UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION AND DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS SUBTYPES

KONG LIH LING

FPV 2009 4
DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION AND DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS SUBTYPES

By

KONG LIH LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MARCH 2009
Dedication to:

My beloved husband Fu Siaw Liung
My daughter Alysa Fu Han Yun
My son Bryan Fu Jia Wei
My parents and family
DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR THE DETECTION AND DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS SUBTYPES

By

KONG LIH LING

MARCH 2009

Chair: Professor Dr Abdul Rahman Omar, PhD

Faculty: Veterinary Medicine

Two different real-time polymerase chain reaction (PCR) detection approaches based on SYBR Green I dye and Taqman probe based assays were developed for the detection and differentiation of infectious bursal disease virus (IBDV) subtypes. Both approaches were able to detect and differentiate IBDV subtypes based on the use of subtype-specific primers or subtype-specific probes where the primers were designed based on single nucleotide polymorphism (SNP) concept. After optimization of the primer combinations and PCR parameters, very virulent-specific primer, IF & IVIR, and classical-specific primer, IF & RCLA were used in the SYBR Green I real-time RT-PCR assay. Plasmid DNA carrying the VP4 gene of the references IBDV strains: very virulent strain UPM94/273 and classical strain D78 were established and used as positive controls in the real
time RT-PCR. The developed assay had a dynamic detection limit which spans over 5 \( \log_{10} \) concentration range for very virulent and spans over 7 \( \log_{10} \) concentration range for classical strain, respectively. The correlation coefficient for amplification of very virulent and classical strain was \( R^2 = 0.9918 \) and \( R^2 = 0.9977 \), respectively. No amplification was found when the subtype-specific primers were used to amplify other avian RNA viruses. The performance of the SYBR Green I based assay was tested on various IBDV isolates including 10 previously characterized IBDV and 11 commercial vaccine strains. The very virulent-specific primer only detected and amplified the very virulent IBDV with threshold cycle (CT) ranged from 14.93 to 21.52 and melting temperature (Tm) between 85.6°C to 88.0°C. The classical-specific primer was only able to amplify the classical IBDV with CT value ranged from 11.99 to 20.89 and Tm between 85.6°C to 86.8°C. The diagnostic efficacy of the developed assay was also evaluated using bursal samples obtained from experimentally infected chickens. Bursal samples collected from D78 vaccine infected chickens at day 3 and 5 p.i were positive for IBDV with average CT of 23.05±1.31, Tm of 85.8±0.17°C and average CT 21.82±1.42, Tm of 86.0±0.28°C, respectively. Bursal samples collected at day 10 p.i from this group were also found positive for IBDV with average CT of 24.42±1.20 and Tm of 85.9±0.18°C. On the other hand, only bursal samples collected at day 3 and 5 p.i were found positive for very virulent IBDV with average CT 19.39±0.72, Tm of 86.6±0.14°C and average CT 23.55±1.39, Tm of 86.5±0.19°C, respectively. In the case of samples from dual infection with different IBDV subtypes, viral RNA was detectable only on day 3.
and 5 p.i. In general, majority of the bursal samples have higher very virulent virus with an average CT value ranged from 21.24±0.68 to 22.19±0.97 compared to vaccine virus with Ct value ranged from 23.88±0.74 to 25.36±1.19.

