UNIVERSITI PUTRA MALAYSIA

CLONING AND EXPRESSION OF THE HAEMAGGLUTININ-NEURAMINIDASE GENE OF NEWCASTLE DISEASE VIRUS STRAIN AF2240 INTO *Eschericia coli*

SUDANI BT. HJ. SUDIN

FSAS 1997 15

CLONING AND EXPRESSION OF THE HAEMAGGLUTININ-NEURAMINIDASE GENE OF NEWCASTLE DISEASE VIRUS STRAIN AF2240 INTO *Eschericia coli*

by

SUDANI BT. HJ. SUDIN

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Enviromental Studies, Universiti Putra Malaysia.

May 1997

ACKNOWLEDGEMENTS

In the name of ALLAH swt, for His most gracefulness and most mercifulness.

I would like to express my deepest appreciation to Assoc. Prof. Dr. Khatijah Mohd. Yusoff for her valuable guidance and knowledge throughout this project. Thanks a million for being a very patient and understanding supervisor. I am also indebted to Assoc. Prof. Dr. Norani Abd. Samad and Assoc. Prof. Dr. Abdullah Sipat for the access to use all the facilities in Lab 143 and Lab 202.

I would also like to thank Encik Husin, Pak Pin, Kak Zuridah, Kak Raja Nor, Najah, Mazidah, Kak Norwati, Aizan, Geok Yong, Ng Ban Kim, Nafizah and Corina for always coming to my aid when I needed help. Not forgetting, Mr. Ng Chong Sin and Jullian from Bio-Diagnostic Sdn. Bhd. who were always ready to solve any technical problems.

To my dearest husband, Khusairi bin Mohamed, my children, Nur Quraishah and Muhammad Syafiq, and my parents, thanks for all the support, faith and love. Always being there for me has given me the courage and

strength to complete this course.

This study was supported by IRPA grants 1-07-05-027 and 01-02-04-0107.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

cDNA - complementary deoxyribonucleic acid

°C - degrees centrigrade

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphate

ddNTP - dideoxynucleotide triphosphates

dsDNA - double-stranded DNA

dUMP - deoxyuridine monophosphate

EDTA - Ethylenediaminetetraacetic acid disodium salt

F - fusion protein

g - gram

h - hour

HA - haemagglutinating activity

HN - haemagglutinin-neuraminidase

IPTG - Isoprophylthio-β-D-galactoside

kb - kilobase

kDa - kilodalton

kPa - kilopascal

l - litre

M - Molar

Mab - monoclonal antibody

MDT - mean death time

min - minute

ml - mililitre

mM - milimolar

Mr - molecular weight

NA - neuraminidase activity

Ncm - nitrocellulose membrane

ND - Newcastle disease

NDV - Newcastle disease virus

ORF - open reading frame

PBS - phosphate saline buffer

PCR - Polymerase chain reaction

pH - *Puissance hydrogene*

RBC - red blood cells

RNA - ribonucleic acid

RNAsin - RNase inhibitor

RNA-PCR - reverse transcription of RNA followed by the Polymerase chain reaction

s - second

SDS - sodium dodecyl sulphate

SDS-PAGE - SDS-polyacrylamide gel electrophoresis

ssDNA - single-stranded DNA

T_a - annealing temperature

T_m - melting temperature

Taq - *Thermus aquaticus*

TBE	-	Tris-boric-EDTA buffer
TEMED	-	tetramethylethylenediamine
UPM	-	Universiti Putra Malaysia
U	-	unit
ml	-	microlitre
V	-	volt
v	-	volume
vRNA	-	viral RNA
v/v	-	volume/volume
W	-	Watt
xg	-	centrifugal force
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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

CLONING AND EXPRESSION OF THE HAEMAGGLUTININ-NEURAMINIDASE GENE OF NEWCASTLE DISEASE VIRUS STRAIN AF2240 INTO *Eschericia coli*

By

SUDANI BT. HJ. SUDIN

MAY 1997

Chairman : Assoc. Prof. Dr. Khatijah Mohd. Yusoff

Faculty : Science and Enviromental Studies

A 1815 bp sequence of the open reading frame (ORF) of the haemagglutinin-neuraminidase (HN) gene of velogenic-viscerotropic Newcastle disease virus (NDV) strain AF2240 was amplified reaction (PCR) using primers S1 (5'CACCAATAGCAGACTCCA3') and S2 (5'CCTTGGCATTGCAGAAG3'). Both primers had dUMP extensions which allowed direct cloning of the amplified product into the pAMP1 vector, which also has the complementary protruding termini.

