UNIVERSITI PUTRA MALAYSIA

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FPV 2003 9

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MASTER OF SCIENCE
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2003

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

July 2003

Dedicated to my parents,
who show me the meaning of love and patience.

To my brother, James, sister, Sheau Yunn and my lovely nephew, Edward.
You all are the lights of my life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

CHARACTERISATION OF NEWCASTLE DISEASE VIRUS (NDV) ISOLATES AND DEVELOPMENT OF REAL-TIME PCR FOR THE DIAGNOSIS OF NDV

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July 2003

Chairman : Professor Aini Ideris, Ph.D.

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A sudden upsurge of Newcastle disease (ND) outbreaks occurred in Malaysia with high mortality among vaccinated birds started in August 2000 and peaked between November 2000 and March 2001. Four isolates; 00/IKS, 01/C, 01/TM and 01/GNS were isolated from the NDV outbreaks in different states in Peninsular Malaysia. Mean death time (MDT) assay was carried out to determine the pathogenicity of the isolates in embryonated chicken eggs. All isolates had MDT of less than 60 hours, indicating that the isolates are velogenic. The nucleotide sequence in the region of F cleavage site for the four NDV isolates were determined and the deduced amino acid sequence showed that all isolates possessed two pairs of basic amino acids (^{112}RRQKR^{116}) and Phe residue at position 117 of the F cleavage site. These amino acid sequences correlated with the MDT results which confirmed that the four NDV isolates are velogenic strains.

Analysis of the partial sequence of the F gene was carried out and the results suggested that the four recent isolates can be grouped under the genotype VII viruses.

All the isolates possess K^{101} and V^{121} at the F gene sequence, a characteristic of genotype VII viruses. Isolates 01/C had an N residue at position 101, which is unique among these four isolates. The phylogenetic analysis of the four isolates based on the partial sequence of M gene showed that their nucleotide similarities varied between 92.7% and 100%, where isolate 00/IKS shared 100% nucleotide sequence similarity with isolate 01/GNS. The four isolates were found to be phylogenetically related to pigeon isolate 1307/US/75 and goose isolate ZJI with similarity ranging from 93.29% to 98.38%. This suggests that the recent isolates might have originated from domestic and free-living birds. There was no evidence to show that the recent isolates evolved from local velogenic NDV strain AF2240, which was isolated in 1960s. The nucleotide similarities of the four NDV isolates with strain AF2240 were 79.88% to 82.63%.

Recent development of real-time PCR has offered the opportunity of developing a sensitive and accurate method to detect NDV. A two-step real-time RT-PCR procedure using the SYBR Green I dye was used as a detection signal. Beside the four isolates, 8 other isolates of NDV were used in this study. They were grouped into 7 velogenic, 1 mesogenic and 4 lentogenic strains. All isolates showed positive results in amplification. A melting curve was obtained immediately after amplification to distinguish specific product from non-specific and primer-dimer. From the melting curve analysis, no primer-dimer and non-specific products were detected and all the isolates had melting temperature (T_m) ranging from 86°C to 87°C. The detection limits of the real-time PCR were compared with RT-nested PCR ELISA and agarose gel electrophoresis by preparing serially ten-fold dilutions of the cDNA. The real-time PCR

could detect up to 1:10^5 dilution of cDNA with the concentration of 1.1 x 10^{-5} μg/μl. However, ELISA assay detection limit reached 1:10^3 with the concentration of 1.1 x10^{-3} μg/μl, whereas the agarose gel electrophoresis can only detect up to 1:10^2 dilution of the cDNA (1.1 x 10^{-2} μg/μl) with a faint band. The SYBR Green I real-time PCR was found to be 100-fold more sensitive than the PCR-ELISA detection method. The study has therefore successfully developed a sensitive, rapid and convenient method for NDV diagnosis using real-time PCR with SYBR Green I as a detection signal.

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

PENCIRIAN ISOLAT VIRUS PENYAKIT NEWCASTLE (NDV) DAN PEMBANGUNAN REAL-TIME PCR UNTUK DIAGNOSIS NDV

Oleh

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Julai 2003

Pengerusi : Profesor Aini Ideris, Ph.D.

