UNIVERSITI PUTRA MALAYSIA

STRUCTURAL GENE CHARACTERIZATION AND DEVELOPMENT OF REAL-TIME PCR DETECTION METHOD FOR INFECTIOUS BRONCHITIS VIRUS

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MASTER OF SCIENCE
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ZARIRAH MOHAMED ZULPERI

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

August 2008

Dedicated to

My lovely dad and mum,
Mr. Mohamed Zulperi Zakaria and Mrs. Samirah Ismail

My beautiful sis,
Ms. Dzarifah Mohamed Zulperi

And to all my wonderful families and friends

Thank you very much; you all are the light of my life

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

STRUCTURAL GENE CHARACTERIZATION AND DEVELOPMENT OF REAL-TIME PCR DETECTION METHOD FOR INFECTIOUS BRONCHITIS VIRUS

By

ZARIRAH MOHAMED ZULPERI

August 2008

Chairman: **Abdul Rahman Omar, PhD**

Faculty: **Veterinary Medicine**

Infectious bronchitis virus (IBV) causes an acute, highly contagious disease that associated with significant economical losses to the poultry industry worldwide. Since IB vaccine induced immunity is serotype-specific, vaccine failures are often associated with the emergence of antigenic variants that differ from the vaccine viruses. Hence, structural genes characterization of IBV isolates is required for developing strategies to improve currently used vaccines. This study described the sequence analysis of structural genes S1, S2, M and N of two Malaysian IBV strains, MH5365/95 and V9/04. The structural genes were amplified using gene-specific primers in reverse-transcriptase polymerase chain reaction (RT-PCR) and cloned into TOPO TA cloning vector. The cloned products were then sequenced and complete nucleotide and amino acid sequences of the genes were determined and analyzed using bioinformatics tools. Nucleotide sequence alignments revealed many point mutations, short deletions and insertions in S1 region of both IBV strains. Sequence and phylogenetic analysis of S1 and M genes

showed that MH5365/95 and V9/04 are variant strain since it does not relate to any Massachusetts vaccine strains of IBV. Analysis based on other structural genes (S2, M and N), indicated both strains are highly related to each other and there were less mutation occurred in the respective genes.

A one step real-time PCR procedure using SYBR Green I dye was used in this study. The developed PCR was performed using a pair of newly designed primers against N gene. Amplification was detected when primer was used with vaccine strains, H120, Volvac® ND+IB MLV, Cevac® BI L, Poulvac® IB H-120, Cevac® Bron 120 L, Beaudette and M4 and non-vaccine strains, T-strain, MH5365/95 and V9/04 with T_M ranging from 85.2°C to 86.0°C. The sensitivity of the assay was compared to the conventional method for IBV detection, virus isolation test using 18 clinical samples from IB suspected cases. Six out of 18 suspected IB cases were positive when analyzed using the real-time PCR assay while none of the samples showed consistent results through lesion observation when analyzed using the virus isolation method followed by HA test. Therefore, the SYBR Green I real-time PCR was found to be more sensitive than virus isolation method. In addition, it was found the assay could detect up to $1:10^3$ dilution of RNA with the concentration $1X\ 10^{-1}$ ng/µl. The assay was also proven to be specific in IBV detection since no specific amplification signal was detected when tested avian viruses such as AIV H9N2, IBDV isolate MB067/05 and NDV isolate 00/IKS. Therefore, the study has successfully developed a rapid, sensitive and specific method for IBV detection using SYBR Green I based real-time PCR when compared to virus isolation and HA test. However, further experiment for improvement of the assay is needed to evaluate the reliability of the system in order to replace the traditional diagnostic method for IBV detection.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat untuk ijazah Master Sains

PENCIRIAN STRUKTUR GEN DAN PEMBANGUNAN KAEDAH PCR MASA NYATA BAGI PENGESANAN VIRUS PENYAKIT BRONKITIS

Oleh

ZARIRAH MOHAMED ZULPERI

Ogos 2008

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Fakulti: **Perubatan Veterinar**

Penyakit virus bronkitis (IB) menyebabkan penyakit berjangkit yang kronik dan ia menyebabkan kerugian yang besar dalam industri penternakan ayam di seluruh dunia. Oleh sebab vaksin yang terdapat pada masa kini adalah terhad kepada sesetengah serotip, kebanyakan kes kegagalan vaksin IB adalah disebabkan wujudnya serotip IB baharu yang berlainan dengan vaksin IB yang terdapat di pasaran. Oleh itu, kajian terhadap penstrukturan gen virus IB adalah penting untuk menyusun, membangun dan memperbaiki strategi dalam penggunaan dan penyediaan vaksin IB. Dalam kajian ini, gen struktur S1, S2, M dan N bagi dua isolat IB dari Malaysia, MH5365/95 dan V9/04 telah dianalisis. Gen struktur tersebut telah diamplifikasi menggunakan pencetus yang spesifik dalam tindak balas berantai polimerase (PCR) transkriptase berbalik dan hasil PCR telah diklon ke dalam vektor TOPO TA. Klon tersebut disahkan melalui analisis penjujukan nukleotida dan asid amino menggunakan perisian bioinformatik. Didapati, jujukan nukleotida pada gen S1 bagi isolat MH5365/95 dan V9/04 mempunyai banyak

