UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF SYBR GREEN 1 BASED REAL-TIME POLYMERASE CHAIN REACTION FOR DETECTION AND DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS

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By

HAIRUL AINI BT. HAMZAH

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

March 2005

Dedicated to:

My beloved husband Zaizy bin Taib
My son Mohd Athif Izzat
My parents and family
Whoever has provided me with care
and compassion throughout my life

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

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Chairman: Associate Professor Abdul Rahman Omar, PhD

Faculty: Veterinary Medicine

The current available method to differentiate very virulent and vaccine strains of infectious bursal disease virus (IBDV) is by restriction fragment length polymorphism of VP2 gene. However, this method is time consuming, error-proned and less sensitive. The newly developed TaqMan real-time PCR is very sensitive but not suitable for routine test as it is expensive. Additionally, the application of the assay in detecting very virulent and vaccine strains of IBDV has not been reported. In this study the performances of SBYR Green 1 real-time, ELISA and conventional agarose detection methods in detecting nested PCR products were compared. It was found that the real-time PCR was at least 100 times more sensitive than ELISA detection method with a detection limit of 250 $\rho g/\mu l$. The developed assay detects both very virulent and vaccine strains of IBDV but not other RNA viruses such as Newcastle

disease virus and infectious bronchitis virus. However, the assay was unable to differentiate the different strains of IBDV. In the subsequent studies, strain-specific primer (match primer) combinations were used for the detection and differentiation of IBDV strains using two steps SYBR Green 1 based real-time PCR. The primers and PCR condition were optimized and validated using both very virulent and vaccine strains. By using the strain-specific primer combinations, specific amplification based on measurement of C_T and Tm were detected. In an optimized PCR condition, specific amplification associated with early amplification with C_T value between 19 to 28 and Tm between 86 to 88°C meanwhile nonspecific amplification from mismatch primer was associated with late amplification with C_T value > 29 and Tm < 82°C or no amplification (C_T value 0 and Tm < 82°C). These characteristic C_T and Tm values were consistently detected following amplification with 4000 ng/ul of cDNA. Hence, the differentiation of IBDV strains was based on the detection of C_T values whilst detection of Tm was for confirmation of the specific amplification. The detection of Tm value alone was not sufficient to differentiate IBDV strains. Even though the detection limit of the real-time PCR to detect IBDV strains was between 6.6 to 7.7 ng/μl, it is recommended that for testing of clinical samples, the cDNA concentrations be maintained between 4000 ng/μl to 66 ng/μl for PCR amplification, since amplification from insufficient primer-template concentration promote amplification of mismatch PCR product. In this study, it showed for the first time application of SYBR Green 1 based real-time PCR for the detection and differentiation of very virulent and vaccine strains of

IBDV. The assay was found to be sensitive, specific, less expensive and has less turn around time compared to the current available diagnostic methods.

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

PEMBANGUNAN TINDAK BALAS RANTAI POLIMERASE MASA-NYATA BERASASKAN SYBR GREEN 1 UNTUK PENGESANAN DAN PEMBEZAAN VIRUS PENYAKIT BURSA BERJANGKIT

Oleh

HAIRUL AINI BT. HAMZAH

Mac 2005

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Kaedah semasa yang digunakan untuk membezakan strain sangat virulen dan vaksin virus penyakit bursal berjangkit (IBDV) adalah dengan fragmen pembatasan polimorfisme panjang terhadap gen VP2. Bagaimanapun, kaedah ini memakan masa, mudah berlaku kesilapan dan kurang sensitif. Pembangunan terbaru PCR masa nyata TaqMan adalah sensitif tetapi tidak sesuai sebagai ujian rutin kerana ujian tersebut mahal. Tambahan pula, aplikasi asai tersebut dalam mengesan strain sangat virulen dan vaksin IBDV tidak pernah dilaporkan. Dalam kajian ini perlaksanaan kaedah PCR masa nyata SYBR Green 1, ELISA dan konvensional agaros dalam mengesan produk nested PCR telah dibandingkan. PCR masa nyata didapati sekurang-kurangnya 100 kali lebih sensitif daripada kaedah pengesanan ELISA dengan had pengesanan pada 250 ρg/μl. Asai yang telah dibangunkan

