UNIVERSITI PUTRA MALAYSIA

CLONING AND EXPRESSION OF THE HAEMAGGLUTININ-NEURAMINIDASE (HN) GENE FROM NEWCASTLE DISEASE VIRUS (NDV) STRAIN AF2240 IN BACULOVIRUS (AcMNPV)

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By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

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LIST OF ABBREVIATIONS

AcMNPV	-	Autographa californica multiple nuclear polyhedrosis Virus
BEVS	-	Baculovirus Expression Vector System
BV	-	Budded virus
cDNA	-	complementary deoxyribonucleic acid
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
ddNTP	-	dideoxynucleotide triphosphates
EDTA	-	Ethylenediaminetetraacetic acid disodium salt
EM	-	Electron Microscope
F	-	fusion protein
HA	-	haemagglutinin activity
HN	-	haemagglutinin-neuraminidase
kb	-	kilobase
kDa	-	kilodalton
MAb	-	monoclonal antibody
Mr	-	molecular weight
NA	-	neuraminidase activity
NDV	-	Newcastle disease virus
OV	-	occlusion virus

ORF	-	open reading frame
PBS	-	phosphate buffer saline
PCR	-	polymerase chain reaction
RBC	-	red blood cells
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcriptase-polymerase chain reaction
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sf9	-	*Spodoptera frugiperda*
Taq	-	*Thermus aquaticus*
TBE	-	Tris-boric-EDTA buffer
VVNDV	-	viscerotropic-velogenic NDV
wt	-	wild type

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

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By

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September 1999

Chairman : Assoc. Prof. Khatijah Mohd. Yusoff, Ph.D.

Faculty : Science and Environmental Studies

Newcastle disease virus strain AF2240 is a major threat to the poultry industry as it causes 100% mortality to susceptible flocks The haemagglutinin-neuraminidase (HN) gene encodes for the HN surface glycoprotein which is known for virus attachment and contains immunogenic properties Therefore, the HN gene was cloned into a Baculovirus Expression Vector system (BEVS) for the development of a subunit vaccine against NDV as well as to study its expression in isolation from the other NDV structural genes

The approach taken involved the amplification (RT-PCR) of the 1 8 kb HN gene, from NDV strain AF2240 genomic RNA and cloning it into a BEVS The

recombinant baculovirus protein RecHNAF2240 expressed in *Sf9* cells was shown to be positive for the haemagglutinin test (HA), neuraminidase test (NA), indirect immunofluorescence (IIF) as well as in SDS-PAGE Western blot analysis indicated the distinct ~ 63 kDa and ~ 75 kDa protein bands as HN specific which corresponded to the unglycosylated and glycosylated HN glycoproteins respectively This observation was confirmed by a time course study using pulsed-labeled [^{35}S] methionine of the HN glycoprotein in the recombinant virus infected cells with tunicamycin The recombinant protein was expressed not only on the surfaces of the infected cells and the viral coat protein but also appears to be biologically active and functional The physical nature of the viruses was also studied using electron microscopy (EM) and it indicated various physical differences

Optimisation study on the recombinant protein RecHNAF2240 production showed that a late log phase infection of the recombinant virus (recHNAF2240) at an m o i of 1 was the most appropriate Based on HI, ELISA and western blot analysis the recombinant subunit vaccine was able to elicit a protective immune response The route of vaccination and a second dose were crucial to illicit an immune response from the chickens However, this protective feature of the recombinant subunit vaccine remains inconclusive and more work should be carried out to bring about the fullest potential of this recombinant subunit vaccine

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

PENGKLONAN DAN PENGEKSPRESAN GEN HEAMGGLUTININ-NEURAMINIDASE (HN) DARIPADA VIRUS PENYAKIT NEWCASTLE (NDV) STRAIN AF2240 KE DALAM BACULOVIRUS (AcMNPV)

Oleh

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Virus penyakit Newcastle strain AF2240 merupakan penyakit virus utama dalam industri penternakan ayam kerana boleh menyebabkan 100% kematian ayam Gen HN yang mengkodkan glikoprotein permukaan HN memainkan peranan dalam perlekatan virus dan juga mempunyai nilai-nilai keimunan Oleh itu, gene HN telah diklonkan dalam Sistem pengekspresan vektor Baculovirus (BEVS) untuk penghasilan vaksin subunit terhadap NDV dan juga untuk mengkaji pengekspresan gen HN berasingan dengan gen-gen NDV yang lain

Pendekatan yang digunakan adalah dengan melibatkan gen HN (1 8 kb) yang telah diamplifikasikan daripada jujukan RNA (RT-PCR) dan diklonkan ke dalam

