



UNIVERSITI PUTRA MALAYSIA

**DEVELOPMENT OF SYBR GREEN I REAL-TIME PCR METHOD FOR
DETECTION AND DIFFERENTIATION OF NEWCASTLE DISEASE
VIRUS PATHOTYPES**

TAN SHEAU WEI

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By

TAN SHEAU WEI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

AUGUST 2008



This dissertation specially dedicated to,

*My late father, Tan Ke Eng,
who always lives in my heart.*

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

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AUGUST 2008

Chairman: Professor Aini Ideris, PhD

Faculty: Veterinary Medicine

Newcastle disease (ND) which is caused by Newcastle disease virus (NDV) is a highly contagious viral disease of domestic poultry, cage, aviary and wild birds. ND outbreaks have led to substantial losses in the poultry industry. NDV can be classified into three major pathotypes: velogenic, mesogenic and lentogenic. Velogenic strains are highly virulent and may lead to 100% mortality in infected chicken whilst mesogenic and lentogenic strains cause mild clinical or inapparent infections, respectively. Early detection and differentiation of NDV pathotypes are very important during monitoring of suspected ND cases or during disease outbreaks. In this study, SYBR Green I real-time polymerase chain reaction (PCR) was developed for detection and differentiation of NDV pathotypes. Velogenic-specific primers (NDVIF2 & NPV2N) and lentogenic-specific primers (NDVIF2 & NPL2N) were designed to detect specific sequence of velogenic strains and lentogenic/vaccine strains, respectively.



After establishing the optimum condition of the real-time PCR, the assay was performed on 22 previously characterized NDV strains. All the velogenic strains were only detected by using velogenic-specific primers (NDVIF2 & NPV2N) with threshold cycle (Ct) ranged from 12.92 to 22.76 and melting temperature between 85.6°C to 86.4°C. Similarly, all the lentogenic/vaccine strains were only successfully detected when lentogenic-specific primers (NDVIF2 & NPL2N) were used. All the lentogenic/vaccine strains amplified with the lentogenic-specific primer had a Ct value ranged from 11.93 to 18.73 and T_m between 87.2°C to 87.6°C. No amplification was found when the NDV velogenic-specific primers and lentogenic-specific primers were used to amplify avian influenza virus (AIV), infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV). This revealed that both velogenic- and lentogenic-specific primers were pathotype specific and no unrelated viral RNA can be amplified. The newly developed assay had a dynamic detection limit which spans over a 5 log₁₀ concentration range. The velogenic and lentogenic amplifications showed high PCR efficiency of 98.8% and 103%, respectively. Mean coefficient variation (CV) of reproducibility tests for velogenic amplification and lentogenic amplification was around 1% and 2%, respectively. The SYBR Green I real-time PCR was 10-fold more sensitive when compared to the conventional detection method using agarose gel electrophoresis. Turnaround time for the developed assay was approximately 2.5 hours including reverse transcription, PCR amplification and melting curve analysis.

Clinical samples from the experimental infected chickens as well as the suspected field cases were collected and then tested on the developed assay. In the experimental infection with lentogenic NDV F strain, virus could be detected 3 days post infection

(p.i.), followed by day 4, 5 and 10 p.i. For the SPF chickens infected with high doses of velogenic NDV strain AF2240 (10^5 to 10^3 ELD₅₀/0.1 ml), the virus can be detected as early as day 2 p.i., followed by day 3 and 4 p.i. All the infected chickens were dead on day 4 p.i. For the chickens group infected with low doses (10^2 to $10^{0.5}$ ELD₅₀/0.1 ml), the virus can be detected starting on day 4 p.i., followed by day 5, 7, 10, 11 and 12 p.i. All the infected chickens were dead on day 12 p.i. The assay was able to detect the viruses as early as day 2 before the observation of clinical signs. This is an important achievement as early detection can prevent further spread of the disease. A total of 41 suspected NDV field cases were tested with the developed assay, 33 cases were NDV negative and 8 cases were positive for velogenic NDV. The results were correlated well with the virus isolation method and F cleavage site sequence analysis. All these 8 isolates possess two pairs of dibasic amino acids at the position 112 to 116 of the F cleavage site, and a phenylalanine residue at the position 117. This F cleavage site analysis revealed that all of the 8 NDV isolates belonged to velogenic group.