The recombinant plasmid was transformed into competent *Eschericia coli* strain DH5α. A total of 174 transformants were obtained and the transformation efficiency was 3.8×10^4 CFU/μg. The presence of the HN specific inserts in the transformants were detected by colony hybridization, Southern blotting and finally nucleotide sequence analysis.

The HN protein was shown to be expressed as an unglycosylated protein of size 63.0 kDa which is comparable to that predicted from the nucleotide sequence. Thus, this shows that the HN protein was succesfully expressed from the *E. coli* and the protein obtained could be used for further analysis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan Ijazah Master Sains.

PENGKLONAN DAN PENGEKSPRESAN GEN HAEMAGGLUTININ-NEURAMINIDASE DARIPADA VIRUS NEWCASTLE DISEASE STRAIN AF2240 KE DALAM *Eschericia coli.*

Oleh

SUDANI BT. HJ. SUDIN

MAY 1997

Pengerusi : Prof. Madya Dr. Khatijah Mohd Yusoff

Fakulti : Sains dan Pengajian Alam Sekitar

Sepanjang 1815 nukleotida rangka pembacaan terbuka bagi gen haemagglutinin-neuraminidase (HN) dari virus Newcastle disease strain AF2240 telah diamplifikasikan melalui teknik tindakbalas rantai polimerase (PCR) dengan menggunakan primer-primer S1 (5'CACCAATAGCAGACTCCA3') dan S2 (5'CCTTGGCATTGCAAGAAG3'). Kedua-dua primer mempunyai jujukan tambahan yang terdiri daripada residu-residu dUMP yang membolehkan pengklonan secara terus hasil-hasil amplifikasi ke dalam vector pAMP1, yang juga mempunyai hujung-hujung yang berkomplementari dengan hasil-hasil PCR yang diperolehi.

Plasmid-plasmid rekombinan yang terbentuk telah ditransformasikan ke dalam *Eschericia coli* strain DH5α. Sejumlah 174 transforman telah diperolehi yang mencatatkan kejayaan transformasi sebanyak 3.8×10^4 CFU/μg. Kaedah-kaedah penghibridan koloni, pemblotan Southern dan penjujukan nukleotida telah dijalankan untuk mengenalpasti kehadiran DNA selitan di dalam vektor-vektor tersebut.

Protein HN yang telah diekspreskan daripada *E. coli* merupakan protein yang tidak menjalani proses glikosilasi dan mempunyai berat molekul 63.0 kDa, sepertimana yang telah dianggarkan daripada jujukan nukleotida gen tersebut. Maka, ini menunjukkan bahawa protein HN ini telah berjaya diekspreskan dan analisa-analisa selanjutnya boleh dijalankan ke atas protein tersebut.

CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is one of the most important viral disease of poultry in many parts of the world, including South East Asia and it has a devastating effect on commercial poultry production. In 1971 an outbreak of ND in California, USA, inflicted US$56 million worth of damage -about US$400 million in today's terms - on the local poultry industry. The outbreak resulted in the slaughter of 12 million birds. The same disease also costs Indonesia more than US$100 million per year in control programmes and losses to the local industry. In Malaysia, this disease is known as `sampar ayam'. Hashim (1993) reported that the total losses due to this disease in the village chicken poultry was 15%. The eggs produced were small in size and with low albumin quality.

The causative agent of this highly infectious disease is the Newcastle disease virus (NDV)which possesses two glycoprotein spikes : the haemagglutinin - neuraminidase (HN) and fusion (F) proteins on its surface. These glycoproteins play an important role in infection, as well as elicitation of protective immunity in hosts (Walsh et al., 1987). The F protein is synthesized as a precursor, F_o, and is subsequently cleaved into F_1

and F_2 components which are held together by disulphide bonds. Generation of F_1 and F_2 is required for the F function in cell-to-cell fusion, hemolysis and virus penetration (Nagai et al., 1976, Hsu et al., 1979; Merz et al., 1981). The HN glycoprotein which carries both haemagglutinating and neuraminidase activities (Sugawara et al., 1982), is involved in virus binding to host cell receptors (Tozawa et al., 1973; Shimizu et al., 1984).

