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IDENTIFICATION OF INFECTIOUS BRONCHITIS VIRUS ISOLATES FROM MALAYSIA

BALKIS BINTI HAJI A. TALIP

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IDENTIFICATION OF INFECTIOUS BRONCHITIS VIRUS ISOLATES FROM MALAYSIA

By

BALKIS BINTI HAJI A. TALIP

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

2007

DEDICATED TO………………..

My Father,

HAJI A TALIP BIN MD NOH

My Mother,

HAJJAH ZAHARAH@ROHANI BINTI JA'AMAT@YA'AMAT

My sisters,

BAHIYAH

BAKHREZA

BAZILAH

BAIYINAH

My very best friend,

LATIFAH BTE MD ARIFFIN

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

IDENTIFICATION OF INFECTIOUS BRONCHITIS VIRUS ISOLATES FROM MALAYSIA

By

BALKIS BINTI HAJI A. TALIP

December 2006

Chairman: Associate Professor Dr. Siti Suri Arshad, PhD

Faculty: Veterinary Medicine

Infectious bronchitis (IB) is a highly contagious respiratory, urogenital and reproduction disease of chickens and it is distributed worldwide. The disease is caused by infectious bronchitis virus (IBV). IBV is a member of the genus *Coronavirus*, family *Coronaviridae* and it has a single-stranded, positive-sense RNA genome of 27.6 kb. It possesses prominent surface spikes and has three major structural proteins; the spike (S) glycoprotein, the small integral membrane (M) glycoprotein and nucleocapsid (N) protein. In the commercial poultry industry, vaccination is used to control the disease. Despite vaccination program the disease continues to occur because IBV can exist in many serotypes. In many incidences, the existing vaccines are not able to provide full protection to the chickens against infectious bronchitis disease. The immune response stimulated to one serotype does not offer cross protection to another serotype. Moreover, the avian coronavirus capable of mutating and many IBV variants has been reported in many countries. Thus, it is crucial to develop a fast, sensitive and specific diagnostic technique to diagnose and identify the causative agent in order to control the disease

spread. In recent years the reverse transcriptase-polymerase chain reaction (RT-PCR), reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP), cloning and genes sequencing had been used increasingly to detect and analyses IBV isolates. The objectives of these studies were to compare and optimize reverse transcriptase-polymerase chain reaction (RT-PCR) to diagnose IBV, to differentiate the Mass strain, and to characterize variant IBV isolated from this study. In this study, one-step and two-step RT-PCR techniques were used to amplify the conserved gene region of IBV by using universal and designed primers. This study was conducted on IBV isolates from year 1991 until 2003. In differentiation studies, isolates were group into serotype using reverse transcriptase-polymerase chain reaction (RT-PCR). Isolates recognized to be non Mass was further amplified their hypervariable region of S gene by using RT-PCR followed by RFLP technique to screen for nephropathogenic strain. Out of 31 IBV isolates, nine Mass strain were found. Following RT-PCR-RFLP, only one isolate showed different fragment pattern compared to nephropathogenic origin (MH5365/95). This particular isolates designated as V9/03 was neither Mass nor non-nephropathogenic serotype. The S1 region of V9/03 was further cloned, sequenced and its nucleotide and amino acid were compared to nephropathogenic MH5365/95 and Mass derivatives as well other references strains obtained from Gene bank. The V9/03 showed sequence homology to Taiwan (AY606321) and Korean (AY257060) strain with 82.5% and 81.6% identities respectively. The V9/03 has lower sequences homology (less than 80%) with nephropathogenic (MH5365/95) and Mass derivatives. The phylogenetic studies indicate that the strain V9/03 could be a local variant IBV which is different from local

nephropathogenic MH5365/95 and vaccine strain. This study showed that variant IBV is circulating in the field as result of mutation of IBV due to the prolonged use of live virus vaccines and the immunological pressure of the virus to keep on survival in immune birds.

