



UNIVERSITI PUTRA MALAYSIA

**DETECTION AND DIFFERENTIATION OF MALAYSIAN
NEWCASTLE DISEASE VIRUS ISOLATES BY
RNA-POLYMERASE CHAIN REACTION
AND CYCLE SEQUENCING**

NG BAN KIM

FSAS 1995 3

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RNA-POLYMERASE CHAIN REACTION
AND CYCLE SEQUENCING

By

NG BAN KIM

Thesis Submitted in Fulfilment of the Requirements for
the Degree of Master of Science in the Faculty of
Science and Environmental Studies,
Universiti Pertanian Malaysia

June 1995



**DEDICATED TO MY PARENT,
BROTHERS AND SISTERS**



ACKNOWLEDGEMENTS

I sincerely thank my supervisory committee members, Assoc. Prof. Dr. Khatijah Mohd. Yusoff (chairman), Assoc. Prof. Dr. Aini Ideris and Assoc. Prof. Dr. Norani Abd. Samad for their guidance and helpful discussion and advice throughout the course of my study.

I am very much grateful to Lau Chin Hoon for teaching me the cycle sequencing technique, for his encouragement and thoughtful comments during the process of research.

I appreciate the assistance of Adam Ahmad in preparing the chicken tissues and Abd Ghani Hashim for excellent photography.

This study was supported by IRPA Grant No. 1-07-05-027 and TWAS Grant No. 93-074 RG/BIO/AS.



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LIST OF ABBREVIATIONS

EID ₅₀	-	Egg infective dose 50%
ELISA	-	Enzyme linked immunosorbent assay
F	-	Fusion
HA	-	Haemagglutination
HI	-	Haemagglutination-inhibition
HN	-	Haemagglutinin-neuraminidase
ICPI	-	Intracerebral pathogenicity index
IVPI	-	Intravenous pathogenicity index
L	-	Large
Le	-	Lentogenic
M	-	Membrane
MDT	-	Mean death time
Me	-	Mesogenic
NAP	-	Nucleocapsid-associated protein
ND	-	Newcastle disease
NDV	-	Newcastle disease virus
NP	-	Nucleocapsid protein
PCR	-	Polymerase chain reaction
RNA-PCR	-	RNA-polymerase chain reaction
RNasin	-	RNase inhibitor
UPM	-	Universiti Pertanian Malaysia
Ve	-	Velogenic



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

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By

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June 1995

Chairman: Dr. Khatijah Mohd. Yusoff

Faculty: Science and Environmental Studies

This study was undertaken to develop diagnostic tests for Newcastle disease virus (NDV) based on RNA-polymerase chain reaction (RNA-PCR) and cycle sequencing techniques as a supplement to the presently available tests.

Two RNA-PCR cycle sequencing systems each targeted at the haemagglutinin-neuraminidase (HN) and fusion (F) genes were developed. RNA-PCR amplification of a 398 base pairs (bp) HN gene fragment was performed on total RNAs extracted from infected allantoic fluid of 9 Malaysian NDV field isolates, vaccine strain V4-UPM and velogenic strain AF2240. Sequence analysis over 113 bases in the amplified fragment showed variations among these isolates/strains. However, identical sequences were obtained from some of the field isolates within a particular pathotype and these were thought to be of the same strain of NDV.



RNA-PCR amplification of a 242 bp F gene fragment was performed on total RNAs which were isolated from several types of tissues (spleen, brain and lung) from six non-vaccinated chickens which were challenged with the velogenic strain AF2240. The spleen was found to have the highest number of samples positive by PCR. Spleens from uninfected chickens as well as those from vaccinated and challenged chickens which were slaughtered were all PCR negatives. The challenged virus was thought to have been neutralised by the antibodies produced by the chickens. The identity of the PCR products amplified from the spleens were confirmed by cycle sequencing. Similarly, this test was also performed on RNAs from infected allantoic fluid of 11 different NDV strains/isolates. These samples were also PCR positives.

The 18 nucleotide at the cleavage site of the F gene amplified fragment was sequenced for 4 NDV strains. The deduced amino acid sequences for strains AF2240 (velogenic) and S (mesogenic) were ¹¹²Arg-Arg-Gln-Arg/Lys-Arg-Phe¹¹⁷ and ¹¹²Gly-Arg/Lys-Gln-Gly-Arg-Leu¹¹⁷ for strains F (lentogenic) and V4-UPM (avirulent). Therefore, the velogenic and mesogenic strains could be distinguished from the lentogenic and avirulent strains by sequencing the cleavage site.