Various vaccination strategies have been used for the prevention and control of the disease. These include immunization with inactivated virus ; infection with avirulent strains and infection with naturally occuring mild lentogenic viruses. One of the main thrusts of molecular biology is the development of subunit vaccines or vaccines which consist of viral proteins or peptides free from genomic nucleic acids. This technology , in conjunction with several others, has allowed the identification sites on the virion surface that are important for inducing protective immune response. These protective antigens may be produced in bulk quantities by transferring and cloning the corresponding genes into suitable hosts or by chemical synthesis if the amino acid sequences are known. These genetically engineered vaccines are potentially purer, safer, cheaper and posseses greater efficacy than many currently used vaccines.

Velogenic viscerotropic NDV strain AF2240 which is used throughout this study is one of the most virulent strain, often causing 100% mortality in

susceptible chicken flocks (Lai, 1985). This strain is a local isolate which has been adapted to the tropical environment and is resistant to 60°C. Therefore it has the potential to be used in the production of a recombinant vaccine against ND in Malaysia. The nucleotide sequence of the HN gene of strain AF2240 was determined by direct RNA sequencing and confirmed by cycle sequencing (Tan et al., 1995). It is 1998 nucleotides long with a single open reading frame that encodes a putative protein of 581 amino acids with a calculated M*r* of 63.8 kDa and five asparagine glycosylation sites. Comparisons of the AF2240 HN protein sequence with other previously published sequence showed 88% homology. This HN protein is unique because of its length and cannot be grouped under the proposed three sizes classes of HN proteins in NDV (Sakaguchi et al., 1989).

This study was carried out to clone the HN gene of NDV strain AF2240 as a preliminary step to an alternative vaccine preparation to live attenuated strains of NDV and it will also provide further opportunities to study the structure and function of the HN protein. Thus, the objectives of this study are :

1. to amplify the HN gene via the Polymerase Chain Reaction (PCR)
2. to clone the HN gene into an expression vector and transform it into *Eschericia coli* strain DH5α; and
3. to express the HN protein

CHAPTER II

LITERATURE REVIEW

Newcastle Disease Virus

Newcastle disease virus (NDV) is the causative agent of ND. It is designated as avian paramyxovirus 1 (A-PMV1), formerly the prototype in the genus *Paramyxovirus* but it is now classified under the genus *Rubulavirus* in the *Paramyxoviridae* family (Table 1).

First reported on a farm near Newcastle-upon-Tyne early this century (Alexander, 1988), NDV has since been known to cause three global pandemics : the first from 1926 to 1934 which spread to most countries in the world, the second from 1965 to 1972 in the Middle East and the third occurred recently in the 1980's and related to a mainly neurotropic disease of racing pigeons, caused by an NDV strain which is distinguishable from other strains by monoclonal antibodies (Alexander, 1985).

ND occurs in domestic fowls, turkeys, pheasants, pigeons, quail and guinea fowl. Ducks and geese are also susceptible, though less so, as are wild

Table 1

Classification of viruses in the family *Paramyxoviridae*; subfamily *Paramyxovirinae*

GENUS	HUMAN PATHOGENS	ANIMAL PATHOGENS
Paramyxovirus	Parainfluenza virus types 1, 3 & 4	Bovine parainfluenza virus 3 Sendai virus Simian parainfluenza virus 10
Morbillivirus	Measles virus	Canine distemper virus Rinderpest virus Peste-des- petits-ruminants virus
Rubulavirus	Mumps virus Parainfluenza virus types 2, 4a & 4b	Avian paramyxovirus 2,3,4,5,6,7,8 & 9 Newcastle disease virus (A-PMV 1) Porcine rubulavirus Simian parainfluenza virus 5 & 41

Source : Murphy et al., 1995.

bird species. The virus can cause mild conjunctivitis in humans although sometimes it may be severe in poultry farmers, abattoir staff, vaccinators and laboratory workers. However, there is no evidence of transmission from humans-to-birds (Alexander, 1988).

Different strains of NDV exhibit a full range of symptoms, from asymptomic infection to almost 100% mortality of infected birds (Alexander, 1985). Initial symptoms of highly virulent isolates include loss of appetite, drop in egg production, green diarrhoea, swelling of the head and blue-ish comb. Mortality is close to 100% and many birds die within a day or two; birds that survive the initial phase often develop nervous signs. Lower virulence isolates cause coughing, gasping, wing and leg paralysis, weight loss, drop in egg production, head tremors and perhaps nervous signs at later stage. Avirulent NDV outbreaks may manifest as respiratory symptoms, lack of appetite and a drop in egg production.

Morphological and Physical Properties

Plate 1 shows negatively stained NDV virions and Fig. 1 shows the schematic diagram of the probable arrangement of the viral structural components. The virion can be viewed as consisting of two structural units : i) the ribonucleoprotein or the nucleocapsid and ii) the envelope proteins with its

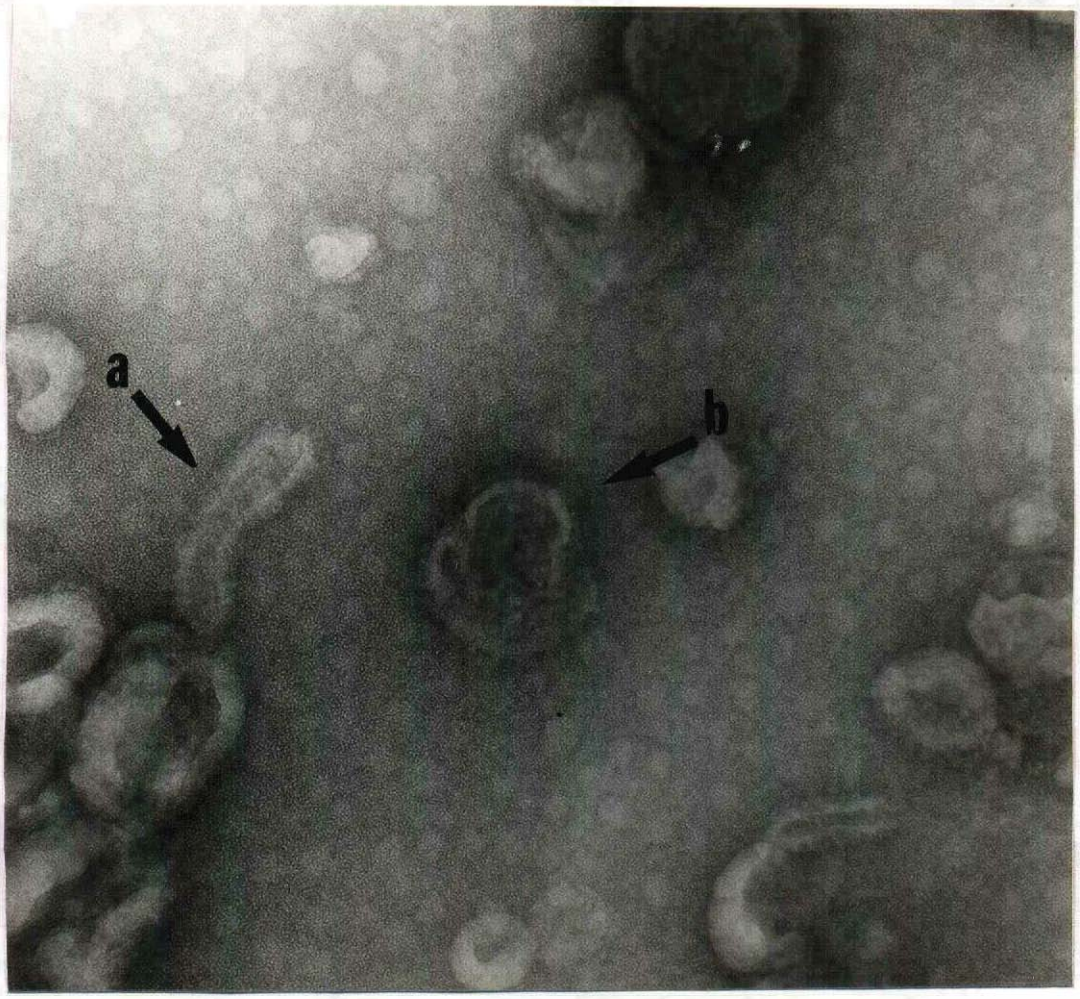

Plate 1 : The pleomorphic forms of negatively stained NDV strain AF2240.
(a) Filamentous
(b) Spherical