In the attempt to improve the efficacy of the developed assay, internal amplification control (IAC) was incorporated into the developed real-time PCR assay for detection of PCR inhibitors. The potential of simultaneous detection of IAC and NDV target was investigated. The simultaneous detection was achieved based on the melting curve analysis. The co-amplified products exhibited two distinguished melting peaks at $86.36 \pm 0.13^\circ\text{C}$ and $91.42 \pm 0.21^\circ\text{C}$ which corresponded to NDV NP gene product and IAC KanR gene product, respectively.

In conclusion, this study successfully developed a SYBR Green I real-time PCR for NDV pathotypes detection and differentiation. The virus can be detected directly from clinical samples without the need of virus propagation in chicken embryonated eggs. Owing to these advantages, the developed assay will contribute significantly in the control and prevention of the spread of the disease. ND-infected birds can be rapidly isolated from the healthy bird in the case of field outbreaks, if the causal agent is detected at the early stage of the outbreak. Consequently, spread of the disease and economical losses can be prevented.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PEMBANGUNAN PCR MASA NYATA SYBR GREEN I UNTUK
PENGESANAN DAN PEMBEZAAN PATOTIP VIRUS PENYAKIT
NEWCASTLE**

Oleh

TAN SHEAU WEI

OGOS 2008

Pengerusi : Profesor Aini Ideris, PhD

Fakulti : Perubatan Veterinar

Penyakit Newcastle (ND) yang disebabkan oleh virus penyakit Newcastle (NDV) merupakan penyakit yang mudah menjangkiti ternakan ayam, burung-burung peliharaan dan burung-burung liar. Penyakit Newcastle telah menyebabkan kerugian yang serius dalam industri ternakan ayam. NDV boleh dibahagikan kepada 3 patotip utama: velogenik, mesogenik dan lentogenik. Strain velogenik adalah sangat virulen dan boleh menyebabkan 100% kematian pada ayam yang dijangkiti manakala strain mesogenik dan lentogenik masing-masing hanya menyebabkan jangkitan yang biasa atau jangkitan yang tidak ketara. Pengesanan dan pembezaan patotip NDV pada peringkat awal adalah sangat penting dalam mengawal wabak ND. Dalam kajian ini, PCR masa nyata SYBR Green I telah dibangunkan untuk mengesan dan membezakan patotip NDV. Primer velogenik (NDVIF2 & NPV2N) dan primer lentogenik (NDVIF2 & NPL2N) telah direka untuk pengesanan jujukan nukleotida yang spesifik pada strain velogenik dan strain lentogenik/vaksin masing-masing.



Selepas menetapkan keadaan optimum bagi PCR masa nyata, 22 strain NDV di mana patotipnya telah ditentukan dahulu digunakan dalam kajian ini. Primer velogenik (NDVIF2 & NPV2N) hanya dapat mengesan strain velogenik dengan had kitaran (Ct) berjulat antara 12.92 ke 22.60 dan suhu peleburan (Tm) di antara 85.6°C dan 86.4°C. Manakala, primer lentogenik (NDVIF2 & NPI2N) hanya mengesan strain lentogenik/vaksin dengan Ct berjulat antara 11.93 ke 18.73 dan Tm di antara 87.2°C dan 87.6°C. Kedua-dua velogenik dan lentogenik primer tidak mengesan virus-virus lain seperti virus selsema burung (AIV), virus penyakit berjangkit bursa (IBDV) dan virus penyakit berjangkit bronkitis (IBV). Ini menunjukkan primer-primer tersebut adalah sangat spesifik dan tidak menghasilkan amplifikasi apabila RNA daripada virus lain digunakan. Kaedah yang dibangunkan dalam kajian ini mempunyai had pengesanan meliputi julat kepekatan sebanyak 5 log₁₀. Amplifikasi velogenik dan lentogenik masing-masing menunjukkan kecekapan PCR 98.8% dan 103%. Purata koefisien varian (C.V.) bagi ujian reproduksibiliti dalam amplifikasi velogenik dan lentogenik masing-masing adalah 1% dan 2%. PCR masa nyata SYBR Green I yang dibangunkan ini adalah 10-kali lebih sensitif daripada kaedah pengesanan tradisional iaitu gel agarose elektroforesis. Kaedah ini boleh disiapkan dalam masa kira-kira 2.5 jam termasuk transkripsi berbalik, amplifikasi PCR dan analisis lengkungan peleburan.

