



**UNIVERSITI PUTRA MALAYSIA**

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

**TAN SHEAU WEI**

**FPV 2003 9**

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

**TAN SHEAU WEI**

**MASTER OF SCIENCE  
UNIVERSITI PUTRA MALAYSIA**

**2003**



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

**By**

**TAN SHEAU WEI**

**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**

**By**

**TAN SHEAU WEI**

**July 2003**

**Chairman : Professor Aini Ideris, Ph.D.**

**Faculty : Veterinary Medicine**

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 (<sup>112</sup>RRQKR<sup>116</sup>) 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<sup>101</sup> and V<sup>121</sup> 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<sup>5</sup> dilution of cDNA with the concentration of 1.1 x 10<sup>-5</sup> µg/µl. However, ELISA assay detection limit reached 1:10<sup>3</sup> with the concentration of 1.1 x10<sup>-3</sup> µg/µl, whereas the agarose gel electrophoresis can only detect up to 1:10<sup>2</sup> dilution of the cDNA (1.1 x 10<sup>-2</sup> 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**

**TAN SHEAU WEI**

**Julai 2003**

**Pengerusi : Profesor Aini Ideris, Ph.D.**

**Fakulti : Perubatan Veterinar**

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 patogenesis 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 (<sup>112</sup>RRQKR<sup>116</sup>) 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<sup>101</sup> dan V<sup>121</sup> 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 ini 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 diperoleh 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  $1 \times 10^5$  pecairan cDNA dengan kepekatan  $1 \times 10^{-5} \mu\text{g}/\mu\text{l}$ , tetapi had pengesanan ELISA mencapai  $1 \times 10^3$  sahaja, manakala elektroforesis gel agarosa hanya boleh mengesan sehingga  $1 \times 10^2$  pecairan 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.



I certify that an Examination Committee met on 23<sup>rd</sup> July 2003 to conduct the final examination of Tan Sheau Wei on her Master of Science thesis entitled "Characterisation of Newcastle Disease Virus (NDV) Isolates and Development of Real-time PCR For Diagnosis of NDV" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (High Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

**RAHA ABDUL RAHIM, Ph.D.**

Associate Professor,  
Department of Biotechnology,  
Faculty of Food Science and Biotechnology,  
Universiti Putra Malaysia.  
(Chairman)

**AINI IDERIS, Ph.D.**

Professor,  
Department of Veterinary Clinical Studies,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Member)

**ABDUL RAHMAN OMAR, Ph.D.**

Associate Professor,  
Department of Veterinary Pathology and Microbiology,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Member)

**DATIN KHATIJAH YUSOFF, Ph.D.**

Professor,  
Department of Biochemistry and Microbiology,  
Faculty of Science and Environmental Studies,  
Universiti Putra Malaysia.  
(Member)

**TAN WEN SIANG, Ph.D.**

Associate Professor,  
Department of Biochemistry and Microbiology,  
Faculty of Science and Environmental Studies,  
Universiti Putra Malaysia.  
(Member)



**GULAM RUSUL RAIMAT ALI, Ph.D.**

Professor/Deputy Dean,  
School of Graduate Studies,  
Universiti Putra Malaysia.

30 SEP 2003

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

**AINI IDERIS, Ph.D.**

Professor,  
Department of Veterinary Clinical Studies,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Chairman)

**ABDUL RAHMAN OMAR, Ph.D.**

Associate Professor,  
Department of Veterinary Pathology and Microbiology,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Member)

**DATIN KHATIJAH YUSOFF, Ph.D.**

Professor,  
Department of Biochemistry and Microbiology,  
Faculty of Science and Environmental Studies,  
Universiti Putra Malaysia.  
(Member)

**TAN WEN SIANG, Ph.D.**

Associate Professor,  
Department of Biochemistry and Microbiology,  
Faculty of Science and Environmental Studies,  
Universiti Putra Malaysia.  
(Member)



**AINI IDERIS, Ph.D.**  
Professor/Dean,  
School of Graduate Studies,  
Universiti Putra Malaysia.

Date : **14** NOV 2003



## DECLARATION

I here declare that the thesis is based on my original work except for quotation 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.



TAN SHEAU WEI

Date : 10/11/2003

## TABLE OF CONTENTS

	Page
<b>DEDICATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	vi
<b>ACKNOWLEDGEMENTS</b>	ix
<b>APPROVAL SHEETS</b>	x
<b>DECLARATION FORM</b>	xii
<b>LIST OF TABLES</b>	xvi
<b>LIST OF FIGURES</b>	xvii
<b>LIST OF ABBREVIATIONS</b>	xviii
<b>CHAPTER</b>	
<b>I INTRODUCTION</b>	1
<b>II LITERATURE REVIEW</b>	5
Newcastle Disease	5
Newcastle Disease Virus Classification	5
Clinical Signs and Pathogenicity	6
Distribution and Genotyping of NDV	8
NDV Genome, Encoded Proteins and Virion Structure	11
Fusion Protein	14
Matrix protein	16
Diagnosis of NDV	
Isolation and Detection of NDV	17
Serology	18
Hemagglutination (HA) Test	18
Hemagglutination Inhibition (HI) Test	18
Characterization of NDV	18
Mean Death Time (MDT)	19
Intracerebral Pathogenicity Index (ICPI) and Intravenous Pathogenicity Index (IVPI)	19
Detection and Identification of NDV by RT-PCR	20
Reverse Transcription Polymerase Chain Reaction (RT-PCR)	21
Real-time PCR	22
Chemistry Development for Real-time PCR	22
Molecular Beacon	23
Double-stranded DNA-binding Dye	25
Hydrolysis Probe	25
Hybridization Probe	26
Applications of Real-time PCR	30