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

**PENGESANAN DAN PEMBEZAAN ISOLAT VIRUS NEWCASTLE DISEASE
MALAYSIA MELALUI TINDAKBALAS RANTAI POLIMERASE-RNA DAN
PENJUJUKAN KITARAN**

Oleh

NG BAN KIM

Jun 1995

Pengerusi: Dr. Khatijah Mohd. Yusoff

Fakulti: Sains dan Pengajian Alam Sekitar

Kajian ini telah dijalankan untuk menghasilkan
ujian diagnosis bagi virus Newcastle disease (NDV)
berdasarkan teknik tindakbalas rantai polimerase-RNA
(RNA-PCR) dan penjujukan kitaran sebagai tambahan kepada
ujian yang sedia ada..

Dua sistem RNA-PCR penjujukan kitaran setiap satu
ditujukan pada gen hemagglutinin-neuraminidase (HN) dan
pertaupan (F) telah dihasilkan. Amplifikasi melalui
RNA-PCR serpihan gen HN yang bersaiz 398 pasangan bes
(bp) dijalankan ke atas RNA total yang diekstrak
daripada cecair alantoik yang ada jangkitan dari 9
isolat NDV Malaysia, strain vaksin V4-UPM dan strain
velogenik AF2240. Analisis jujukan terhadap 113 bes
dalam serpihan yang diamplifikasi menunjukkan variasi di
antara isolat/strain. Bagaimanapun, jujukan yang
identikal didapati pada sesetengah isolat dari patotaip
tertentu dan difikirkan adalah strain NDV yang sama.



Amplifikasi melalui RNA-PCR serpihan gen F yang bersaiz 242 bp dijalankan ke atas RNA total yang diasingkan daripada beberapa jenis tisu (limpa, otak dan paru-paru) dari enam ayam tanpa diberi vaksin yang dicabar dengan strain velogenik AF2240. Limpa didapati mempunyai bilangan sampel yang PCR positif yang tertinggi. Limpa dari ayam yang tanpa jangkitan serta limpa dari ayam yang disembelih setelah diberi vaksin dan dicabar didapati PCR negatif kesemuanya. Virus pencabar difikirkan telah dineutralkan oleh antibodi yang dihasilkan oleh ayam tersebut. Identiti produk PCR yang diamplifikasi dari limpa disahkan melalui penjujukan kitaran. Ujian ini juga telah dilakukan ke atas RNA daripada cecair alantoik yang ada jangkitan dari 11 strain/isolat NDV yang berlainan. Kesemua sampel tersebut juga didapati PCR positif.

18 nukleotida pada tapak pemotongan gen F telah dijujuk untuk 4 strain NDV. Jujukan asid amino yang ditentukan untuk strain AF2240 (velogenik) dan S (mesogenik) adalah $^{112}\text{Arg-Arg-Gln-Arg/Lys-Arg-Phe}^{117}$ dan untuk strain F (lentogenik) dan V4-UPM (avirulen) adalah $^{112}\text{Gly-Arg/Lys-Gln-Gly-Arg-Leu}^{117}$. Oleh itu, strain velogenik dan mesogenik dapat dibezakan dari strain lentogenik dan avirulen dengan menjujuk tapak pemotongan tersebut.



CHAPTER I

INTRODUCTION

Newcastle disease virus (NDV), a member of the *Paramyxoviridae* family, is an economically important pathogen of poultry. The viral genome consists of a negative sense single-stranded RNA molecule of approximately 15 kilobase (kb) in length. The six gene products encoded by the genome are the nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN) protein and the large (L) protein (Samson, 1988).