Sampel klinikal daripada eksperimen jangkitan ayam dan beberapa kes daripada ladang yang disyaki telah dikumpulkan dan diuji menggunakan kaedah yang dibangunkan ini. Dalam eksperimen jangkitan dengan strain lentogenik F, virus boleh dikesan pada hari ke-3 selepas jangkitan (p.i.), diikuti dengan hari ke-4, ke-5 dan ke-10 p.i. Bagi kumpulan ayam SPF yang dijangkiti dengan dos tinggi strain velogenik

AF2240 (10^5 to 10^3 ELD₅₀/0.1 ml), virus boleh dikesan pada hari ke-2 p.i., diikuti dengan hari ke-3 dan hari ke-4 p.i. Semua ayam dalam kumpulan ini mati selepas hari ke-4 p.i. Bagi kumpulan ayam SPF yang dijangkiti dengan dos rendah strain velogenik AF2240 (10^2 to $10^{0.5}$ ELD₅₀/0.1 ml), virus boleh dikesan bermula pada hari ke-4 p.i., diikuti dengan hari ke-5, hari ke-7, hari ke-10, hari ke-11 dan hari ke-11 p.i. Semua ayam dalam kumpulan ini mati selepas hari ke-12 p.i. Kaedah PCR masa nyata ini dapat mengesan virus seawal hari ke-2 p.i. sebelum tanda-tanda klinikal ditunjukkan. Ini merupakan suatu pencapaian penting kerana pengesanan pada peringkat awal dapat mencegah penyakit Newcastle merebak. Sejumlah 41 kes NDV dari ladang yang disyaki telah diuji menggunakan kaedah yang dibangunkan, 33 kes adalah NDV negatif dan 8 kes positif bagi NDV velogenik. Keputusan tersebut adalah sejajar dengan keputusan-keputusan daripada kaedah pencilan virus dan analisis jujukan nukleotida di bahagian pencelahan protein F. Kesemua 8 isolat NDV yang positif dalam PCR nyata masa memiliki dua pasang asid amino basik di kedudukan 112 ke 116 dan residu Phe di kedudukan 117 pada bahagian pencelahan protein F. Jujukan asid amino pada bahagian pencelahan protein F ini menunjukkan kesemua 8 isolat NDV adalah velogenik.

Kawalan amplifikasi dalaman (IAC) telah dimasukkan dalam kaedah PCR masa nyata yang dibangunkan untuk mengesan perencat PCR. Potensi pengesanan IAC dan target NDV secara serentak telah diuji. Pengesanan secara rentak ini dicapai melalui analisis lengkungan peleburan. Produk bagi amplifikasi serentak ini mempamerkan 2 tahap peleburan yang berbeza pada $86.36 \pm 0.13^\circ\text{C}$ dan $91.42 \pm 0.21^\circ\text{C}$, masing-masing mewakili produk gen NP bagi NDV dan gen KanR bagi IAC.

Kesimpulannya, kajian ini telah berjaya membangunkan satu kaedah PCR masa nyata SYBR Green I untuk pengesanan dan pembezaan patotip NDV. Virus dapat dikesan secara langsung daripada sample klinikal dan pertumbuhan virus dalam telur tidak diperlukan. Kaedah yang dibangunkan ini akan memberi sumbangan dalam mengawal dan mencegah kemerebakan penyakit Newcastle. Ayam yang dijangkiti penyakit Newcastle dapat dipisahkan daripada ayam-ayam yang sihat dengan segera, sekiranya agen jangkitan dapat dikesan pada peringkat awal berlakunya wabak penyakit. Dengan demikian, kemerebakan penyakit and kerugian ekonomi dapat di cegah.