tersebut mengesan kedua-dua strain sangat virulen dan vaksin IBDV tetapi tidak virus lain seperti virus penyakit sampar, dan virus berjangkit bronkitis. Walau bagaimanapun, asai tersebut tidak boleh membezakan strain IBDV yang berbeza. Dalam kajian seterusnya, gabungan primer strain-khusus (primer sepadan) digunakan untuk mengesan dan membezakan strain IBDV menggunakan dua langkah PCR masa nyata berasaskan SYBR Green 1. Primer tersebut dan keadaan PCR telah dioptimumkan dan disahkan menggunakan kedua-dua strain sangat virulen dan vaksin IBDV. Dengan menggunakan gabungan primer strain-khusus, amplikasi khusus berdasarkan ukuran C_T dan Tm telah dikesan. Dalam keadaan PCR yang optimum, amplifikasi khusus telah dikaitkan dengan amplifikasi awal dengan nilai C_T antara 19 hingga 28 dan nilai Tm di antara 86°C hingga 88°C manakala amplifikasi tidak khusus dari primer tidak sepadan dikaitkan dengan amplifikasi lewat dengan nilai C_T > 29 dan Tm < 82°C atau tiada amplifikasi (nilai C_T 0 dan Tm < 82°C). Nilai ciri C_T dan Tm dikesan secara konsisten berikutan amplifikasi dengan 4000 ng/µl cDNA. Maka, pembezaan strain IBDV adalah berdasarkan pada pengesanan nilai C_T manakala pengesanan nilai Tm adalah untuk pengesahan dari amplifikasi khusus. Pengesanan nilai Tm sahaja tidak mencukupi untuk membezakan strain IBDV. Walaupun had pengesanan PCR masa nyata untuk mengesan strain IBDV adalah antara 6.6 hingga 7.7 ng/µl, adalah disyorkan bahawa untuk menguji sampel klinikal, kepekatan cDNA dikekalkan antara 4000 ng/µl ke 66 ng/µl untuk amplifikasi PCR kerana amplifkasi daripada ketidakcukupan kepekatan primer-templat menggalakkan penghasilan produk PCR yang

tidak sepadan. Dalam kajian ini, dibentangkan buat kali pertama aplikasi PCR masa nyata berasaskan SYBR Green 1 untuk pengesanan dan pembezaan strain sangat virulen dan vaksin IBDV. Asai tersebut didapati sangat sensitif, khusus, lebih ekonomi dan masa pusing balik yang lebih pendek berbanding dengan kaedah diagnostik yang boleh didapati sekarang.

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I certify that an Examination Committee met on 29th March 2005 to conduct the final examination of Hairul Aini Hamzah on her Master of Science thesis entitled "Development of SYBR Green 1 Based Real-Time Polymerase Chain Reaction for Detection and Differentiation of Infectious Bursal Disease Virus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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

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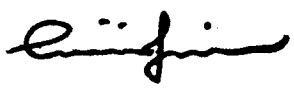

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Date: 15 JUL 2005

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 or concurrently submitted for any other degree at UPM or other institutions.

HAIRUL AINI HAMZAH

Date: 25/05/05

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LIST OF ABBREVIATIONS

AC-ELISA	Antigen-capture Enzyme-linked Immunosorbant Assay
AGPT	Agar Gel Diffusion Precipitin Test
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary Deoxyribonucleic Acid
°C	Degree Celcius
CAM	Chorioallantoic Membrane
C_T	Threshold Cycle
CV	Coefficient Variation
DNA	Deoxyribonucleic Acid
ddH_2O	Double Distilled Water
ddNTP	Dideoxynucleotide Triphosphate
dNTP	Deoxynucleotide Triphosphate
ds	Double Stranded
DTT	Dithrothreitol
dH_2O	Distilled Water
DIG	Digoxigenin
DMSO	Dimethysulphoxide
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
ELISA	Enzyme-linked Immunosorbant Assay
F	Fluorescence
FRET	Fluorescence Resonance Energy Transfer

HCl	Hydrochloric Acid
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
IBV	Infectious Bronchitis Virus
Kb	Kilobase
KCl	Kalium Chloride
kDA	Kilodalton
Mg	Magnesium
Mg_2Cl	Magnesium Chloride
min	Minute
mins	Minutes
ml	Mililiter
mM	Milimolar
NCBI	National Center Biotechnology Information
µg	Microgram
µM	Micromolar
ng	Nanogram
NDV	Newcastle Disease Virus
OD	Optical Density
OD_{405}	Optical Density at 405nm wavelength
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
ρg	Picogram
ρmole	Picomole

PTC	Peltier Thermal Cycler
qcPCR	Competitive Quantitative PCR
R^2	Regression Coefficient
RBC	Red Blood Cell
RE	Restriction Endonuclease
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse Transcriptase
SD	Standard Deviation
Secs	Seconds
SPF	Specific-Pathogen-Free
ss	Single Stranded
T	Temperature
TAE	Tris-Acetate-EDTA
Taq	Thermus aquaticus
Tm	Melting Teperature
TAE	Tris-Acetate-EDTA Buffer
Tris	2-amino-2(hydroxymethy)-1,3 propandiol
ul	Microlitre
UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
w/v	Weight/Volume

v/v	Volume/Volume
vv	Very virulent

Amino Acid	Single/Three Letter Amino Acid Code	
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Glutamine Acid	Q	Gln
Glutamic Acid	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Valine	V	Val