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

PENGENALPASTIAN ISOLAT-ISOLAT VIRUS BRONKITIS BERJANGKIT DI MALAYSIA

Oleh

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December 2006

Pengerusi: **Profesor Madya Dr.Siti Suri Arshad, PhD**

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Bronkitis berjangkit merupakan penyakit pernafasan ayam yang menular ke serata dunia. Ianya disebabkan oleh virus bronkitis berjangkit (IBV). Virus bronkitis berjangkit (IB) merupakan ahli genera *Coronavirus*, famili *Coronaviridae*. Virus ini merupakan virus bebenang tunggal, RNA genom berpancaindera-positif bersaiz 27.6 kb. Ianya mempunyai permukaan tajam yang menonjol dan mempunyai tiga protein berstruktur yang utama; glikoprotein pepaku (S), glikoprotein membran kecil perlu (M) dan protein nukleokapsid (N). Dalam industri penternakan ayam, vaksinasi digunakan untuk mengawal penyakit. Walaupun program vaksinasi, penyakit terus berlaku kerana IBV boleh wujud di dalam pelbagai serotip. Dalam kebanyakkan kejadian, kewujudan vaksin-vaksin sahaja tidak mencukupi untuk menyediakan perlindungan sepenuhnya kepada ayam-ayam bagi menentang IBV. Tindakbalas imuniti terhadap satu serotip tidak boleh menawarkan perlindungan bersilang kepada serotip yang lain. Lagipun, coronavirus avian berkebolehan untuk mutasi dan banyak varian IBV telah dilaporkan di beberapa

negara. Demikian, adalah sangat genting agen penyebab dikenalpasti untuk mengawal penyebaran penyakit. Sejak akhir-ahir ini, RT-PCR, RT-PCR-RFLP, pengklonan dan penjujukan gen telah banyak digunakan untuk mengesan dan analisis isolat IBV. Objektif-objektif dalam kajian ini adalah untuk membina dan optimasi RT-PCR untuk diagnosa IBV, mengasingkan strain Mass dan untuk mengenalpasti IBV varian terpencil dalam kajian ini. Dalam kajian ini, teknik satu langkah dan 2-langkah RT-PCR telah digunakan untuk menggandakan gen yang majmuk bagi IBV dengan menggunakan primer universal dan direka. Kajian ini telah dijalankan ke atas isolat-isolat IBV dari tahun 1991-2003. Dalam kajian pengasingan, isolat-isolat telah dibahagikan kepada strain Mass dan bukan Mass. Isolat bukan Mass telah digandakan gennya dan dilakukan RFLP untuk mengenalpasti strain nefropatogenik. Sembilan daripada 30 isolat IBV adalah strain Mass, selebihnya dijangkakan nefropatogenik (MH5365/95) kecuali satu isolat. Isolat tersebut adalah V9/03. Isolat ini telah dijujukkan dan mempunyai sedikit kesamaan dengan isolat dari Taiwan (AY) dan Korean (AY257060) with 82.5% dan 81.6%. The V9/03 mempunyai persamaan yang rendah (kurang dari 80% dengan nefropatogenik MH5365/95 dan terbitan Mass. Analisis filogenetik menunjukkan V9/03 adalah varian berbeza daripada nefropatogenik MH5365/95 and strain vaksin. Kajian ini menunjukkan bahawa varian IBV berkitaran di ladang, sama seperti mutasi IBV disebabkan penggunaan vaksin virus hidup dalam tempoh yang berpanjangan dan tekanan imunologi virus untuk terus kekal dalam ayam-ayam yang imun.

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I certify that an Examination Committee met on the 19th December 2006 to conduct the final examination of Balkis Binti Haji A Talip on her Master of Science thesis entitled "Molecular Characterization of Infectious Bronchitis Virus Isolates from Malaysia" 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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DECLARATION

I hereby 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 is not concurrently, submitted for any other degree at UPM or other institutions.