The performance of the developed SYBR Green I based assay was analyzed with other standard diagnostic methods. In the uninfected control group, no obvious microscopic lesions were found in the bursa and the lesions score was less than 1.0. However, mild bursal lesions without signs of inflammation with lesions score less than 3.0 was detected from bursal tissue obtained from chickens inoculated with vaccine strain D78. Based on the lesion score, it was clear that bursal pathology developed rapidly, with complete loss of tissue architecture by day 3 p.i. when the chickens were infected with virulent IBDV. The correlation between ELISA antibody titers and real-time CT values were inversely related, where the lower titers of antibodies associated with higher level of viral RNA as found in chickens infected with very virulent strain UPM94/273. On the other hand, vaccine strain D78 induced higher detectable antibody titers than UPM94/273, which indirectly support less virus replication with late positive amplification in real-time RT-PCR. Thus, the level of viral RNA in bursal samples obtained from D78 infected chickens was lower than UPM94/273 infected chickens. A total of 37 bursal samples from IBD suspected field cases were collected and then tested on the developed assay. The developed SYBR Green I based PCR assay was able to detect 9 samples positive for very virulent, 4 positive for classical IBDV and 12 samples positive for both very virulent and
vaccine strains of IBDV. Sequence analysis of the hypervariable region of the VP2 gene of the IBDV samples revealed that the residues involved in determining the virulence of VV IBDV and CL IBDV were highly conserved. For the Taqman based duplex real-time PCR assay development, a new set of primers FWDC and RVSC were designed from the conserved region of VP4 of both very virulent and classical strains. A dual-labeled fluorescent probe each specific for very virulent IBDV (ProVV) and vaccine IBDV (ProCL) were designed. The performance of the developed Taqman assay was compared with other PCR methods namely conventional RT-PCR and previously developed SYBR Green I assay. The Taqman assay was found far more superior in terms of turn around time and sensitivity. With the aid of β-actin gene, the Taqman assay was also used to determine the viral load fold changes in bursal samples that were positive for both vaccine and very virulent IBDV. Majority of these samples have higher viral load fold change in very virulent than the classical strain except for three samples MB078/04, MB001/05 and MB033/05 which showed higher fold change in classical strain than very virulent strain. In conclusion, this study has successfully developed SYBR Green I based and Taqman based one-step real-time PCR assays for rapid detection and differentiation of IBDV subtypes in particular very virulent and classical IBDV strains.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN ASAI TINDAK BALAS RANTAI POLIMERASE UNTUK PENGESANAN DAN PERBEZAAN SUBTIP VIRUS PENYAKIT BURSAL BERJANGKIT

Oleh

KONG LIH LING

MAC 2009

Pengerusi: Professor Dr Abdul Rahman Omar, PhD

Fakulti: Perubatan Veterinar

Dua pendekatan berlainan tindak balas rantai polimerase berdasarkan pewarna SYBR Green I and probe Taqman telah dibangunkan bagi mengesan dan membezakan subtip IBDV. Kedua-dua pendekatan ini berupaya mengesan dan membezakan subtip IBDV berdasarkan kepada primer spesifik subtip atau probe spesifik subtip di mana reka bentuk primer adalah berdasarkan konsep polimorfisme nukleotida tunggal (SNP). Selepas pengoptimuman gabungan primer dan parameter PCR, primer spesifik sangat virulen, IF & IVIR, dan primer spesifik klasik, IF & RCLA telah digunakan dalam asai PCR SYBR Green I masa nyata. Plasmid DNA dengan gen VP4 dari strain sangat virulen UPM94/273 dan strain klasik D78 telah dibangunkan dan digunakan sebagai kawalan positif dalam RT-PCR masa nyata. Ujian yang dibangunkan menunjukkan had pengesanan yang dinamik di mana ia meliputi julat kepekatan sebanyak 5 log untuk strain sangat virulen dan julat kepekatan sebanyak 7 log untuk strain
klasik, masing-masing. Koefisien korelasi ($R^2$) bagi amplifikasi strain sangat virulen dan strain klasik adalah 0.9918 dan 0.9977, masing-masing. Tiada amplifikasi dikesan apabila primer spesifik subtip tersebut digunakan ke atas virus RNA unggas yang lain. Prestasi asai SYBR Green I diuji ke atas pelbagai isolat IBDV yang terdiri daripada 10 isolat IBDV yang telah dikaji dahulu dan 11 strain vaksin komersial. Primer spesifik sangat viru len hanya mengesan strain IBDV sangat virulen dengan had kitaran (CT) berjulat antara 14.93 ke 21.52 dan suhu peleburan (Tm) di antara 85.6°C dan 88.0°C. Manakala, primer spesifik klasik hanya dapat mengesan strain IBDV klasik dengan CT berjulat antara 11.99 ke 20.89 dan Tm di antara 85.6°C dan 86.8°C. Keberkesanan diagnostik asai yang dibangunkan ini juga dinilai menggunakan sample bursa daripada ayam yang dijangkiti secara eksperimen. Sample bursa dari ayam yang disuntik vaksin D78 pada hari 3 dan 5 selepas jangkitan (p.i) adalah positif untuk IBDV dengan purata CT 23.05±1.31, Tm 85.8±0.17°C dan purata CT 21.82±1.42, Tm 86.0±0.28°C, masing-masing. Sampel bursa yang dikumpulkan pada hari ke-10 didapati positif untuk IBDV dengan purata CT 24.42±1.20 dan Tm 85.9±0.18°C. Namun, hanya sampel bursa yang dikumpulkan pada hari 3 dan 5 p.i. adalah positif untuk IBDV amat virulen dengan purata CT 19.39 ± 0.72, Tm 86.6±0.14°C dan purata CT 23.55±1.39, Tm 86.5±0.19°C. Dalam kes sampel dari jangkitan berganda dengan subtip IBDV berlainan, RNA virus hanya dapat dikesan pada hari ke-3 dan ke-5 p.i. Secara keseluruhan kebanyakan sampel bursa mempunyai tahap tinggi virus sangat virulen dengan purata CT di antara
21.24±0.68 ke 22.19±0.97 dibandingkan dengan virus vaksin dengan purata CT di antara 23.88±0.74 ke 25.36±1.19.