BEVS. Protein rekombinan baculovirus RecHNAF2240 yang diekpreskan dalam sel *Sf9* menunjukkan keputusan yang positif untuk ujian hemaglutinin (HA), neuraminidase (NA), imunopendarfluoran (IIF) serta SDS-PAGE. Analisis "western blot" pula menunjukkan jalur protein ~ 63 kDa dan ~ 75 kDa sebagai specifik kepada protein HN yang bersamaan dengan glikoprotein HN yang masing-masing tidak mengalami glikosilasi dan yang mengalami glikosilasi. Keputusan tersebut juga telah didapati dengan menggunakan protein rekombinan yang dilabelkan dengan [^{35}S] methionin dengan kehadiran antibiotic tunikamisin. Penemuan sedemikian membuktikan bahawa protein rekombinan diekspreskan pada permukaan sel-sel yang dijangkiti virus rekombinan, pada permukaan protein perlindung virus dan adalah aktif secara biologikal serta berkebolehan berfungsi. Ciri-ciri fizikal virus yang berlainan juga telah dikaji dengan bantuan mikroskop electron (EM).

Jangkitan virus rekombinan recHNAF2240 pada fasa log akhir pada m.o.i. 1 merupakan nilai yang paling sesuai untuk penghasilan protein yang optimum. Penghasilan tindakan keimunan oleh vaksin subunit berdasarkan ujian HI, ELISA and analisis "Western blot" menunjukkan bahawa tempat vaksin disuntik and penggunaan dos kedua adalah perlu untuk menghasilkan tindakbalas imun daripada ayam-ayam yang dikaji. Namun demikian, ujian awal ini memberi maklumat yang penting tentang persoalan perlindungan ayam daripada jangkitan virus NDV dan ujian yang lebih menyeluruh adalah digalakan supaya dapat meningkatkan potensi vaksin rekombinan yang dihasilkan.

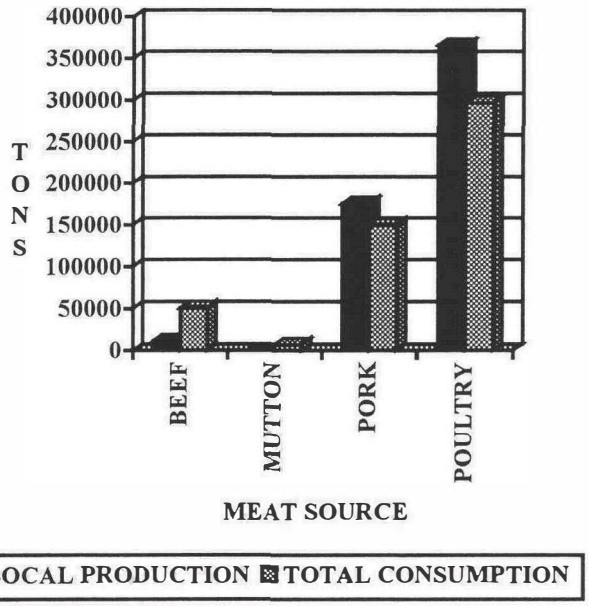
CHAPTER I

INTRODUCTION

The production of chicken in Malaysia ranks among the highest in the world in proportion to its population A 1992 survey reported in Asiaweek (20 April, 1994) on the various poultry producing countries in Asia, put Malaysia in 4th placing after China, India and Indonesia, the three highest populated countries in Asia Furthermore, the 1990 livestock statistics prepared by the Department of Veterinary Services (DVS) showed that the consumption and production of chicken was the highest among animal products (Figure 1a) The popularity of poultry products lies mainly on their nutritive values as both poultry meat and eggs production with regards to energy is similar to milk and is low in saturated fats Also, in Malaysia, the price of chicken among other common food sources appears to be the lowest (Figure 1b)

The poultry industry in Malaysia was worth 1 5 billion ringgit in 1992 (more than 55% of the total value of livestocks) and in 1997, it rose to 4 5 billion ringgit With the intensification of the poultry industry, Malaysia is currently self-sufficient in poultry eggs and meat but at the same time, is exposed to the inevitable increase in the prevalence of disease and loses

a)



b)

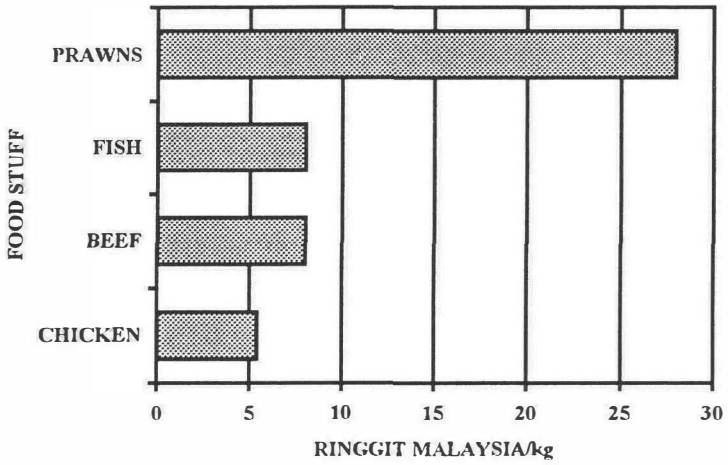


Figure 1: Comparison of Production, Consumption and Prices of Local Meat Sources. a) Production and Consumption of Animal Products in 1990 (DVS Livestock Statistics, 1992) and b) Prices of Common Food Source in 1998 (The Star, 1998).

