



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

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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

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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 (T_m) 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 T_m 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 , T_m of $85.8 \pm 0.17^\circ\text{C}$ and average CT 21.82 ± 1.42 , T_m of $86.0 \pm 0.28^\circ\text{C}$, 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 T_m of $85.9 \pm 0.18^\circ\text{C}$. 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 , T_m of $86.6 \pm 0.14^\circ\text{C}$ and average CT 23.55 ± 1.39 , T_m of $86.5 \pm 0.19^\circ\text{C}$, 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.



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

Oleh

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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 virulen hanya mengesan strain IBDV sangat virulen dengan had kitaran (CT) berjulat antara 14.93 ke 21.52 dan suhu peleburan (T_m) 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 T_m 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 , T_m $85.8 \pm 0.17^\circ\text{C}$ dan purata CT 21.82 ± 1.42 , T_m $86.0 \pm 0.28^\circ\text{C}$, masing-masing. Sampel bursa yang dikumpulkan pada hari ke-10 didapati positif untuk IBDV dengan purata CT 24.42 ± 1.20 dan T_m $85.9 \pm 0.18^\circ\text{C}$. 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 , T_m $86.6 \pm 0.14^\circ\text{C}$ dan purata CT 23.55 ± 1.39 , T_m $86.5 \pm 0.19^\circ\text{C}$. 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 \pm 0.68 ke 22.19 \pm 0.97 dibandingkan dengan virus vaksin dengan purata CT di antara 23.88 \pm 0.74 ke 25.36 \pm 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 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 subtipe IBDV terutamanya strain amat virulen dan klasik.



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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.

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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.

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Date: 15 April 2009



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