DNA Sequencing	31
Phylogenetic Analysis	32
<b>III ISOLATION AND CHARACTERIZATION OF NDV ISOLATES</b>	<b>34</b>
Introduction	34
Materials and Methods	38
Virus Isolation	38
Chicken Tissue Specimens	38
Processing of Samples	38
Egg Passage and Haemagglutination Spot Test	40
Haemagglutination (HA) End Point Titration Assay	41
Haemagglutination Inhibition (HI) Test	41
Virus Propagation	42
Virus Harvesting	42
Mean Death Time (MDT) Test	43
Viral RNA Extraction	43
Determination of RNA Concentration and Purity	44
Primer Used	45
RT-PCR Amplification	46
Using Primer BK1/BK2	46
Using Primer M1/M2	47
Agarose gel electrophoresis	47
Ethidium Bromide Staining	48
Extraction and Purification of RT-PCR Product	48
DNA Sequencing	49
Sequence Assembly and Analysis	49
Phylogenetic Analysis	50
Results	52
Egg Passage	52
Haemagglutinin Activity (HA) End Point Titration Assay	52
Haemagglutination Inhibition (HI) Test	52
Virus Propagation	53
Mean Death Time (MDT) Test	53
RT-PCR Amplification	54
Nucleotides and Amino Acids Sequence Analysis	57
F protein gene	57
M protein gene	59
Phylogenetic analysis	61
Discussion	66



<b>IV</b>	<b>DEVELOPMENT OF REAL-TIME PCR FOR DIAGNOSIS OF NDV ISOLATES</b>	<b>71</b>
	Introduction	71
	Materials and Methods	73
	NDV Isolates	73
	Viral RNA Extraction	73
	Determination of RNA Concentration and Purity	74
	Primers Design	74
	cDNA synthesis	75
	SYBR Green I real-time PCR	75
	Melting curve analysis	76
	Specificity determination	76
	Sensitivity Test	76
	SYBR Green I real-time PCR	76
	RT nested-PCR ELISA	77
	Analysis of the PCR Products	78
	Agarose gel electrophoresis	78
	Colourmetric detection (ELISA) of PCR products	79
	Results	80
	SYBR Green I real-time PCR	80
	Melting Curve Analysis	80
	Sensitivity Test	83
	Discussion	88
<b>V</b>	<b>GENERAL DISCUSSION AND CONCLUSION</b>	<b>91</b>
	<b>BIBLIOGRAPHY</b>	<b>97</b>
	<b>APPENDICES</b>	<b>107</b>
	<b>BIODATA OF THE AUTHOR</b>	<b>117</b>



## LIST OF TABLES

<b>Table</b>		<b>Page</b>
2.0	Functions of the NDV proteins	13
3.0	List of NDV infected tissue samples from the ND outbreaks during 2000-2001	39
3.1	Primers used for amplification and sequencing of F protein gene and M protein gene	45
3.2	Published sequences of F gene used in alignment	50
3.3	NDV isolates examined phylogenetically	50
3.4	HA and HI titer of NDV isolates	53
3.5	MDT result of NDV isolates	54
3.6	Distance matrix of the 4 recent NDV isolates and other published NDV strains	63
4.0	List of NDV used in the study	73
4.1	List of primers used in the study	74
4.2	Sensitivity of SYBR Green I assay	83
4.3	Comparison of the sensitivity of the SYBR Green I, the RT-nested PCR ELISA and agarose gel electrophoresis detection methods	85



## LIST OF FIGURES

<b>Figure</b>		<b>Page</b>
2.0	Schematic representation of the virion structure of NDV	12
2.1	A schematic diagram of F glycoprotein	15
2.2	The molecular beacon assay	24
2.3	Dye incorporation and Hybridisation probe method	27
2.4	The TaqMan assay	29
3.0	RT-PCR product of the 242 bp F gene of recent NDV isolates	55
3.1	RT-PCR product of the 232 bp F gene of recent NDV isolates	56
3.2	Predicted amino acid sequence alignment of NDV isolates	58
3.3	Predicted amino acid sequence alignment of NDV isolates	60
3.4	Phylogenetic relationship of NDV isolates based on nucleotide sequences from a portion of the M protein gene	65
4.0	Amplification plot of all NDV isolates	81
4.1	Melting curve profiles of the SYBR Green I for the NDV isolates	82
4.2	Performance of SYBR Green I real-time PCR on a serial of 10-fold dilution of cDNA	84
4.3	Detection of nested PCR products by ELISA	86
4.4	Agarose gel electrophoresis detection method	87



## LIST OF ABBREVIATIONS

bp	- base pair
cDNA	- complementary deoxyribonucleic acid
°C	- 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
<i>Taq</i>	- <i>Thermus aquaticus</i>
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.