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

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

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 yery 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 specifik sangat virulen 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$\pm$1.31, Tm 85.8$\pm$0.17°C dan purata CT 21.82$\pm$1.42, Tm 86.0$\pm$0.28°C, masing-masing. Sampel bursa yang dikumpulkan pada hari ke-10 didapati positif untuk IBDV dengan purata CT 24.42$\pm$1.20 dan Tm 85.9$\pm$0.18°C. Namun, hanya sampel bursa yang dikumpulkan pada hari 3 dan 5 p.i. adalah positif untuk IBDV amat virulen dengan purata CT 19.39 ± 0.72, Tm 86.6$\pm$0.14°C dan purata CT 23.55$\pm$1.39, Tm 86.5$\pm$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.

Prestasi asai SYBR Green I yang dibangunkan ini juga diuji dengan kaedah diagnostik yang lain. Dalam kumpulan kawalan tanpa jangkitan, tiada lesi mikroskop pada tisu bursa dengan skor lesi kurang daripada 1.0. Namun, ayam yang telah diinokulasi dengan D78 hanya mempunyai lesi yang sederhana tanpa tanda-tanda keradangan dan skor lesi kurang daripada 3.0. Berdasarkan pada skor lesi, ia jelas bahawa patologi bursa terjadi dengan cepat, dengan kemusnahan lengkap seni bina tisu pada hari ke-3 p.i bagi ayam yang dijangkiti virus strain virulent. Kolerasi antara titer antibodi ELISA dan nilai CT masa nyata adalah berkaitan secara songsang, di mana titer antibodi terendah berkaitan dengan paras tertinggi virus RNA sebagaimana dijumpai dalam ayam yang telah dijangkiti dengan strain sangat virulen UPM94/273. Tambahan pula, strain vaksin D78 telah mencetuskan titer antibodi yang tinggi berbanding UPM94/273, yang mana secara tidak langsung tidak menyokong replikasi virus dan amplifikasi positif lewat dalam RT-PCR masa nyata. Oleh itu, paras RNA virus dalam D78 adalah lebih rendah berbanding UPM94/273. Sejumlah 37 bursa sampel daripada kes lapangan disyaki IBD dikumpulkan dan diuji ke atas asai yang dibangunkan. Asai SYBR Green I yang dibangunkan berjaya mengesan 9 sampel positif untuk strain amat virulen, 4 sampel positif untuk strain klasik dan 12 sampel positif untuk kedua-dua strain IBDV amat virulen dan klasik. Analisis jujukan bahagian hiper-bolehubah gen VP2 sampel IBDV tersebut menunjukkan

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 konventional dan asai SYBR Green I. Asai Taqman tersbut 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, Zarirah 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

LIST OF TABLES