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NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS AS AN ANTIGENIC CARRIER

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By

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July 2002

Chairman : **Khatijah Mohd Yusoff , Ph.D**

Faculty: **Science and Environmental Studies**

Newcastle disease virus (NDV) is an economically important avian virus that causes loss to the poultry industry. It has a wide host range infecting 27 of the 50 orders of birds. Generally, the virus consists of six structural proteins: nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuramidase (HN) and large (L). The NP protein resembles the classical herringbone morphology when observed under electron microscope. However, the morphology changed into individual ring-like particles when the *myc* epitope and six histidine residues were fused to the C-terminal end of the protein. Further investigation showed that the C-terminus of this protein derivative is exposed on the surface of the ring-like particles. In this project, several chimeric proteins have been constructed in which the antigenic regions of the HN or F protein of NDV strain *AF2240*, *myc* epitope

and six histidine residues were linked to the C-terminus of the NP protein. The chimeric proteins were expressed efficiently in *Escherichia coli* as detected by Western blot analysis. Electron microscopic analysis on these proteins revealed that they assembled into ring-like particles. These chimeric NP proteins exhibited antigenicity of the *myc* epitope suggesting that the foreign sequences were exposed on the surface of the particles. Chickens vaccinated with the chimeric particles exhibited an immune response against NDV. However, no protection was observed when the vaccinated chickens were challenged by the virus.

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

PROTEIN NUCLEOKAPSID VIRUS PENYAKIT NEWCASTLE SEBAGAI PEMBAWA ANTIGEN

Oleh

AMIR RABU

Julai 2002

Pengerusi: **Khatijah Mohd. Yusoff, Ph.D**

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Virus penyakit Newcastle (NDV) merupakan virus avian yang penting yang menyebabkan kerugian terhadap perusahaan penternakan ayam. Virus ini mempunyai hos yang luas serta berupaya untuk mengjangkiti 27 daripada 50 order burung. Secara amnya, virus ini mempunyai enam jenis protein: nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuramidase (HN) dan large (L). Pemerhatian di bawah mikroskop elektron menunjukkan protein NP bagi virus ini bergabung membentuk morfologi tulang 'herring'. Walaubagaimanapun, morfologi ini berubah menjadi bentuk gegelang individu apabila epitop *myc* dan enam residu histidina dicantumkan kepada terminal-C protein tersebut. Kajian seterusnya mendapati terminal-C ini mengunjur keluar dari permukaan bentuk gegelang tersebut. Di dalam projek ini, beberapa protein 'chimeric' yang

mengandungi protein NP telah dibentuk dengan menggabungkan beberapa bahagian antigen dari protein HN atau F, epitop *myc* dan histidina residu ke terminal-C protein itu. Protein 'chimeric' ini telah berjaya diekspres dalam *Escherichia coli* dan boleh dikesan dengan pemblotan Western. Pengamatan dengan menggunakan mikroskop elecktron menunjukkan protein 'chimeric' ini juga membentuk gegelang individu itu. Kajian terhadap sifat antigen menunjukkan epitop *myc* pada protein ini dapat dikesan dengan antibodi. Ini mencadangkan protein yang digabungkan terdedah pada permukaan gegelang tersebut. Selain itu, ayam-ayam yang disuntik dengan protein 'chimeric' ini dapat menghasilkan antibodi terhadap virus penyakit Newcastle. Walaubagaimanapun, ayam-ayam ini tidak dapat dilindungi daripada jangkitan penyakit Newcastle ini.

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I certify that an Examination Committee met on 29th July 2002 to conduct the final examination of Amir Rabu on his Master of Science thesis entitled "Nucleocapsid Protein of Newcastlse Disease Virus as an Antigenic Carrier" 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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The thesis submitted to the Senate of Universiti Putra Malaysia 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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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

AMIR RABU

Date: 16 AUG 2002

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ABBREVIATIONS

A_{600}	absorbance at wavelength 600 nm
A_{540}	absorbance at wavelength 540 nm
ATP	adenosine triphosphate
α	alpha
β	beta
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
C-terminus	carboxy terminus
dH_2O	distilled water
dNTP	deoxyribonucleotide phosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELD_{50}	50% of egg lethal dose
ELISA	enzyme-linked immunosorbent assay
h	hour
HA	haemagglutination
HBcAg	hepatitis B core antigen
HI	haemagglutination inhibition
H_2O	water
IgG	immunoglobulin G
kb	kilobase
kDa	kilodalton
M	molar
MHC	major histocompatibility complex
min	minutes
mRNA	messenger RNA
µl	microliter (10^{-6} m)

nm	nanometer (10^{-9} m)
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
RNA	ribonucleic acid
s	seconds
SDS	sodium deocyl phosphate
SPF	specific pathogen free
$TCID_{50}$	50% tissue culture infectious dose
U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is a major disease that can cause huge losses to the poultry industries. The disease is caused by the Newcastle disease virus (NDV). Other diseases that threaten the industries are infectious bursal disease, influenza A, infectious bronchitis disease and Marek's disease. ND is controlled by using vaccines. This method of control is effective, reliable and has been used for quite a long time (Hilgers *et al.*, 1998).