residu-residu yang terlibat dalam menentukan virulen VV IBDV dan CL IBDV adalah amat terpelihara. Bagi pembangunan asai masa-nyata Taqman duplex, satu set primer baru FWDC dan RVSC telah direka bentuk berpandukan pada kawasan VP4 terpelihara bagi kedua-dua strain sangat virulen dan klasik. Satu probe label berganda pendarfluor masing-masing spesifik untuk IBDV strain amat virulen (proVV) dan strain klasik (ProCL) telah dibangunkan. Prestasi asai Taqman yang dibangunkan tersebut telah dibandingkan dengan kaedah PCR lain iaitu RT-PCR konvensional dan asai SYBR Green I. Asai Taqman tersebut didapati lebih efisien dalam konteks masa asai tersebut tamat dan sensitif. Dengan bantuan gen β-actin, asai Taqman tersebut juga digunakan untuk menentukan perubahan gandaan dalam sampel bursa yang didapati positif untuk kedua-dua IBDV amat virulen dan klasik vaksin. Majoriti sampel bursa tersebut mempunyai perubahan gandaan virus bagi strain sangat virulen adalah lebih tinggi berbanding strain klasik kecuali bagi sampel MB078/04, MB001/05 dan MB033/05 menunjukkan bahawa perubahan gandaan dalam strain klasik lebih tinggi berbanding strain sangat virulen. Kesimpulannya, kajian ini telah berjaya membangunkan dua asai PCR berdasarkan SYBR Green I dan Taqman berdasarkan asai PCR satu-langkah bagi pengesanan dan pembezaan pantas subtip IBDV terutamanya strain amat virulen dan klasik.
ACKNOWLEDGEMENTS

I would like to express my heartfelt thank and appreciation to my supervisor, Prof. Dr. Abdul Rahman Omar, co-supervisors, Prof. Datin Paduka Dr. Aini Ideris and Prof. Dr. Mohd Hair Bejo. The completion of this dissertation would not be achieved without the constructive advices, continuous supports and constant guidance from my supervisory committee. They are most responsible for helping me complete the writing of this dissertation as well as the challenging research that lies behind it. They are my mentor and inspiration. Their wisdom, knowledge and commitment to the highest standard inspired and motivated me. They showed me the best role model of a diligent scientist, versatile and intense thinker, and the need to be persistent to accomplish any goal. Thank you for believing in me and willing to accept both my strength and weakness. I am very grateful to Prof. Rahman, who gave me insightful comments and proofread my chapters despite with many other academic and professional commitments.

Special thanks go to my dearest friend: Sheau Wei, for always standing by me, and for sharing all my happiness and sorrow. It has been a great experience and a joy working with my colleagues in the Biologics laboratory. I would like to express my gratitude to all my lab-mates, who involved directly or indirectly in my doctoral research: Siti Khatijah Muhamad, Zairah Zulperi, Nurulfiza Mat Isa, Tan Ching Giap, Wan Keng Fei, Nurul Hidayah Abdullah Zawawi and Lim Kian Lum. Thanks for their invaluable supports and genuine friendship. Special thanks go to
Sheau Wei, Ching Giap and Wan, for helping me in my experimental trial. I am also grateful that during the course of PhD study, I was supported by National Science Fellowship (NSF) from the Ministry of Science, Technology and Innovation (MOSTI) Malaysia.