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I certify that an Examination Committee has met on 29 August 2008 to conduct the final examination of Tan Sheau Wei on her Doctor of Philosophy thesis entitled “Development of SYBR Green I real-time PCR method for Detection And Differentiation of Newcastle Disease Virus Pathotypes” 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 Doctor of Philosophy.

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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, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

TAN SHEAU WEI

Date:

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vii
ACKNOWLEDGEMENTS	xi
APPROVAL	xiii
DECLARATION	xv
LIST OF TABLES	xx
LIST OF FIGURES	xxiii
LIST OF ABBREVIATIONS	xxvi
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	7
Newcastle Disease	7
Newcastle Disease Virus Classification	7
Clinical Signs and Pathogenicity	8
Worldwide Distribution of NDV	10
NDV Genome and Virion Structure	12
Molecular Determinant of NDV Pathogenicity	14
Nucleocapsid Protein	15
Diagnosis of ND	16
Isolation and Detection of NDV	17
Serology	17
Hemagglutination Test	18
Hemagglutination Inhibition Test	18
Biological Characterization of NDV	18
Mean Death Time	19
Intracerebral Pathogenicity Index and	19
Intravenous Pathogenicity Index	
Molecular Based Techniques in the Diagnosis of NDV	19
Real-time Polymerase Chain Reaction	21
Hydrolysis Probe	22
Hybridization Probe	24
Double-stranded DNA-binding Dye	26
Applications of Real-Time PCR	28
Applications of Real-Time PCR	29
in Diagnosis of NDV	
Implementation of Internal Amplification Control	30
in Real-Time PCR Based Diagnostic Methods	



III	DEVELOPMENT OF SYBR GREEN I BASED TWO-STEP REAL-TIME PCR FOR DETECTION AND DIFFERENTIATION OF NDV PATHOTYPES	33
	Introduction	33
	Materials and Methods	36
	Propagation of NDV	36
	Primer Design	37
	Viral RNA Extraction	41
	Determination of RNA Concentration and Purity	42
	Reverse Transcription and First Strand cDNA Synthesis	42
	Optimization of SYBR Green I based Real-Time PCR	43
	Melting Curve Analysis of the Amplified PCR Products	44
	Agarose Gel Electrophoresis	45
	Ethidium Bromide Staining	45
	Extraction and Purification of Real-Time PCR Product	46
	DNA Sequencing	46
	Sequence Analysis of the Real-Time PCR Product	47
	Development of Real-Time PCR Assay to Detect Velogenic and Lentogenic Strains of NDV	47
	Specificity Test	48
	Construction of Plasmid DNA	49
	TOPO Cloning Reaction	49
	Plasmid DNA Extraction and Purification	50
	Detection Limit and Reproducibility Test	51
	PCR Efficiency	51
	Results	52
	Optimization of SYBR Green I Real-Time PCR with Different Primer Combinations	52
	Primer Set 1A: NDVIF2 & NPV, Set 1B: NDVIF2 & NPL	52
	Primer Set 2A: NDVIF2 & NPV2N, Set 2B: NDVIF2 & NPL2N	56
	Amplification Using Different Concentration of cDNA	60
	Agarose Gel Electrophoresis	64
	Sequence Analysis of the PCR Amplified Product	66
	Development of Real-Time PCR Assay to Detect Velogenic and Lentogenic Strains of NDV	67
	Specificity Test	69
	Detection Limit, Reproducibility Test and PCR Efficiency	71
	Discussion	78