The greatest threat in the poultry industry, not only in Malaysia but also in Asia is Newcastle disease virus (NDV) that is responsible for 314 outbreak cases recorded in Malaysia between 1985 to 1987. In 1976, NDV caused the US a loss of around 215 million US dollars wherelse in Malaysia between 1973 to 1977, 9% of all mortality costing the country 3.88 million ringgit annually was due to NDV.

Vaccination appears to be the main control of poultry viral disease in Malaysia and other developed as well as developing countries but NDV being endemic in this region, still poses a threat despite routine vaccination programmes which cost 18 to 50 % of the DVS annual budget (1992). It cannot be denied that current vaccines (live attenuated or killed) and local feed-delivered vaccines for village chickens; a common source of NDV epidemics as well as the readily available poultry health like vaccines and production technology from overseas have contributed towards improving the control of the disease. Nevertheless, these approaches alone may prove inadequate for Asia's rapid developing poultry industry to achieve long term stability and self-sustainability.

Therefore, efforts are being made with the aide of the ever advancing recombinant DNA technology and molecular biology for the development of more suitable vaccines derived from local NDV strains which would further complement the needs of the country's poultry industry. The approach would also cater for a more effective improvement of viral disease control through research by utilising available local resources. The growing interest and application of this technology has made possible the identification of specific sites on the surface of the virus which are crucial for inducing protective immune response. Taking advantage of this fact,

the production of vaccines from infectious agent which would not replicate as the pathogenic agent but able to induce immunity without causing any risk of increased or altered pathogenicity upon exposure to infection may bring about a more effective vaccine

In this study, a subunit vaccine was developed through the cloning of the HN gene from NDV strain AF2240, isolated from the viral genomic nucleic acid, into a Baculovirus Expression Vector System (BEVS) The BEVS is capable of expressing high levels of recombinant viral protein complete with post-translational modification in insect cells It will also facilitate the study of the HN gene expression in isolation from the rest of the NDV structural genes Thus the objectives of this study are -

1 to isolate the HN gene using PCR methods,

2 to clone the HN gene into BEVS,

3 to express the recombinant HN protein in *SF9* insect cells, and

4 to determine the functionality of the recombinant protein

CHAPTER II

LITERATURE REVIEW

Newcastle Disease Virus

Taxonomic Classification

Newcastle disease virus (NDV) is the aetiological agent of the Newcastle disease, one of the most serious infectious disease of poultry in many parts of the world including Malaysia The reclassification of virus taxa in 1993 by the International Committee in the Taxonomy of Viruses (ICTV) grouped the NDV species into the *Rubulavirus* genus of the *Paramyxoviridae* family in the order of *Mononegavirales* (Murphy *et al.*, 1995, Pringle, 1998) The other genera under the *Paramyxoviridae* family are the *Morbillivirus* and the *Paramyxovirus* where NDV was earlier classified together with mammalian parainfluenza virus type 1-5 and mumps virus NDV is the prototype of the genus and is the only member of the avian paramyxovirus -1 (PMV-1) serotype Various other groups which are serologically distinguishable from NDV have been isolated from avian species and grouped into eight other serotypes called PMV-2 to PMV-9 (Alexander, 1986)

History and Distribution of NDV

The first outbreak of NDV is known to occur in 1926 on the island of Java, Indonesia called Batavia showing high mortality (Brandly, 1964) At the same period, Doyle (1927) and Konno *et al* (1929) (cited in Brandly, 1964) reported a similar disease in Korea and in Newcastle-on-Tyne, where the common name of the disease was coined. Three years later, NDV spread throughout the entire Southeast Asia (Lancaster, 1966) where it is now endemic (Aini, 1993), Australia (Johnstone, 1931) and India (Brandly, 1964) Within 10 years, ND had been reported in Japan, Africa and subsequently in various other countries such as the Middle East as well as in Europe (Lancaster, 1966) Apparently, ND was only first reported in the US in 1944 (Beach, 1944), Canada in 1948 and in South America in the 1950s (cited in Beard, 1984)

Newcastle Diease Virus and the Malaysia Scenario

In Peninsular Malaysia, the incidence of an ND outbreak was reported at Parit Buntar, Perak in 1934 causing high mortalities in susceptible chickens with necrotic lesions, respiratory signs, diarrhoea and eventually death (Whitworth, 1934, cited in Lim, 1994) Within a few years, it had spread to other states like Selangor, Melaka, Kedah and Johor (Wallace, 1939, cited in Lim, 1994) In contrast, the virulent ND is known to be associated with two sources namely wild bird species like psittacine birds and chickens The highly virulent virus termed as velogenic viscerotropic Newcastle disease (VVND) virus is from the earlier source that killed domestic chickens within 2 to 4 days upon exposure (Lim, 1994) Pearson *et al* (1975) (cited