BALKIS BINTI HAJI A TALIP

Date : -2-2007

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LIST OF ABBREVIATION

%	percentage
µg	Microgram
µl	microlitre
AF	allantoic fluid
AGPT	agar gel precipitin test
AMV	Avian myeloblastosis virus
ATP	adenosine triphosphate
BEL	Beaudette embryo lethal
bp	base pair
cDNA	copy deoxyribonucleoase acid
CF	complement fixation
cp	connecting peptide
CTP	cytidine triphosphate
ddH_20	distilled deionized water
DNA	deoxyribonuclease acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E.coli	*Escherichia coli*
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	England Molecular Biology Laboratory
GTP	guanosine triphosphate
HA	haemagglutination
HCl	hydrochloride acid
HI	haemagglutination-inhibition
IB	infectious bronchitis
IBV	infectious bronchitis virus
kb	kilobase pair
KCl	kalium chloride
kDa	kilodalton
l	litre
LB	Luria Bertani
M	membrane
MAb	monoclonal antibody
MVK	Makmal Veterinar Kawasan Petaling Jaya
$MgCl_2$	Magnesium chloride
MHV	murine hepatits virus
mM	miliMolar
Mo-MLV	Moloney murine leukemia virus
MOPS	3-*N*-morpholino propanesulfonic acid
mRNA	messenger RNA
MVP	Malaysian Vaccine Pharmaceutical
N	Nucleocapsid

NaCl	natrium chloride
NDV	New Castle disease virus
NS	Nonstructural
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pmol	picomole
RE	restriction enzyme
RNA	ribonuclease acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RT-PCR-RFLP	reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism
S	spike
SDS	Sodium Doedecyl Sulfate
SgRNAs	subgenomic mRNA
sp	signal peptide
TBE	Tris-borate EDTA buffer
TTP	thymidine triphosphate
U	unit
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United States of America
UTR	untranslated region
UV	ultraviolet
V/60	volt per 60 minutes
VN	virus neutralization
VNT	virus neutralization test
VRI	Veterinary Research Institute
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

Infectious bronchitis virus (IB) is an acute, highly contagious respiratory and urogenital disease of chickens. The disease, also called avian infectious bronchitis virus is of significant to the economic importance to the poultry industry as it causes poor weight gain and feed efficiency. The disease was first reported in 1931 in the United State of America (Beach and Schalm, 1936). It is one of the organisms isolated and produces air-sacculitis resulting in condemnations of broilers at processing (King and Cavanagh, 1991).

IBV together with Turkey and pheasant coronaviruses, belongs to Group 3 of the genus *Coronavirus*, family *Coronaviridae* (Cavanagh, 2001). All the members of this genus have a linear, non-segmented, positive sense, single stranded RNA genome of approximately 27 kb in length. The first 20 kb encode the viral RNA-dependent RNA polymerase and proteases. The remainder of the genome encodes five structural proteins, the spike (S) consisting S1 and S2, envelope (E), membrane (M) and nucleocapsid (N) proteins, four small non-structural proteins 3a, 3b, 5a and 5b and a 3' untranslated region (UTR).

Variations in the S1 and N genes, in particular, are believed to be critical for emergence of variants because of their role in virus replication and immunity. Moreover, S1 and N

have been used most frequently to determine the relatedness of emerging IBV. The S1 glycoprotein is located on the surface of the virion ad carries epitopes and determinants for virus neutralizing antibodies, protective immunity and cell tropism (Casais *et al*., 2003 ; Cavanagh *et al*., 1986 ; Cavanagh *et al*., 1988). The N protein, located in the capsid of the virion in involved in RNA replication and carries group-specific antigenic determinants (Ignjatovic and Galli, 1995). The 3'UTR region is involved in initiation of negative-strand RNA synthesis and has also been used to assess variation in emerging IB strains and other members of coronavirus Group 3 (William *et al*., 1993).

IBV evolution is currently considered to be driven by three factors such as the inherent propensity of its RNA genome to mutate, the continuous use of live, often multiple vaccines and the immunological pressure exerted on circulating viruses by the enduring presence of immune bird populations. These three factors probably role in performance and involve various mechanisms, such as point mutations, deletion, insertions and recombination to generate new variants (Cavanagh *et al*., 1992a ; Kuster *et al*., 1990 ; Wang *et al*., 1994).

Serotyping of IBV isolate is essential in the design of prevention and control strategies as well as in epidemiological studies. Several serotyping methods have been used including virus neutralization (VN) (Zwaagstra *et al*., 1992), reverse transcription-polymerase chain reaction-restriction fragment length polymorphism (RT-RFLP) (Kwon *et al*., 1993), serotype-specific reverse transcriptase-polymerase chain reaction (RT-PCR) (Lai and Cavangh, 1997) and multiplex PCR (Wang and Khan, 1999). For the control of disease