IV	EVALUATION OF PERFORMANCE OF THE DEVELOPED SYBR GREEN I REAL-TIME PCR	82
	Introduction	82
	Materials and Methods	84
	NDV Strains	84
	Determination of Median Embryo Lethal Dose	84
	Experimentally Infected Chickens and Tissue Samples	85
	Virus Inoculation in SPF Chickens	85
	Post-Inoculation Observations and Collection of Infected Tissue Samples	86
	Processing of Tissue Samples	87
	Viral RNA Extraction	87
	Determination of RNA Concentration and Purity	87
	cDNA Synthesis and SYBR Green I Real-Time PCR	87
	Results	88
	Determination of Median Embryo Lethal Dose (ELD ₅₀) of Velogenic NDV Strain AF2240	88
	Clinical Signs	88
	Detection of NDV in Sample from Experimentally Infected Chicken	91
	Detection of Lentogenic NDV Strain F	91
	Detection of Velogenic NDV Strain AF2240	96
	Discussion	110
V	COMPARISON OF THE DEVELOPED REAL-TIME PCR METHOD WITH VIRUS ISOLATION AND SEQUENCING USING SUSPECTED NDV CASES	112
	Introduction	112
	Materials and Method	115
	Chicken Tissue Samples	115
	Processing of Sample	115
	Viral RNA Extraction	118
	Detection of NDV Using SYBR Green I Real-Time PCR	118
	Egg Passage and Hemagglutination Spot Test	118
	Hemagglutination End Point Titration Assay	119
	Determination of Fusion Protein Cleavage	119
	Site Analysis	
	RT-PCR Amplification	119
	Gel Purification and DNA Sequencing	120
	Results	121
	Detection of NDV Using SYBR Green I Real-Time PCR	121
	Validation of Accuracy of SYBR Green I Real-Time PCR in Detection and Differentiation of NDV Pathotypes	133
	Egg Passage and Haemagglutination Spot Test	133
	Hemagglutination End Point Titration Assay	135
	Fusion Protein Cleavage Site Analysis	135
	Discussion	138

VI	USE OF INTERNAL AMPLIFICATION CONTROL IN NDV DIAGNOSTIC REAL-TIME PCR METHOD	141
	Introduction	141
	Materials and Methods	143
	Construction of DNA Plasmid as Internal Amplification Control	143
	Plasmid Extraction and Purification	144
	Determination of DNA Concentration and Purity	144
	Primer	145
	Coamplification of IAC and NDV	145
	Optimization of Real-time PCR and Melting Curve Analysis	145
	Agarose Gel Electrophoresis	147
	Comparison of NDV Real-Time PCR Incorporating With and Without IAC	147
	Assay Reproducibility	147
	Results	148
	Optimization of Duplex Real-Time PCR for IAC and NDV Detection	148
	Melting Curve Analysis	148
	Agarose Gel Electrophoresis	150
	Comparison of NDV Real-Time PCR Incorporating With and Without IAC	153
	Differences of Ct Values	153
	Standard Curve Analysis	153
	Melting Curve Analysis	155
	Assay Reproducibility	158
	Discussion	159
VII	GENERAL DISCUSSION AND CONCLUSION	162
	REFERENCES	169
	APPENDICES	179
	BIODATA OF STUDENT	189
	LIST OF PUBLICATIONS	190



LIST OF TABLES

Table		Page
3.0	Primers designed for two-step real-time PCR	38
3.1	Primer combinations and their relationship to template (NDV) used in real-time PCR	41
3.2	NDV strains used in this study	48
3.3	Threshold cycle (Ct) values and melting temperatures (Tm) of velogenic strain AF2240 when tested with matched and mismatched primer (Primer set 1A)	54
3.4	Threshold cycle (Ct) values and melting temperatures (Tm) of lentogenic strain F when tested with matched and mismatched primer (Primer set 1B)	55
3.5	Threshold cycle (Ct) values and melting temperatures (Tm) of velogenic strain AF2240 when tested with matched and mismatched primer (Primer set 2A)	58
3.6	Threshold cycle (Ct) values and melting temperatures (Tm) of lentogenic strain F when tested with matched and mismatched primer (Primer set 2B)	59
3.7	Amplification of velogenic strain AF2240 using velogenic-specific primer and mismatched primer tested on different concentration of cDNA	61
3.8	Amplification of lentogenic strain F using lentogenic-specific primer and mismatched primer tested on different concentration of cDNA	63
3.9	Detection of velogenic and lentogenic NDV strains with the developed real-time PCR using different primer combinations	68
3.10	Specificity of velogenic- and lentogenic-specific primer evaluated with other avian disease virus	71
3.11	Reproducibility of the developed SYBR Green I real-time PCR	75
4.0	Clinical signs of infected chickens infected with different doses of velogenic NDV AF2240	90