NDV strains are known to display variations in many biological properties including pathogenicity towards their hosts. These strains are being classified into three major groups depending on the severity of the disease in susceptible chickens. Strains causing high mortality are termed velogenic. The mesogenic strains are those that cause up to 50% mortality in young and susceptible chickens and seriously decreasing egg production. Strains that produce mild disease in infected hosts are known as lentogenic. In addition



to these three groups, some strains cause no disease at all and are classified as avirulent. The strains variations in virulence are primarily due to differences in susceptibilities of the F_0 precursor protein to cleavage by host cell proteases into its active form. Recent studies have revealed a relationship between strain pathogenicity and the amino acid composition at the cleavage site of the F_0 protein. The amino acid sequence at the cleavage site is Arg-Arg-Gln-Arg(Lys)-Arg-Phe for velogenic and mesogenic strains and Gly-Arg(Lys)-Gln-Gly-Arg-Leu for lentogenic and avirulent strains (Alexander, 1990b). Thus, the velogenic and mesogenic strains could be distinguished from the lentogenic and avirulent strains by sequencing the cleavage site.

Newcastle disease (ND) can cause great economic losses to the poultry industry. It is thus important to have methods which can provide rapid and specific diagnosis in order to prevent and control the disease. Clinical signs typical of ND produced by a bird is not a definitive proof that the agent responsible for the disease is NDV. There is a possibility that the infecting NDV is of an avirulent or vaccine strain which is asymptomatic and the observed clinical symptoms similar to ND are in fact caused by other pathogens (Alexander, 1988). Therefore, birds showing clinical



signs of ND should be diagnosed to demonstrate the presence of a virulent NDV strain.

The detection and pathotyping of NDV isolates is routinely carried out through a time consuming process that involves isolation, identification and virulence characterisation by one or more of the following biological tests: mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) (Alexander, 1988). In recent years, alternative methods based on anti-peptide antibodies (Hodder et al., 1993) or oligonucleotide probes (Jarecki-Black et al., 1992; Jarecki-Black and King, 1993) have been developed. Apart from confirming NDV infections, further tests for distinguishing virus strains are also important in tracing the origin of a particular strain from an infected flock. For this purpose, diagnostic procedures using monoclonal antibodies (Srinivasappa et al., 1986; Erdei et al., 1987) and oligonucleotide fingerprinting (McMillan and Hanson, 1982) have been developed.

The polymerase chain reaction (PCR) technique which is specific, rapid and sensitive has been used for the detection of many veterinary important viruses including NDV. Jestin and Jestin (1991) have developed a PCR test which could specifically detect NDV isolates from the infected allantoic fluid of embryonated chicken



eggs. The target sequence for amplification was located within the F gene.

In a previous study, the author had developed an RNA-polymerase chain reaction (RNA-PCR) assay based on the HN gene for detecting NDV in infected allantoic fluid (Ng, 1993). This RNA-PCR assay was used to amplify a segment of the HN gene from several reference NDV strains. If positive results were obtained for all the reference NDV strains, the assay should also be applicable to the local NDV isolates. Also, diagnosis of NDV directly from chicken tissue would be faster than that from allantoic fluid as virus growing is not required. This project was thus carried out with the following objectives:

1. to study the feasibility of using the HN gene RNA-PCR assay to detect Malaysian field isolates of NDV;
2. to differentiate between various local NDV isolates by sequencing the HN gene PCR amplified-DNAs; and
3. to develop an RNA-PCR cycle sequencing system based on the F gene for the rapid detection and pathotyping of NDV directly from infected chicken tissues.

CHAPTER II

LITERATURE REVIEW

Newcastle Disease Virus (NDV)

NDV is the agent causing ND in avian species. The virus also called avian paramyxovirus type I, is a member of the *Paramyxovirus* genus from the *Paramyxoviridae* family. To date, many NDV isolates with wide variations in biological properties have been identified worldwide.

Morphology

NDV is a large pleomorphic enveloped virus which varies in size from 150 to 400 nm. Its genome consists of a single stranded negative polarity RNA of approximately 15 kilobase (kb) in length. The genome encodes six structural proteins namely: nucleocapsid (NP) protein, phosphoprotein (P), large (L) protein, matrix (M) protein, fusion (F) protein and haemagglutinin-neuraminidase (HN) protein. The function of each of the protein is summarised in Table 1.



Table 1

Function of NDV Coded Proteins

Protein	Abbrev.	Function
nucleocapsid	(NP)	major structural component of nucleocapsid: complexed with RNA genome
phosphoprotein	(P)	associated with nucleocapsid, role in transcription / replication.
large	(L)	RNA directed RNA polymerase.
matrix	(M)	virus assembly organiser, moderates transcription.
fusion	(F)	fusion of virus and host membrane.
haemagglutinin-neuraminidase	(HN)	attachment to cellular receptor and receptor destroying activity.