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Wabak penyakit Newcastle (ND) telah dilaporkan di Malaysia baru-baru ini dengan kadar kematian yang tinggi di kalangan ayam yang telah diberi vaksin. Wabak penyakit ini bermula pada Ogos 2000 dan mencapai kemuncak di antara November 2000 dan Mac 2001. Isolat 00/IKS, 01/C, 01/GNS dan 01/TM yang dipencilkan daripada wabak penyakit Newcastle dari beberapa negeri di Semenanjung Malaysia telah digunakan dalam kajian ini. Masa kematian purata (MDT) telah dijalankan untuk menentukan patogenisiti isolat NDV dalam telur ayam yang berembrio. Keempat-empat isolat mempunyai MDT kurang daripada 60 jam, menunjukkan bahawa semua isolat adalah velogenik. Jujukan nukleotida di bahagian pencelahan F untuk keempat-empat isolat NDV juga dikenalpasti. Jujukan asid amino menunjukkan bahawa kesemua isolat memiliki dua pasang asid amino basik (^{112}RRQKR^{116}) dan residu Phe di kedudukan 117 pada bahagian pencelahan F. Jujukan asid amino adalah sama dengan keputusan MDT dan memastikan keempat-empat isolat NDV adalah strain velogenik.

Analisis sebahagian jujukan nukleotida gen F telah dijalankan dan keputusan mencadangkan bahawa keempat-empat isolat boleh dikumpulkan di bawah virus genotip VII. Semua isolat memiliki K^{101} dan V^{121} pada jujukan gen F, ciri-ciri bagi virus genotip VII. Isolat 01/C mempunyai residu N di kedudukan 101, di mana ianya adalah unik di antara empat isolat. Analisis filogenesis untuk keempat-empat isolat berdasarkan sebahagian jujukan gen M menunjukkan persamaan nukleotid bagi empat isolat adalah di antara 92.7% dan 100%. Di antara empat isolat ini, isolat 00/IKS berkongsi 100% persamaan jujukan nukleotida dengan isolat 01/GNS. Semua empat isolat didapati berkaitan secara filogenik dengan isolat burung merpati 1307/US/75 dan isolat angsa ZJI dengan persamaan berjulat antara 93.29% dan 98.38%. Ini mencadangkan bahawa isolat-isolat baru ini mungkin berasal daripada burung domestik dan hidupan bebas. Tiada bukti menunjukkan isolat-isolat baru in berkembang secara beransur-ansur daripada NDV velogenik tempatan strain AF2240 yang diasingkan pada 1960an. Persamaan nukleotida keempat-empat isolat NDV dengan isolat AF2240 adalah 79.89% ke 82.63%.

Perkembangan baru real-time PCR telah menawarkan peluang untuk pembangunan cara pengesanan NDV yang sensitif dan tepat. Satu prosedur RT-PCR masa sebenar dua langkah dengan penggunaan pewarna SYBR Green I telah digunakan sebagai tanda isyarat pengesanan. Selain daripada keempat-empat isolat, 8 isolat lain juga digunakan dalam kajian ini. Mereka digolongkan sebagai 7 velogenik, 1 mesogenik dan 4 strain lentogenik. Semua isolat menunjukkan keputusan positif dalam amplifikasi. Untuk membezakan produk spesifik daripada produk tidak spesifik dan

primer-dimer, suatu lengkungan peleburan diperolehi segera selepas amplifikasi Analisis lengkungan peleburan menunjukkan tiada primer-dimer dan produk tidak spesifik dikesan dan semua isolat mempunyai takat peleburan pada suhu 86°C hingga 87°C Had pengesanan real-time PCR dibandingkan dengan RT-nested PCR ELISA dan elektroforesis gel agarosa dengan menyediakan siri pencairan sepuluh ganda cDNA Real-time PCR boleh mengesan sehingga 1 10^{5} pecairan cDNA dengan kepekatan 1 1 x 10^{-5} µg/µl, tetapi had pengesanan ELISA mencapai 1 10^{3} sahaja, manakala elektroforesis gel agarosa hanya boleh mengesan sehingga 1 10^{2} pencairan cDNA dengan jalur kabur Real-time PCR SYBR Green I didapati 100 kali ganda lebih sensitif daripada pengesanan PCR-ELISA Oleh itu, kajian ini telah berjaya membangun satu cara diagnosis NDV yang sensitif, cepat dan mudah, dengan menggunakan real-time PCR serta SYBR Green I sebagai isyarat pengesanan

ACKNOWLEDGEMENTS

I would like to express my heartiest gratitude and appreciation to Professor Dr. Aini Ideris, Chairman of the Supervisory Committee for providing invaluable advice and support in this Master research.