Table	Page	
4.1	Results of SYBR Green I real-time PCR from chickens experimentally infected with lentogenic NDV strain F	91
4.2	SYBR Green I real-time PCR results of lentogenic infection on different days post-infection	95
4.3	Results of SYBR Green I real-time PCR from chickens experimentally infected with velogenic NDV strain AF2240	97
4.4	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group A (10^5 ELD ₅₀ /0.1 ml)	98
4.5	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group B (10^4 ELD ₅₀ /0.1 ml)	100
4.6	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group C (10^3 ELD ₅₀ /0.1 ml)	101
4.7	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group D (10^2 ELD ₅₀ /0.1 ml)	103
4.8	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group E (10^1 ELD ₅₀ /0.1 ml)	106
4.9	SYBR Green I real-time PCR results of infected tissue samples from chickens in Group F ($10^{0.5}$ ELD ₅₀ /0.1 ml)	108
5.0	List of NDV suspected cases used in this study	116
5.1	Suspected NDV cases tested with SYBR Green I real-time PCR	123
5.2	SYBR Green I real-time PCR result of clinical case MB047/05	124
5.3	SYBR Green I real-time PCR result of clinical case MB064/05	126
5.4	SYBR Green I real-time PCR result of clinical case MB076/05	127
5.5	SYBR Green I real-time PCR result of clinical case MB085/05	128
5.6	SYBR Green I real-time PCR result of clinical case MB091/05	129
5.7	SYBR Green I real-time PCR result of clinical case MB093/05	130

Table		Page
5.8	SYBR Green I real-time PCR result of clinical case MB095/05	131
5.9	SYBR Green I real-time PCR result of clinical case MB128/04	132
5.10	Virus isolation method using egg passage	134
5.11	Hemagglutinin (HA) titer of NDV isolates isolated from the clinical cases	135
6.0	Primer for amplification of kanamycin resistance (KanR) gene in IAC-NDV duplex real-time PCR	145
6.1	Comparison of NDV real-time PCR incorporated with or without IAC	154
6.2	Reproducibility of IAC-NDV duplex real-time PCR	158

LIST OF FIGURES

Figure		Page
2.0	Schematic representation of the virion structure of NDV	13
2.1	The hydrolysis (TaqMan) assay	23
2.2	Hybridization probe method	25
2.3	DNA-binding dye method	27
3.0	Nucleotide sequence comparisons of the developed primers with 20 published sequences of velogenic and lentogenic NDV strains	39
3.1	Amplification plot and melting curve analysis of real-time PCR using velogenic strain AF2240 as template (Primer set 1A)	54
3.2	Amplification plot and melting curve analysis of real-time PCR using lentogenic strain F as template (Primer set 1B)	55
3.3	Amplification plot and melting curve analysis of real-time PCR using velogenic strain AF2240 as template (Primer set 2A)	58
3.4	Amplification plot and melting curve analysis of real-time PCR using lentogenic strain F as template (Primer set 2B)	59
3.5	Determination of effective concentration of cDNA in detecting velogenic strain AF2240 using different primer combinations	61
3.6	Determination of effective concentration of cDNA in detecting lentogenic strain F using different primer combinations	63
3.7	Agarose gel electrophoresis using real-time PCR products of velogenic strain AF2240 and lentogenic strain F, respectively	65
3.8	Sequence analysis of PCR products from NDV strain AF2240 and F amplified with primers NDVIF2 & NPV2N and NDVIF2 & NPL2N, respectively	66
3.9	Specificity of NDV velogenic-specific primers (NDVIF2 & NPV2N) evaluated with other avian disease viruses	69
3.10	Specificity of NDV lentogenic-specific primers (NDVIF2 & NPL2N) evaluated with other avian disease viruses	70