There are several forms of vaccines used in controlling ND. The most widely used are the live and killed vaccines. These vaccines are usually made from the lentogenic viruses which do not cause any illness to the adult chicken. The strains of NDV that are usually used as vaccines are La Sota (Goldhaft, 1980), Mustekwar, Clone30 and B1 (Hitchner and Johnson, 1948). These vaccines are not only cheap but they can provide sufficient protection to the chicken against the virus. The vaccines are delivered through several ways. For example, the killed vaccines are usually delivered through injections. Simpler routes of vaccination are applied for the live vaccines. These vaccines are usually delivered orally, through aerosol, eye drop, piercing and even vent brush (Gallili and Nathan, 1998).

Even though the live and killed ND vaccines are successful and widely utilized around the world, studies are being conducted to enhance their efficiency and quality of these vaccines (Maas *et al.*, 1999). Such studies were carried out to improve numerous aspects of the vaccines such as the routes and doses of vaccination given to the chicken as well as production cost. The efficiency of most of the vaccines can be enhanced by delivering them with adjuvants. These adjuvants, however, must be safe and cheap. Examples of adjuvants normally used in the vaccination are oil emulsion and lipopolysaccharides (Hilgers *et al.*, 1998; Aucouturier *et al.*, 2001). Even though these live vaccines are effective, its safety is of high concern, due to the sporadic ND outbreak in Australia within vaccinated chicken flocks and also at locations near them. These outbreaks were often found to be caused by new isolates that originated from the lentogenic viruses (Gould *et al.*, 2001).

In order to improve these problems, the potential of using genetically engineered vaccines in controlling ND and other poultry diseases have been carried out. The vaccines involved in such studies are usually proteins as well as DNA. Sakaguchi *et al.* (1996) constructed a plasmid expressing the fusion (F) protein of NDV capable of inducing protective immunity when injected into chickens. Several recombinant vaccines have also been developed that provide protection against ND. Poxvirus-type vectors such as vaccinia (Meulemans *et al.*, 1988a; Nishino *et al.*, 1991), fowlpox (Boursnell *et al.*, 1990) and pigeon pox (Letellier *et al.*, 1991) expressing either the F or haemagglutinin-neuramidase (HN) proteins have been shown to be protective. In addition, a chimeric herpes virus of turkeys expressing the F and HN of NDV is protective against both

Marek's disease and ND (Morgan *et al.*, 1992; Hecket *et al.*, 1996; Reddy *et al.*, 1996). Most of these vaccines, which were developed for ND and other chicken diseases, reduced the vaccination time, cost and stress put on the chicken. Besides, there are research on the use of carriers to produce vaccines. The carriers can be fused to obtain chimeric antigens, possessing desired properties derived from the fusion partner which are added to the target antigen (LaVallie and McCoy, 1995; Nilsson *et al.*, 1997). The main objectives of using carriers are to simplify the production and purification of the recombinant vaccines (Nygren *et al.*, 1994).

Recently, the nucleocapsid (NP) protein of NDV was successfully obtained by expressing it in bacterial system (Kho *et al.*, 2001). The derivative of this NP protein, NP_{cfus}, which contains the *myc* epitope and six histidine residues fused to the C-terminus, was expressed in the same system. These fusion proteins were expressed in abundance as highly soluble and stable proteins which can be purified easily by sucrose gradient centrifugation. Both the NP and NP_{cfus} proteins self–assembled into ring-like particles. However, the NP_{cfus} did not form the herringbone-like structure as the NP protein, suggesting that the fusion of the *myc* epitope and histidine residues inhibited the herringbone-like particles formation. Further examination by immunogold labelling on the NP_{cfus} particles revealed that its C-terminus is exposed on the surface of the ring-like particles (Kho *et al.*, 2001).

Based on the above finding, it is obvious that the NP_{cfus} has the potential to be developed as a carrier for chimeric antigens. This protein has all the criteria needed to

become such a carrier. In addition, this protein is stable and its C-terminus is exposed on the surface of the protein, thus it is ideal for carrying any antigen.