I would like to express my sincere thanks and appreciation to Associate Professor Dr. Abdul Rahman Omar, Professor Datin Dr. Khatijah Yusoff and Associate Professor Dr. Tan Wen Siang, members of the Supervisory Committee for their constructive suggestions, proper guidance and encouragement throughout my study.

It has been a great experience and a joy working with my colleagues in the Biologics laboratory. Special thanks go to the staff of Biologics Laboratory, Puan Rodiah Husin, En. Adam Ahmad, En. Mohd. Redha Izwan and everybody who has helped me in this study. My heartiest appreciation also goes to my dear friends, Lih Ling, Siti, Shila, Siow Kian, Hui Min, Mee Hoong, Mei Yen, Pei Yun and Jiunn Jye, for their invaluable friendship and love.

Finally, I would like to thank God for His spiritual guidance and for blessing me with all those wonderful people mentioned above. This Master thesis would not have come true had it not been for the love and blessings from God.

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LIST OF ABBREVIATIONS

bp	- base pair
cDNA	- complementary deoxyribonucleic acid
ºC	- degree Celcius
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleotide triphosphate
ddNTP	- dideoxynucleotide triphosphate
ds	- double stranded
EDTA	- Ethylenediaminetetraacetic acid disodium salt
F	- fusion protein
h	- hour
HA	- haemagglutinin activity
HN	- haemagglutinin-neuraminidase
kb	- kilobase
kDA	- kilodalton
M	- matrix protein
min	- minutes
ml	- millilitre
mM	- millimolar
µg	- microgram
µM	- micromolar
NDV	- Newcastle disease virus
ng	- nanogram
PBS	- phosphate buffer saline
PCR	- polymerase chain reaction
PTC	- Peltier thermal cycler
RBC	- red blood cell
RNA	- ribonucleic acid
RT-PCR	- reverse transcriptase-polymerase chain reaction
RT	- room temperature
S	- seconds
ss	- single stranded
TAE	- Tris-acetate-EDTA
Taq	- *Thermus aquaticus*
TAE	-tris-acetic-EDTA buffer
UPMGA	- unweighted pair group method with arithmetic mean
UPM	- Universiti Putra Malaysia
UV	- Ultraviolet
w/v	- weight/volume
v/v	- volume/volume

Amino Acid	Single/Three Letter Amino Acid Code	
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Glutamine	Q	Gln
Glutamic Acid	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Valine	V	Val

CHAPTER I

INTRODUCTION

The poultry industry forms a major component of the livestock industry in Malaysia. According to the Department of Veterinary Services, Malaysia, the ex-farm value of poultry production in Malaysia is estimated to be RM4.0 billion (Livestock Products Statistics, 2001). Poultry meat and eggs are consumed chiefly as the main source of protein for Malaysians. The total local consumption of poultry meat has increased by 73% from 373,330 metric tonne in 1991 to 644,560 metric tonne in 2001. The production of chicken contributed about 80% of the total output of animal products.

The greatest threat in the poultry industry, not only in Malaysia but also in Asia is Newcastle disease (ND) caused by the Newcastle disease virus (NDV). ND is a highly contagious viral disease of poultry and has greater than 95% morbidity and mortality in susceptible chickens (Errington *et al*., 1995). The disease is classified as List A disease that requires reporting to the Office of International Epizootics (OIE) and outbreaks result in strict trade embargoes (Seal *et al.,* 2000a). In Malaysia, ND is still endemic throughout the country despite routine vaccination programmes in commercial poultry farms. Data concerning ND compiled from regional diagnostic laboratories showed that the disease still remains the most frequently diagnosed disease in poultry (Lo, 1993).