mutasi titik, mutasi pendek dan penambahan nukleotida. Analisis filogenetik bagi gen struktur S1 dan M menunjukkan isolat MH5365/95 dan V9/04 adalah strain varian kerana tidak mempunyai persamaan dengan vaksin IBV strain Massachusetts. Analisis gen struktur yang lain (S2, M dan N) menunjukkan terdapat persamaan di antara kedua-dua isolat dan hanya sedikit proses mutasi yang berlaku di dalam gen tersebut.

PCR masa nyata (real-time PCR) menggunakan pewarna SYBR Green I telah digunakan dalam kajian ini. PCR masa nyata yang dibangunkan ini menggunakan sepasang pencetus baru yang direka berdasarkan kepada gen N. Amplifikasi telah dikesan apabila pencetus tersebut diuji dengan strain vaksin, H120, Volvac® ND+IB MLV, Cevac® BI L, Poulvac® IB H-120, Cevac® Bron 120 L, Beaudette dan M41 dan strain bukan vaksin, T-strain, MH5365/95 dan V9/04 dengan T_M pada suhu di antara 85.2°C sehingga 86.0°C. Perbandingan sensitiviti asai tersebut telah dijalankan dengan menggunakan kaedah tradisional untuk mengesan IB iaitu kaedah pengasingan virus melalui ujian sampel klinikal yang disyaki positif IB. Daripada eksperimen tersebut, enam daripada lapan belas sampel klinikal tersebut didapati positif IB apabila kaedah PCR masa nyata digunakan tetapi keputusan adalah tidak konsisten apabila analisis menggunakan kaedah pengasingan virus diikuti dengan ujian HA. Oleh itu, asai PCR masa nyata didapati adalah lebih sensitif berbanding kaedah pengasingan virus. Di samping itu, PCR masa nyata didapati dapat mengesan virus sehingga 10^{-3} pencairan RNA iaitu bersamaan dengan $1x10^{-1}$ ng/µl RNA. Ia juga adalah spesifik terhadap virus IB kerana tiada amplifikasi dapat dikesan apabila diuji menggunakan virus unggas yang lain seperti AIV H9N2, IBDV isolat MB067/05 dan NDV isolat 00/IKS. Oleh yang demikian, kaedah PCR masa nyata menggunakan pewarna SYBR Green I adalah sensitif, spesifik, cepat dan mudah bagi mengesan IBV apabila dibandingkan dengan kaedah pengasingan virus

dan ujian HA. Walau bagaimanapun, kajian lanjutan diperlukan untuk menguji tahap keberkesanan asai ini sebelum ia dapat menggantikan kaedah diagnosis tradisional dalam pengesanan virus IB.

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I certify that an Examination Committee has met on 27th August 2008 to conduct the final examination of Zarirah binti Mohamed Zulperi on her Master of Science thesis entitled "Structural Gene Characterization and Development of Real-Time PCR Detection Method for Infectious Bronchitis Virus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

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Date: 23 October 2008

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

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Date: 13 November 2008

DECLARATION

I 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 Universiti Putra Malaysia or other institutions.

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ZARIRAH MOHAMED ZULPERI
Date: 22 September 2008

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%	percent
°C	degree celcius
µg	microgram
µl	microliter
µM	micromolar
µm	micronmeter
AGPT	agar gel precipitin test
AMV	avian myeloblastosis virus
bp	base pair
CAM	chlorioallantoic membrane
cDNA	copied DNA
CEK	chicken embryo kidney
cRNA	copied RNA
C_T	threshold cycle
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double-stranded
E	small membrane protein
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
FDA	Food and Drug Administration
FRET	fluorescence resonance energy transfer
g	gram
HA	haemagglutination test
HI	haemagglutination inhibition
HVR	hypervariable region
HVR	hypervariable
IB	infectious bronchitis
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
kb	kilo basepair
LB	Luria-Bertani
M	membrane glycoprotein
Mass	Massachusetts
MgCl	magnesium chloride
min	minutes
ml	milliliter
mM	milimolar
mRNA	messenger RNA
N	nucleocapsid protein
NDV	Newcastle disease virus
ng	nanogram
NTC	non-template control
OD	optical density