/HN dan pET-43.1a/F (sistem *E. coli*). Plasmid rekombinan ini digunakan untuk mentransfomkan sel perumah masing-masing, yang seterusnya diaruh untuk penghasilan protein rekombinan HN dan F. Namun, tiada pengekspresan protein diperhatikan dalam sel rekombinan *P. pastoris* dan *S. cerevisiae*. Manakala perumah bakteria telah didapati bahawa ianya mengekspreskan protein rekombinan HN dan F (dari plasmid pRSETA/HN and pRSETB/F masing-masing), serta protein yang bergabungan dengan NusA, iaitu NusA-HN dan NusA-F (dari plasmid pET-43.1a/HN dan pET-43.1a/F masing-masing). Protein-protein rekombinan HN dan F dihasilkan sebagai badan inklusi (IB) yang tak terlarutkan, sementara protein NusA-HN dan NusA-F telah diekspreskan dalam bentuk terlarutkan dalam *E. coli*.

Protein rekombinan ini ditulenkan dan seterusnya digunakan untuk mengimunkan ayam bebas patogen spesifik (SPF). Keputusan ELISA menunjukkan bahawa badan inklusi tak terlarutkan dan terlarutkan urea bagi protein-protein rekombinan HN dan F, serta protein NusA-HN dan NusA-F yang terlarutkan berjaya merangsangkan penghasilan antibodi yang mengesan NDV. Antara antigen ini, badan inklusi HN yang dilarutkan dalam urea telah mencetuskan titer antibodi yang tertinggi. Walau bagaimanapun, antibodi ayam ini gagal menuetralkan aktiviti virus sebagaimana yang ditunjukkan dalam ujian seperti perencatan hemaglutinasi (HI), perencatan neuraminidase (NI) dan perencatan hemolisis (HLI). Ini menjelaskan keterjangkitan NDV terhadap ayam yang telah diimunkan itu setelah dicabar dengan virus tersebut. Walaupun terdapatnya antibodi terhadap NDV,

namun tiada ayam yang diimunkan itu terlindung daripada cabaran virus. Analisis immunoblot terhadap interaksi di antara antigen dan antibodi menunjukkan bahawa antibodi anti-F tidak mengenali glikoprotein F virus yang nyahasli, begitu juga dengan serum anti-NDV yang tidak mengesan protein rekombinan F. Namun, antibody anti-HN mengikat glikoprotein HN virus yang nyahasli, begitu juga dengan serum anti-NDV yang dapat mengesan protein rekombinan HN. Penemuan ini menunjukkan potensi kegunaan protein HN yang dihasilkan dalam *E. coli* ini sebagai antigen dalam pengesanan antibodi NDV.

ACKNOWLEDGEMENTS

All the glories and thanksgivings to God almighty in the highest.

My deepest gratitude to my supervisors, Prof. Datin Dr. Khatijah Mohd. Yusoff, Assoc. Prof. Dr. Tan Wen Siang, Dr. Tan Chon Seng and Assoc. Prof. Dr. Abdul Rahman Omar for their invaluable guidance, help and patience throughout the project, and for the critical review in the completion of this thesis.

I am indeed indebted to my seniors in the Virology Lab 143: Omeima, Alan, Subha, Chui Fong, Putery, Vijay, Wei Hong and Chiew Ling for sharing their knowledge and experience with me.

I appreciate the friendship and assistance from all my labmates: Pria, Amir, Kok Lian, Wai Ling, Tang, Majid, Dr. Azri, Geok Hun, Lalita, Eddie, Thong Chuan, kak Raha, Riha, Wawa, Sharifah, Su, Rafidah, Zul, Onie, Eni, Firoozeh, Swee Tin, Kah Fai, Andrew, Shirley Foo, Taznim, Salwa and Yan Peng.

My special thanks to Ina who had shared my burden and worked restlessly in protein purification and field trial. I am deeply grateful to her commitment and assistance.

I would like to express my sincere gratitude to Assoc. Prof. Dr. Abdullah Sipat, Assoc. Prof. Dr. Che Nyonya Abdul Razak, Prof. Abu Bakar Salleh, Assoc. Prof. Dr. Raja Noor Zaliha Raja Abdul Rahman, Assoc. Prof. Dr. Tong Chow Chin and Assoc. Prof. Dr. Raha Abdul Rahim for their approval for me to access the facilities in their laboratories.

I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis.

This study was supported by IRPA grants of The Ministry of Science, Technology and Environment of Malaysia.

Last but not least, I want to take the opportunity to thank my parents, brothers and sister for their endless love, care and encouragement.

Without all of you, it would not be possible for me to complete my project and thesis. May God bless you all for your kindness.

I certify that an Examination Committee met on 31st December 2004 to conduct the final examination of Wong Sing King on his Doctor of Philosophy thesis entitled "Production of Recombinant Envelope Proteins of Newcastle Disease Virus (NDV) in *Escherichia coli* and Analysis of their Immunological Properties" 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:

ABDUL MANAF ALI, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

RAJA NOOR ZALIHA RAJA ABDUL RAHMAN, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

SITI SURI ARSHAD, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

JIMMY KWANG, PhD
Professor
Temasek Life Sciences Laboratory
National University of Singapore
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

DATIN KHATIJAH MOHD. YUSOFF, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

TAN WEN SIANG, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

TAN CHON SENG, PhD
Senior Researcher
Biotechnology Unit
Malaysia Agriculture Research and Development Institute
(Member)

ABDUL RAHMAN OMAR, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

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.

_____

WONG SING KING

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	adenine
ADH1	alcohol dehydrogenase 1
ADH2	alcohol dehydrogenase II
AGR4	argininosuccinate lyase
Ala (A)	alanine
AMV	avian myeloblastosis virus
AOX	alcohol oxidase
APMV-1	avian paramyxovirus type-1
APS	ammonium persulfate
Arg (R)	arginine
ARS	autonomously replicating sequence
Asn (N)	asparagine
Asp (D)	aspartic acid
ATP	adenosine-5'-triphosphate
ATPase	adenosine triphosphatase
BCIP	bromochloroindolyl phosphate
BiP	heavy chain binding protein
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CITE	cap-independent translation enhancer
CMI	cell-mediated immunity
CMV	cytomegalovirus
CTP	cytidine-5'-triphosphate
CYC1	iso-1-cytochrome c

Cys (C)	cysteine
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
ddNTPs	dideoxyribonucleotides or dideoxyribonucleoside-5'-triphosphates
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine-5'-triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides or deoxyribonucleoside-5'-triphosphates
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
EID_{50}	mean egg infectious dose
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmid reticulum
F	fusion (protein)
FPV	fowlpox virus
G	guanine
g	gravity
Gal	galactose
GAP	glyceraldehydes-3-phoshate dehydrogenase
GlcNAc	N-acetyl-glucosamine
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine
gor	gluthathione oxido-reductase
GRAS	generally regarded as safe
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate