UNIVERSITI PUTRA MALAYSIA

SEQUENCING, CLONING AND EXPRESSION OF THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN GENE OF STRAIN AF2240

OMEIMA SALIH MOHAMMED

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SEQUENCING, CLONING AND EXPRESSION OF THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN GENE OF STRAIN AF2240

By

OMEIMA SALIH MOHAMMED

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

August 1999

Especially to……………..

my husband, daughters, parents, sisters and brother

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LIST OF ABBREVIATIONS

AcMNPV	*Autographa califomica* multiple nuclear polyhedrosis virus
BCIP	bromo-4-chloro-3-indolyl phosphate
bp	base pair
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNTP	deoxyribonucleotides
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
h	hour
HAU	haemagglutination unit
kDa	kilodalton
l	liter
mg	miligram
min	minute
ml	mililiter
mM	milimolar
Mab	monoclonal antibody
m o i	multiplicity of infection
NBT	nitroblue tetrazolium
NDV	Newcastle disease virus
ng	nanogram
NTE	natrium chloride-Tris-EDTA buffer
OD	optical density
PBS	phosphate buffer saline
PEG	polyethylene glycol
PFU	plaque forming units
pmol/µl	picomol per microliter
RT-PCR	reverse transcriptase-polymerase chain reaction

SDS-PAGE	sodium dodecyle sulphate-polyacrylamide gel electrophoresis
SF	serum free
SS	serum supplemented
Sf9	*Spodopetra frugiperda* cells
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TEMED	tetramethylethylenediamine
TNM-FH	*Trichoplusia ni* medium-formulation hink
TTBS	tween 20 tris-natrium chloride buffer
U	unit (s)
V	volt
V/V	volume per volume
W	watt
W/V	weight per volume
%	percentage
°C	degrees centigrate

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

SEQUENCING, CLONING AND EXPRESSION OF THE NEWCASTLE DISEASE VIRUS FUSION (F) PROTEIN GENE OF STRAIN AF2240.

By

OMEIMA SALIH MOHAMMED

August 1999

Chairperson: Associate Professor Khatijah Mohamad Yusoff, Ph.D.

Faculty: Science and Environmental Studies

Newcastle disease is a highly contagious viral disease of poultry and has greater than 95 % morbidity and mortality. The etiological agent, Newcastle disease virus (NDV), is a member of the family *Paramyxoviridae* and the genus *Rubulavirus.* Immune responses to the fusion protein (F) antigen of NDV were demonstrated to play an important role in the prevention of infection. Accordingly, the F gene of a Malaysian heat-resistant NDV velogenic-viscerotropic strain AF2240 was amplified and cloned into pCR^{TM}2.1 vector. Its sequence was determined by sequencing of the cloned F gene RT-PCR products. It contains 1791 nucleotides and encodes a large ORF of 553 amino acids. The deduced amino acid sequences of the F_0 precursor protein has a pair of dibasic residues with an intervening glutamine (Q) at the proteolytic site, three hydrophobic regions and six asparginine glycosylation sites. The cleavage-activation site as well as part of the fusion-inducing sequence were compared among a series of virulent and avirulent NDV strains using multiple

sequence alignment program The sequence analysis grouped the AF2240 strain of NDV among the highly virulent strains based on the molecular data

The gene encoding the F protein was subcloned into *baculovirus* expression vectors, pBlueBacHis2 and pCR^{TM}Bac, derived from AcMNPV genomic DNA Upon co-transfection of the respective plasmids with Bac-N-Blue linearized DNA in Sf9 cells, a recombinant rBacF *baculovirus* was verified by PCR, electron micrographs and immunoassay of the F protein At the transcription level, the mRNA of the F protein were detected by RT-PCR analysis using the F gene specific primers In Sf9 cells infected with rBacF recombinant *baculovirus*, the expressed F protein was properly located onto the cell surface as revealed by the immunofluoresence test In SDS-PAGE analysis, a protein band that had a molecular weight of 64 kDa, which corresponds to the precursor F_0 protein, was detected on the immunoblots This result shows that the expressed F protein was not cleaved in Sf9 cells However, sequence analysis of the cleavage site of rBacF cloned F gene revealed 100% homology to the authentic NDV F gene that rules out any mutations at this site

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PENJUJUKAN, PENGKLONAN DAN PENGEKSPRESAN GEN PROTEIN FUSION (F) VIRUS NEWCASTLE DISEASE (NDV) DARI STRAIN AF2240.

Oleh

OMEIMA SALIH MOHAMMED

Ogos 1999

Pengerusi: Profesor Madya Khatijah Mohamad Yusoff, Ph.D.

Fakulti: Sains dan Pengajian Alam Sekitar

Penyakit Newcastle atau pun penyakit sampar ayam adalah suatu penyakit virus ayam yang sangat berjangkit dan menunjukkan lebih daripada 95 % morbiditi dan mortaliti. Agen etiologinya, iaitu virus Newcastle disease (NDV), adalah anggota famili Paramyxoviridae dan genus Rubulavirus. Tindak balas imun terhadap antigen protein pertaupan (F) NDV menunjukkan peranan yang penting di dalam pencegahan jangkitan. Justeru itu, gen F strain AF2240 NDV Malaysia yang berjenis velogenik-viserotropik telah di amplifikasikan dan diklonkan ke dalam vektor pCR™2.1. Jujukannya ditentukan melalui penjujukan produk RT-PCR gen F yang terklon. Ia mengandungi 1791 nukleotida dan mengkodkan satu rangkai pembacaan terbuka (ORF) besar yang mempunyai 553 asid amino. Jujukan asid amino protein prekursor F_0 mempunyai sepasang residu dwibes berdampingan dengan glutamina (Q) pada tapak tindak balas proteolisis, tiga kawasan hidrofobik dan enam tapak glikosilasi asparigina. Tapak potongan-pengaktifan serta sebahagian jujukan pendorong pertaupan dibandingkan di antara siri strain-strain

NDV virulens dan tak virulens melalui program susunan jujukan berganda. Analisis jujukan tersebut meletakkan strain AF2240 NDV diantara strain-strain yang paling virulens berdasarkan data biologi molekulnya.

Gen yang mengkodkan protein F disubklonkan ke dalam vektor pengekspresan bakulovirus, pBlueBacHis2 dan pCRTMBac, yang berasal daripada DNA genom AcMNPV. Selepas ko-transfeksi dengan plasmid DNA bebenang tunggal Bac-N-Blue ke dalam sel Sf9, satu rekombinan bakulovirus rBacF ditentukan melalui PCR, mikrograf elektron dan pengasaian-imuno protein F. Pada tahap transkripsi, mRNA protein F melalui analisis RT-PCR dengan menggunakan primer yang spesifik kepada gen F. Dalam sel Sf9 yang dijangkiti dengan bakulovirus rekombinan rBacF, protein F yang telah diekspreskan itu terletak pada permukaan sel seperti yang ditunjuk oleh ujian imunopendarfluoran. Dalam analisis SDS-PAGE, satu jalur protein yang mempunyai berat molekul 64 kDa, yang berpadan dengan protein prekursor F_0, dikesan melalui sapimuno. Keputusan ini menunjukkan bahawa protein yang telah diekspreskan itu tidak terpotong di dalam sel Sf9. Walaubagaimana pun jujukan yang terdapat pada tapak potongan dalam gen F yang terklon menunjukkan 100 % homologi dengan gen F yang asal dan ini menunjukkan bahawa tiada mutasi yang terlibat pada tapak tersebut.

CHAPTER I

INTRODUCTION

Newcastle disease (ND) is a highly contagious infection of many avian species caused by the Newcastle disease virus (NDV), the only member of the avian *paramyxovirus* I (APMV-I) serotype. The infectious virus may be transmitted by ingestion or inhalation which is the basis of mass application vaccination procedures for poultry (Meulmanns, 1988). The virus has a wide host range infecting 27 of the 50 orders of birds with variable clinical severity and transmissibility (Fenner *et al*., 1987; Kaleta and Baldauf, 1988). NDV occurs worldwide and has a considerable economic impact on the Malaysian poultry industry ranging from losses due to disease and the expense of vaccination to the significant cost of diagnostic laboratory investigations. In Malaysia, the heat resistant viscerotropic-velogenic NDV strain AF2240 was first isolated from a local field outbreak in the 1960's and it was reported to cause high morbidity and mortality in poultry (Lai and Ibrahim, 1987). This strain is resistant to the temperature of 60°C and has the potential to be used in the production of a recombinant vaccine against the disease in the tropics.

In general, NDV has a single-stranded, non-segmented negative-sense genomic RNA of approximately 15 kb that codes for at least six viral proteins. Of these, the fusion glycoprotein (F) appears to be an ideal antigen for diagnostic and immunological purposes, as anti-sera from NDV-infected birds contain high levels of antibody to the F protein (Umino *et al*., 1990). Functionally, the F protein is required for cell-to-cell fusion, haemolysis and virus penetration (Merz *et al*., 1981). Proteolytic cleavage of the inactive F_0 precursor into two disulphide-linked subunits, F_1 and F_2, is necessary for infection *in*

vivo. Monospecific antibodies to this glycoprotein were shown to neutralize viral infectivity (Scheid and Choppin, 1977).

Furthermore, NDV isolates are characterized by pathogenesis in chicken and may be categorized into three main pathotypes, depending on the severity of the isolate (Alexander, 1988; 1991). Lentogenic isolates are of low virulence and cause mild respiratory or enteric infections. Viruses of intermediate virulence that cause primarily respiratory disease are mesogenic, while virulent viruses that cause high mortality are velogenic. Velogenic forms of NDV are further classified either as neurotropic or viscerotropic based on their clinical manifestations (Alexander, 1991). In general, evolution of virulence among the different pathotypes appears to involve a variety of mechanisms including mutations in regulatory regions (Wimmer *et al*., 1993) and viral adaptation for utilization of alternative or expanded repertoires of cellular receptors. There is increasing evidence that viruses utilize coreceptors and alternative receptors for their entry into cells, and that this may be an important determinant of virulence. In general, the mechanisms of RNA virus variation that leads to different pathotypes include mutation, homologous and nonhomologous recombinations, and genome segment reassortment (Wain-Hobson, 1996). This is mainly due to viral RNA polymerase which has a higher chance of jumping onto another template molecule to generate recombinant progeny by a copy-choice mechanism (Lai, 1992). This continuous production of mutants favors adaptability of the virus in the event of environmental changes. Examples are the specific mutations in variant viruses that are resistant to neutralizing antibodies (Borrego *et al*., 1993) or to some antiviral inhibitors. However, the availability of procedures for the amplification and sequencing of viral genomes present in biological specimens has resulted in the production of huge amounts of sequence information, as well as in the definition and establishment of

phylogenetic relationships among virus genera and among individual isolates of a genus. Several lines of evidence suggest that RNA virus evolution is constrained by the complexity of viral functions in interaction with cellular functions (Domingo and Holland, 1997).

Despite their simplicity, viruses are sophisticated organisms that have evolved highly efficient techniques for infecting cells, expressing their genomes and generating new copies of themselves. The development of recombinant DNA techniques has made it possible to exploit these properties. Of these, the *baculovirus* expression vector system is very commonly used for foreign gene expression (Kidd and Emery, 1993). Its attraction lies in the high yields of foreign gene products and the eukaryotic environment for post-translational modification provided by the insect host cell (Luckow, 1991). It is now possible to manipulate *baculoviruses* genetically, and recombinant viruses expressing novel proteins are being used as important research tools to study the structure and function of these proteins; they might also be used to deliver selected genes into higher organisms for use in gene therapy, vaccines and immunotherapy. Accordingly, as a live recombinant vaccine, the *baculovirus* expression system provides a method for the production of large quantities of biologically active and antigenic NDV F protein for both research and diagnostic applications (Mori *et al.*, 1994).

Due to the differences among NDV strains as reflected by their ability to induce disease in chickens and to replicate in different tissues, it is important to further define genetic heterogeneity that may occur and to possibly identify the vaccine types from which this field isolate is derived through gene tree analysis. Furthermore, as a preliminary step in the production of a subunit vaccine from the F glycoprotein, its gene

expression in *baculovirus* will motivate its use as a safe vaccine instead of the commercial live or killed/attenuated NDV vaccines.

Therefore, the objectives of this study are:

1. to determine and analyse the nucleotide sequence of the F glycoprotein gene of NDV strain AF2240;
2. to compare the phylogeny of strain AF2240 with the other NDV strains;
3. to clone the F gene into a *baculovirus* expression vector system; and
4. to partially analyse the expressed F protein.