In the poultry industry, vaccination against ND is a common practice worldwide. Vaccination for ND has been used since the 1940s (Beard & Hanson, 1984). The most widely used vaccines are live virus consisting of lentogenic or selected mesogenic strains (Aini, 1990a; Aini, 1990b; Marin *et al.*, 1996). These vaccines work well when administered correctly, but problems arise in the field when insufficient attention is paid to factors such as age of the birds, route of inoculation, the strain of virus and follow-up serology (Taylor *et al.*, 1989).

A sudden upsurge of ND outbreaks with high mortality among vaccinated birds has been recently reported in Malaysia. Report from Office Internationale de Epizootics (OIE), stated that the recent outbreaks in Malaysia started in August 2000 and peaked between November 2000 and March 2001. The outbreaks declined with only sporadic outbreaks until December 2001 (Multiannual Animal Disease Status, OIE). A total of 44 outbreaks were recorded in 2000 and caused 367,218 deaths. However, from January to December 2001 the number of outbreaks had increased significantly to 84, involving 525,981 cases and about 313,000 deaths (59.6% mortality). Most cases of high mortality (10%-100%) were recorded in broilers aged between 20 and 42 days. Outbreaks also occurred in layer and village chicken farms, but in smaller numbers. The outbreaks were distributed mostly in states with a high density of poultry farms, such as Pulau Pinang, Johor, Negeri Sembilan, Selangor and Perak.

The cause of the recent outbreaks is still unknown. It has been hypothesized that the recent outbreaks of ND were due to the multi-factorial vaccination failure against ND.

This is probably caused by the improper vaccination, such as improper dose/strains and route of administration. The immunosuppressive agents such as avian leukosis virus subgroup J (ALV-J) and infectious bursal disease virus (IBDV) might also contribute to these recent outbreaks (Omar *et al*., 2002; Hair-Bejo *et al*., 2002). Therefore, characterization of these recent NDV isolates is essential for determining the molecular pathogenicity and epidemiology of NDV transmission. Analysis of viral nucleotide sequence, substitutions of deduced amino acid sequence and phylogenetic relationship will provide a better understanding in the investigation of these ND outbreaks.

With the advent in molecular biology based techniques, reverse transcription polymerase chain reaction (RT-PCR) has been developed for the detection of NDV (Jestin & Jestin, 1991). The generated PCR product can be used for nucleotide sequencing of fusion (F) protein cleavage site analysis, the primary molecular determinant for viral pathogenicity. Epidemiological studies have also been carried out based on the nucleotide and amino acid sequences of NDV genes (Toyoda *et al*., 1989; Collins *et al*., 1993, 1994; King & Seal, 1997; Yu *et al*., 2001).

The goal of the development of molecular based test is to achieve a simpler, rapid and highly sensitive test. The use of RT-PCR followed by ethidium bromide staining gel electrophoresis of the PCR product is fairly laborious and therefore not really an option for routine analysis of many steps which would introduce more potential for contamination. Kho *et al*. (2000) reported a RT-nested PCR enzyme-linked

immunosorbent assay technique for NDV detection rather than electrophoretic technique. The authors found that their colorimetric detection system to be 10-fold more sensitive than electrophoresis. The recent development of fluorogenic PCR-based format, termed as 'real-time PCR' promises a wide dimension in diagnostic approach. The introduction of real-time PCR has made it possible to accurately quantify starting amounts of nucleic acid during the PCR reaction without the need for post-PCR analyses (Bustin, 2000; Kearns *et al*., 2001; Komunian-Pradal, 2001). In the study by Aldous *et al.* (2001), a panel of fluorescence-labelled probes was used to differentiate avirulent and virulent NDV rapidly and accurately. However, no studies have been carried out in the detection of NDV using SYBR Green I intercalating dye chemistry.

In this study, four recently isolated NDV isolates (00/IKS, 01/C, 01/TM and 01/GNS) were used. The characterization of the recent NDV isolates will provide an invaluable information about the pathogenicity and epidemiology of NDV. The development of a rapid, specific and sensitive diagnosis of NDV surely would pave way for a more effective control of the disease.

Thus the objectives of this study were :

1) to characterize the recent NDV isolates based on MDT test and F cleavage site analysis,
2) to characterize the evolutionary relationship of the isolates based on phylogenetic analysis, and
3) to develop a real-time PCR diagnostic approach for NDV.