Last but not least, I thank my family especially my husband, for their unconditional supports and encouragement to pursue my interest. They always hold me tight in the midst of storm and replenish me with their unfailing love.
I certify that a Thesis Examination Committee has met on 26 March 2009 to conduct the final examination of Kong Lih Ling on her thesis entitled "Development of Real-Time Polymerase Chain Reaction Assays for the Detection and Differentiation of Infectious Bursal Disease Virus Subtypes" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the candidate be awarded the Doctor of Philosophy.

Members of the Examination Committee are as follows:

**Hassan Hj Mohd Daud, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Siti Suri Arshad, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Tan Wen Siang, PhD**
Professor
Faculty of Biotechnology and Molecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

**Daral J. Jackwood, PhD**
Professor
The Ohio State University
United States
(External Examiner)

_______________________________
BUJANG KIM HUAT, PhD
Professor and Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia.

Date: 21 May 2009
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

**Abdul Rahman Omar, Ph.D.**  
Professor,  
Department of Veterinary Pathology and Microbiology,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Chairman)

**Datin Paduka Aini Ideris, Ph.D.**  
Professor,  
Department of Veterinary Clinical Studies,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Member)

**Mohd Hair Bejo, Ph.D.**  
Professor,  
Department of Veterinary Pathology and Microbiology,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.  
(Member)

HASANAH MOHD. GHAZALI, PhD  
Professor and Dean,  
School of Graduate Studies,  
Universiti Putra Malaysia.

Date: 8 June 2009
DECLARATION

I here 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.

________________
KONG LIH LING

Date: 15 April 2009
TABLE OF CONTENTS

DEDICATION ii
ABSTRACT iii
ABSTRAK vii
ACKNOWLEDGEMENTS xi
APPROVAL xiii
DECLARATION xv
LIST OF TABLES xxi
LIST OF FIGURES xxv
LIST OF ABBREVIATIONS xxxii

CHAPTER

1 INTRODUCTION 1

2 LITERATURE REVIEW 8
  2.1 Infectious Bursal Disease (IBD) 8
  2.2 Infectious Bursal Disease Virus (IBDV) 10
    2.2.1 Description of the Virus 10
    2.2.2 Genome and Proteins 12
    2.2.3 Antigenicity 15
    2.2.4 Pathogenicity 17
  2.3 Diagnosis of IBD 18
    2.3.1 Clinical and Histopathological Diagnosis 19
    2.3.2 Serological Diagnosis 21
    2.3.3 Virological Diagnosis 22
  2.4 Control and Prevention of IBD 23
  2.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR) 25
    2.6 Real-Time RT-PCR (RRT-PCR) 27
      2.6.1 Melting Curve Analysis 28
      2.6.2 Threshold Cycle 29
      2.6.3 Chemistry of RRT-PCR 30
      2.6.4 Application of RRT-PCR in Diagnosis of IBDV 36