UPM

CHAPTER II

LITERATURE REVIEW

Newcastle Disease

Historical Aspects

Paramyxoviruses have a wide geographic distribution and include many important human and animal respiratory viruses, some of which are extremely pathogenic and cause high morbidity and mortality in the infected host species. However, Newcastle disease (ND) remains a major problem for the poultry industry worldwide (Alexander, 1991). This disease was first recognised as a viral infection after the epidemic outbreak at Newcastle-upon-Tyne in England from where it derived its name (Doyle, 1927). Since then both viscerotropic and neurotropic types of velogenic Newcastle disease virus (NDV) have been found worldwide (Spradbrow, 1988; Alexander, 1991).

Asia appears to be the home of NDV, with the first report coming in 1926 from Batavia (now known as Jakarta). Most of the other early reports in 1926 or 1927 were from Asia. In fact, ND has been described as endemic throughout Southeast Asia and the most important of the viral diseases of poultry in the region since 1982 (Shortridge, 1982; Cheng-Yao *et al.,* 1997). In Malaysia, from 1973 to 1977, the different NDV strains were reported to cause 90% mortality in chickens costing the country 3.88 million ringgit Malaysia. However, the velogenic viscerotropic strain AF2240 has been shown to be responsible for highest morbidity

and mortality among the poultry flocks. The virus was isolated from a local field outbreak in the early 1960s (Lai and Ibrahim, 1987).

Newcastle Disease Virus

NDV is the aetiological agent for the devastating disease of poultry more commonly known as fowl pest, characterized by infection of the upper respiratory tract and haemorrhagic enteritis and tracheitis. Velogenic strains have a mortality rate greater than 90% (Errington *et al.*, 1995). The virus family *Paramyxoviridae* consists of enveloped RNA viruses that undergo capsid assembly in the cytoplasm and are budded from the cell surface in an envelope of modified cell membrane (Melnick, 1982). NDV has recently been reclassified into the genus *Rubulavirus* which also includes mumps virus, simian virus 5 (SV5) and human parainfluenza virus (Murphy *et al.*, 1996).

NDV are large pleomorphic membrane enveloped virions of roughly spherical shape ranging in size from 150-400 nm which contain a long helical nucleocapsid structure. The envelope is covered with spike glycoproteins (haemagglutinin neuramindase (HN) and fusion (F) proteins) that are 8-12 nm long (Figure 1) (Choppin and Compans, 1975).

Furthermore, the NDV genome is a single stranded RNA of negative sense and has a molecular weight of 5.2-5.7 x 10^6 daltons which is approximately 15 kilobases (kb) (Dahlberg and Simon, 1969). This RNA genome codes for six main structural proteins. The virion core contains a helical nucleocapsid which comprise

Figure 1: Newcastle disease electron monograph.

NDV electron monograph at a standard magnification of x250000 using UPM EM. Virions are enveloped, 60-300 nm in diameter and 1000-10000 nm long (bar 100 nm). The arrow points to surface projections on the envelope, spikes (HN and F glycoproteins) 9-15 nm long, spaced 7-10 nm apart.

the nucleic acid, the majority nucleocapsid protein (NP), the less abundant phosphoprotein (P) and a few molecules of the large protein (L). The core is surrounded by the matrix protein (M) that interacts with the two membrane glycoproteins, the HN and the F proteins. Additionally, the P gene is edited during transcription via the addition of a single G residue at position 484 to produce the V protein (Steward *et al*., 1993). The NP protein is involved in the packaging of the viral RNA and in RNA replication in conjunction with the L and P proteins. Unlike SV5 and mumps virus, NDV does not encode the small hydrophobic (SH) protein between the F and HN genes (Murphy *et al*., 1996).

The virus carries its own RNA-dependent RNA polymerase, which is responsible for the transcription and replication of the viral genome in the infected cell. The genome of this virus is found in both virions and infected cells to be complexed with the viral nucleoprotein NP or N as ribonucleoprotein (RNP) complexes, and it is these RNP complexes, rather than naked viral RNA, that are the actual templates recognized by the viral RNA polymerase. Replication involves the synthesis of a replicative intermediate consisting of a complementary copy of the genome, known as the antigenome, which is also encapsidated. The genome is also used as template by the viral RNA polymerase to synthesize the viral messenger RNAs (mRNAs), which, in contrast to the antigenomes, are capped and polyadenylated (Figure 2) (Adolfo, 1998).

NDV Glycoproteins

For many *paramyxoviruses*, including NDV, syncitium formation requires the expression of both surface glycoproteins (HN and F) in the same cell and evidence