Therefore, the objectives of this research were :

1. to investigate the potential of NP_{cfus} as an antigen carrier by fusing the epitopes of HN or F glycoproteins to the C-terminus of NP_{cfus};
2. to evaluate the capability of these chimeric NP proteins in inducing immune response in chickens; and
3. to determine whether the antibodies induced by such chimeric NP proteins can induce protection against NDV.

Chapter 2

Literature Review

Newcastle disease (ND) is a very contagious disease which causes the loss of million Ringgit Malaysia every year (Kho *et al.*, 1999). ND is caused by Newcastle disease virus (NDV). The virus is classified as a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* (Seal *et al*, 2000a; Yusoff and Tan, 2001). Initially, NDV was classified as the prototype of *Paramyxovirus*, but it was later classified as the member of *Rubulaviruses* in 1993 (Yusoff and Tan, 2001; Gould *et al*, 2001). In general, other members of the *Rubulaviruses* contain small hydrophobic (SH) gene which is deficient in NDV (Lamb and Kolakofsky, 1996) and based on this dissimilarity, it has been suggested that NDV be grouped as a separate member of *Paramyxovirinae* (de Leeuw and Peeters, 1999). NDV not only can infect chickens but it is known to be infectious to other types of birds such as turkey pigeon, ostrich and wild bird (Alexander, 1989; Samina *et al.*, 1999). Therefore, the virus has a broad range of host or reservoirs that can make it spread rapidly. Generally, chickens are the most susceptible to NDV infection compare to other avian species.

2.1. Occurrence of NDV

ND was first discovered in Jakarta, Indonesia in 1926. However, the first outbreak was reported by Doyle in Newcastle-Upon-Tyne during spring 1926

(Seal *et al.*, 2000a). Subsequently, more outbreaks were reported in several countries such as Philiphines, Korea, Sri Lanka and India (Alexander, 1988). Eventhough ND was only noticed in 1926, it was not a totally new disease. The emergence of the disease is believed to occur earlier than the first reported outbreak, but the occurrences were disregarded due to the lack of expertise in the disease and diagnostic tools for detection. Moreover, ND normally occurs along with other avian diseases making identification very difficult (Alexander, 1988). Since then, several techniques have been employed to overcome the disease and these were successful in its control. Nowadays, outbreaks of ND still occur worldwide, but these can be detected rapidly by various reliable methods such as polymerase chain reactions (PCR) and ELISA (Errington *et al.*, 1995; Makkay *et al.*, 1999).

2. 2. Pathotypes and the Importance

NDV can be divided according to their pathogenicity. The virus is mainly divided into three pathotypes (Gallili and Nathan, 1998; Romer-Obendorfer *et al.*, 1999). Viruses that cause severe disease and 100% mortality are termed velogenic strains. The velogenic strains are further separated into two subgroups namely, viscerostropic strains that induce haemorrhagic intestinal lesions, and the neurotropic strains that are responsible for acute respiratory and nervous disorders (Alexander 1989; Gallili and Nathan, 1998). The strain that causes moderate disease but seriously reduce the egg production are termed mesogenic (Peeters *et al.*, 2001). The strains normally cause only 50% mortality. The strains of low virulence are termed lentogenic which presents extremely low mortality

except for young chicks, where it can still cause deaths. The differences of these strains can be observed by mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) (Gallili and Nathan, 1998). Velogenic strains have an ICPI and IVPI ranging from 1.5 to 3.0 units, whereas mesogenic and lentogenic have an ICPI and IVPI values less than 1.0 unit. The differences are also observed on their mean time required to kill an embryonated egg (MDT). MDT for velogenic strains was less than 60 h. In contrast, MDT for lentogenic is more than 90 h, and for velogenic, the MDT is between 60 to 90 h (Alexander, 1989; Gallili and Nathan, 1998). These differences between the pathogenicity of these strains are shown in Table 2.1.

The pathogenicity is essential to identify the mode of actions of the virus because virus mortality is strain dependent. Furthermore, the virus has a broad host range, which can easily and rapidly transmit. The incubation period of the virus is less than a week. Birds may die without showing any clinical signs especially when infected with velogenic strains. Some of the birds may show gasping, muscular tremors, spasms, and even paralysis upon infections (Sakaguchi *et al.*, 1996). In the infection of the mesogenic strains, respiratory illness may dominate the clinical signs at the early phase. Later on, egg production falls dramatically. In the case of infection by lentogenic strains towards adult birds, no severe disease can be observed (Reynolds and Maraqa, 2000a). NDV is very infectious and chickens are usually very susceptible to the infection. Therefore, chickens can easily be infected and the infection with various strains of NDV not only kills them but also affects the egg production.