3 DEVELOPMENT OF SYBR GREEN 1 BASED ONE-STEP REAL-TIME RT-PCR DETECTION METHOD FOR DETECTION AND DIFFERENTIATION OF IBDV SUBTYPES 38
  3.1 Introduction 38
  3.2 Materials and Methods 41
3.2.1 Viruses and Preparation of Virus Stocks
3.2.2 Primers Design
3.2.3 RNA Extraction
3.2.4 Determination of RNA Concentration and Purity
3.2.5 Conventional RT-PCR
3.2.6 Determination of PCR Products Concentration and Purity
3.2.7 Agarose Gel Electrophoresis
3.2.8 Purification of PCR Products
3.2.9 TOPO Cloning Reaction
3.2.10 Subculture and Analysis of Positive Colonies
3.2.11 Preparation of Stock Culture
3.2.12 Extraction and Purification of Recombinant Plasmids
3.2.13 Restriction Enzyme Analysis of the Plasmids
3.2.14 RRT-PCR Primers Used
3.2.15 SYBR Green 1 Based One-Step RRT-PCR
3.2.16 Melting Curve Analysis
3.2.17 RRT-PCR Assay on Samples Previously Characterized IBDV Field Isolates
3.2.18 Detection Limit of the Developed Method
3.2.19 Specificity of the Developed Method
3.3 Results
3.3.1 Conventional RT-PCR
3.3.2 Analysis of Recombinant Plasmid
3.3.3 Restriction Enzyme Digestion Analysis
3.3.4 Positive Control
3.3.5 Optimization of RRT-PCR
3.3.6 Development of the One-Step RRT-PCR Detection Method for Rapid Differentiation of IBDV Subtypes
  Very Virulent Strain UPM94/273
  Classical Strain D78
  RRT-PCR Assay on Samples Previously Characterized IBDV Field Strains
  IBDV Vaccine Strains
3.3.7 Detection Limit of the Developed Method
3.3.8 Specificity of the Developed Method
3.4 Discussion
4 EVALUATION ON PERFORMANCE OF THE DEVELOPED REAL-TIME PCR ASSAY IN DETECTING IBDV SUBTYPES USING BURSAL SAMPLES FROM EXPERIMENTAL INFECTION

4.1 Introduction 115
4.2 Materials and Methods 117
  4.2.1 Viruses 117
  4.2.2 Propagation of Viruses in SPF Embryonated Chicken Eggs 117
  4.2.3 Titration of IBDV 118
  4.2.4 Experimental Infection in SPF Chickens 118
    Immunogenicity Studies Using ELISA 119
    Histopathology 120
    Bursal to Body Weight Ratio (B/B Ratio) 121
  4.2.5 RNA Extraction 121
  4.2.6 Determination of RNA Concentration and Purity 121
  4.2.7 Primer Used 122
  4.2.8 SYBR Green 1 Based One-Step RRT-PCR 122
  4.2.9 Melting Curve Analysis 122
  4.2.10 Statistical Analysis 122

4.3 Results 123
  4.3.1 Experimental Infection in SPF Chickens 123
    Clinical Signs and Mortalities 123
    Determination of Bursa to Body Weight Ratio 124
    Histopathology and Lesions Scoring 128
    Enzyme-Linked Immunosorbent Assay (ELISA) 135
  4.3.2 Detection and Differentiation of Experimental Samples 137

4.4 Discussion 148

5 ACCURACY OF THE DEVELOPED REAL-TIME PCR ASSAY IN DETECTING IBDV SUBTYPES USING BURSAL SAMPLES FROM SUSPECTED IBD OUTBREAK CASES

5.1 Introduction 152
5.2 Materials and Methods 155
  5.2.1 IBDV Suspected Cases 155
  5.2.2 Preparation of Virus Stocks 156
  5.2.3 RNA Extraction 157
  5.2.4 Determination of RNA Concentration and Purity 157
  5.2.5 Primer Used 157
  5.2.6 SYBR Green 1 Based One-Step RRT PCR 157
  5.2.7 Melting Curve Analysis 158
5.2.8 Negative Amplification Confirmation
5.2.9 Positive Amplification Confirmation
5.2.10 Agarose Gel Electrophoresis and Ethidium Bromide Staining
5.2.11 Purification of PCR Product
5.2.12 DNA Sequencing
5.2.13 Sequence Assembly and Analysis

5.3 Results
5.3.1 Validation on the Performance of the Developed Assay
   Bursal Samples from IBD Suspected Outbreak Cases
5.3.2 Negative Amplification Confirmation
5.3.3 Sequence Analysis of VP2 Gene of Bursal Samples from IBDV Suspected Cases