(Source: Samson, 1988).



The F and HN proteins are synthesised as precursor proteins and are cleaved by host cell proteases to form functional proteins (Samson, 1988).

Virulence Variation

NDV strains can cause distinct clinical signs and different degrees of disease severity in susceptible chickens. Based on this biological characteristic NDV strains have been grouped into three pathotypes:

1. the viscerotropic-velogenic strains which cause either haemorrhagic lesions in the intestinal tract or neurological and respiratory signs with high mortality;
2. the mesogenic strains which cause respiratory and sometimes nervous infection with low mortality; and
3. the lentogenic strains which cause mild or inapparent respiratory infection.

In addition to these three pathotypes, some strains cause inapparent enteric infection and are classified as avirulent. This classification, however, is merely a guide because there is always some degree of overlap in the clinical signs produced (Alexander, 1990a).



Molecular Marker of Virulence of NDV

The variation in virulence among NDV strains has been correlated in part with the variations in susceptibilities of the F_0 precursor protein to proteolytic cleavage to form an active protein (Nagai et al., 1976; 1979). The F_0 protein of velogenic and mesogenic strains is readily cleaved by proteases of a wide range of host cells while the F_0 protein cleavage does not occur in most cell types infected with lentogenic and avirulent strains. Another protein which also plays a role in determining the virulence of NDV strains is the HN protein. There are three different sizes of primary translation HN polypeptides: 616, 577, or 571 amino acids depending on the position of the stop codons (Sakaguchi et al., 1989). Both the smaller HN polypeptides are synthesised by either velogenic, mesogenic or lentogenic NDV strains and are already in their active forms. In contrast, the 616 amino acid HN polypeptide is found only in avirulent strains and the extended amino acids have to be removed for it to become biologically active. Since the F_0 protein of velogenic and mesogenic strains can be activated in a wide range of host cells and their HN protein is already synthesised in their active form, these strains are able to spread through the host and cause a more severe disease than the lentogenic and avirulent strains.



Recent nucleotide sequencing studies covering NDV strains of all pathotypes have revealed a relationship between virulence variations and the amino acid composition at the cleavage site of the F₀ protein. The amino acid sequence at the cleavage site of velogenic and mesogenic strains is Arg-Arg-Gln-Arg(Lys)-Arg-Phe while for lentogenic and avirulent strains the sequence at that site is Gly-Arg(Lys)-Gln-Gly-Arg-Leu (Alexander, 1990b). Thus, the variation in amino acid composition at the cleavage site may be used as a marker to distinguish the velogenic and mesogenic strains from the lentogenic and avirulent strains. The avirulent strains can be further distinguished from the lentogenic strains by determining the position of stop codon in the HN gene.

Diagnosis of Newcastle Disease

The unequivocal method of diagnosing ND involves a rather complex procedure which consists of three stages. Initially, the virus is isolated from suspected clinical specimen in either cell cultures or more commonly in embryonated chicken eggs. Then, allantoic fluid obtained from these eggs between 5 to 7 days post inoculation is tested for haemagglutination (HA) activity with chicken red blood cells. HA positive samples indicate the

presence of NDV or one of the other avian paramyxoviruses or influenza virus. HA negative samples are passaged for at least one more time. Isolation of NDV is subsequently confirmed by a haemagglutination-inhibition (HI) test using known positive antiserum. However, other avian paramyxovirus serotypes particularly viruses of PMV-3 serotype may show some inhibition with NDV antiserum. Monoclonal antibodies directed against highly conserved epitopes of NDV antigens have been produced by several research groups (Russell and Alexander, 1983; Ishida et al., 1985; Meulemans et al., 1987; Lana et al., 1988) to replace the antiserum for confirming NDV isolation. Finally, the virulence of the isolated NDV strain is determined by one or more of the following biological tests: mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI). These are biological tests which measure the seriousness of the disease caused by the isolated NDV strain to embryonated chicken eggs or chickens. NDV strains are classified into three major pathotypes: velogenic, mesogenic and lentogenic based on calculated index value (Alexander, 1988).

Serological tests for NDV which may be used to detect the virus or to monitor immune response in vaccination are also available and these include virus neutralization, single radial immunodiffusion and agar