5.4 Discussion

6 DEVELOPMENT OF TAQMAN BASED REAL-TIME RT-PCR IN COMPARISON TO SYBR GREEN I BASED REAL-TIME RT-PCR FOR THE DETECTION OF IBDV SUBTYPES
6.1 Introduction
6.2 Materials and Methods
   6.2.1 IBDV Samples from IBD Suspected Outbreak Cases
   6.2.2 Experimental Trial Samples
   6.2.3 Primers and Probes Design
   6.2.4 RNA Extraction
   6.2.5 Determination of RNA Concentration and Purity
   6.2.6 Optimization of TaqMan Based Duplex RRT-PCR
   6.2.7 TaqMan Based Duplex RRT-PCR
   6.2.8 Assay Detection Limit
   6.2.9 Assay Specificity
   6.2.10 Assay Reproducibility
   6.2.11 Relative Quantification
6.3 Results
   6.3.1 Primers and Probes Design
   6.3.2 Optimization of TaqMan Assay
   6.3.3 Assay Detection Limit
   6.3.4 Assay Reproducibility
   6.3.5 Performance and Validation of TaqMan Assay
      Bursal Samples Tested Positive as Very Virulent IBDV Strains
      Bursal Samples Tested Positive as Classical IBDV Strains
      Bursal Samples Tested Positive as Dual
6.3.6 Comparisons on the turn out time of different PCR assays
6.3.7 Viral Load Fold Change
   - Beta-Actin House-Keeping Gene
   - Viral Load Fold Change of Dual Positive IBDV Bursal Samples and Experimental Trial Samples

6.4 Discussion

7. GENERAL DISCUSSION AND CONCLUSION

RECOMMENDATIONS FOR FUTURE STUDIES

BIBLIOGRAPHY

APPENDICES

BIODATA OF AUTHOR

LIST OF PUBLICATIONS
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Primers used for amplification of different IBDV subtypes</td>
<td>50</td>
</tr>
<tr>
<td>3.2</td>
<td>Previously characterized IBDV field strains used in the study</td>
<td>53</td>
</tr>
<tr>
<td>3.3</td>
<td>List of IBDV vaccine strains used in the study</td>
<td>54</td>
</tr>
<tr>
<td>3.4</td>
<td>Performance of one-step RRT-PCR in detecting specific amplification of vvIBDV, UPM94/273 using perfect match and mismatch primer combinations</td>
<td>68</td>
</tr>
<tr>
<td>3.5</td>
<td>Performance of one-step RRT-PCR in detecting specific amplification of classical IBDV, D78 using perfect match and mismatch primer combinations</td>
<td>71</td>
</tr>
<tr>
<td>3.6</td>
<td>Detection of previously characterized IBDV field strains with the developed real-time RT-PCR using different primer combinations</td>
<td>75</td>
</tr>
<tr>
<td>3.7</td>
<td>Detection of IBDV vaccine strains with the developed real-time RT-PCR using different primer combinations</td>
<td>82</td>
</tr>
<tr>
<td>3.8</td>
<td>Threshold cycle (CT) and melting temperature (Tm) values of amplification of serially diluted total RNA of very virulent IBDV UPM94/273 using match and mismatch primer combinations</td>
<td>91</td>
</tr>
<tr>
<td>3.9</td>
<td>Threshold cycle (CT) and melting temperature (Tm) values of amplification of serially diluted total RNA of classical IBDV D78 using match and mismatch primer combinations</td>
<td>94</td>
</tr>
<tr>
<td>3.10</td>
<td>Threshold cycle (CT) and melting temperature (Tm) values of amplification of serially diluted plasmid of very virulent IBDV UPM94/273 using match and mismatch primer combinations</td>
<td>100</td>
</tr>
<tr>
<td>3.11</td>
<td>Threshold cycle (CT) and melting temperature (Tm) values of amplification of serially diluted plasmid of classical IBDV D78 using match and mismatch primer combinations</td>
<td>104</td>
</tr>
<tr>
<td>3.12</td>
<td>Threshold cycle (CT) and melting temperature (Tm) values of specific amplification of IBDV reference strains and other avian viruses</td>
<td>110</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1</td>
<td>Mortalities rate for the experimentally groups</td>
<td>124</td>
</tr>
<tr>
<td>4.2</td>
<td>Body weight of experimental chickens in four different groups</td>
<td>125</td>
</tr>
<tr>
<td>4.3</td>
<td>Bursa weight of experimental chickens in four different groups</td>
<td>126</td>
</tr>
<tr>
<td>4.4</td>
<td>Bursa: body weight ratios of experimental chickens in four different groups</td>
<td>127</td>
</tr>
<tr>
<td>4.5</td>
<td>Histological lesions in the bursa of Fabricius of experimental chickens in four different groups</td>
<td>129</td>
</tr>
<tr>
<td>4.6</td>
<td>Number of chicken positive for IBDV antibody detected by ELISA</td>
<td>135</td>
</tr>
<tr>
<td>4.7</td>
<td>IBDV antibody titers in average $\log_{10}$ measured by ELISA</td>
<td>136</td>
</tr>
<tr>
<td>4.8</td>
<td>SYBR Green I based real-time RT-PCR detection of vaccine D78 infection on different days post-infection</td>
<td>140</td>
</tr>
<tr>
<td>4.9</td>
<td>SYBR Green I based real-time RT-PCR detection of very virulent, UPM94/273 infection on different days post-infection</td>
<td>143</td>
</tr>
<tr>
<td>4.10</td>
<td>SYBR Green I based real-time RT-PCR detection of both vaccine (CL) D78 and very virulent (VV) UPM94/273 infection on different days post-infection</td>
<td>147</td>
</tr>
<tr>
<td>5.1</td>
<td>List of bursal samples from IBD suspected cases used in this study</td>
<td>155</td>
</tr>
<tr>
<td>5.2</td>
<td>VP2 primers used in RT-PCR amplification</td>
<td>159</td>
</tr>
<tr>
<td>5.3</td>
<td>Bursal samples from IBD suspected outbreak cases tested with SYBR Green I real-time RT-PCR</td>
<td>169</td>
</tr>
<tr>
<td>5.4</td>
<td>GenBank accession number for the 25 IBDV isolates used in this study</td>
<td>174</td>
</tr>
<tr>
<td>5.5</td>
<td>Comparison of amino substitution at VP2 hypervariable region between classical and very virulent strains and the 25 IBDV isolates isolated from IBD suspected cases</td>
<td>178</td>
</tr>
<tr>
<td>6.1</td>
<td>Primers-probes (114 bp) for IBDV subtypes detection</td>
<td>191</td>
</tr>
<tr>
<td>6.2</td>
<td>Primers-probe (81 bp) for beta-actin house-keeping gene</td>
<td>191</td>
</tr>
</tbody>
</table>
6.3a Threshold cycle values for gradient real-time RT-PCR tested on dual-labeled probes with different annealing temperature

6.3b Threshold cycle values for gradient duplex real-time RT-PCR tested on dual-labeled probes for very virulent IBDV with different annealing temperature

6.4a Intra assay variation for amplification of serially diluted RNA from very virulent IBDV strain

6.4b Intra assay variation for amplification of serially diluted RNA from classical IBDV strain

6.4c Intra assay variation for amplification of serially diluted RNA from $\beta$-actin, house-keeping gene

6.5 Threshold cycle values and variability data of Taqman based duplex real-time RT-PCR assay for bursal samples tested positive for very virulent IBDV detection

6.6 Threshold cycle values and variability data of Taqman based duplex real-time RT-PCR assay for bursal samples tested positive for classical IBDV strains detection

6.7 Threshold cycle values and variability data of Taqman based duplex real-time RT-PCR assay for dual positive IBDV strains detection

6.8 Threshold cycle values and variability data of Taqman based duplex real-time RT-PCR assay for experimental trial dual-infection IBDV detection

6.9 Assay comparison between the developed duplex Taqman RRT-PCR with SYBR Green I RRT-PCR and conventional RT-PCR

6.10 The parameters for equation of standard curves, correlation of coefficient ($R^2$), and difference in slope ($\Delta s$) for $\beta$-actin and IBDVs D78 and UPM94/273

6.11 Threshold cycle values and variability data of $\beta$-actin house-keeping gene for dual positive IBDV bursal samples obtained from suspected IBD cases and experimental trial infections

6.12 Viral load fold change of dual positive IBDV bursal samples using the $2^{-\Delta\Delta Ct}$ Method
6.13 Viral load fold change of experimental trial dual-infection IBDV samples on day 3 and 5 p.i using the $2^{-\Delta\Delta C_t}$ Method