UNIVERSITI PUTRA MALAYSIA

DETECTION AND MOLECULAR IDENTIFICATION OF INFECTIOUS BRONCHITIS VIRUS ISOLATED IN MALAYSIA

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By

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Special dedication to my husband, my children and my parents.

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

DETECTION AND MOLECULAR IDENTIFICATION OF AVIAN INFECTIOUS BRONCHITIS VIRUS ISOLATED IN MALAYSIA

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August 2000

Chairman : Associate Professor Dr. Khatijah Mohd. Yusoff

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Infectious bronchitis is a disease of economic importance in the poultry industry in Malaysia. Currently, natural outbreaks of IB are controlled through the use of vaccine. However, outbreaks still occur in vaccinated flocks. One of the major factors speculated for these outbreaks is the heterologous protection afforded by the current standard IB vaccines against different IB virus (IBV) serotypes and variants present in the country. Rapid and sensitive methods for the detection and identification of serotypes causing these outbreaks would therefore be valuable to the understanding and control of IB. In this study, reverse-transcription polymerase chain reaction (RT-PCR) using the published primers (C2U/C3L, S1oligo3'/S1oligo5', S1oligo3'/S1Newoligo5', IBP1+/IBRP2-, IBVN2+/IBVN1- and UTR2+/UTR1-) were used to detect 14 Malaysian IBV isolates. Only primers UTR2+/UTR1- and IBP1+/IBRP2- were found to be able to detect all of the 14

Malaysian isolates. These primers could therefore be used as universal primers for the detection of Malaysian IBV isolates.

The S1 gene of a Malaysian nephropathogenic IBV (MH5365/95) and the variable region in the S1 gene of the remaining 13 IBV isolates were RT-PCR using primers S1oligo5-1/S1oligo3' and TM897F/TM1328R respectively. The PCR products were cloned, sequenced and compared the sequences with published sequences of the S1 gene of other IBV strains. Based on this sequence comparison, MH5365/95 was found to be different from the other IBV strains. The remaining 13 isolates were classified into 2 general groups. The first group, that was designated as Group A, consists of three isolates and was closely related (more than 95% homology) to Massachusetts type (M41), the most commonly used vaccine strain in this country. The second group which consists of 10 isolates was genetically different from the published IBVs showing not more than 82% homology with the published sequence. These 10 isolates were further subdivided into Groups B and C, comprising 7 and 3 isolates respectively. The 3 isolates of Group C belong to the same group as MH5365/95. The Group B isolates, however, were more closely related to the M41 serotype (about 82% in homology) compared with that of Group C isolates (less than 80%). There was about 78% nucleic acid homology when Groups B and C were compared. It could therefore be concluded that the outbreaks in this country were caused by the new strains of IBV, which were different from the vaccine strains, thereby resulting in the vaccines becoming less efficacious to protect the chickens against the local IBVs.

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PENGESANAN DAN PENGENALPASTIAN MOLEKUL VIRUS BRONKITIS BERJANGKIT AVIAN YANG DIASINGKAN DI MALAYSIA

Oleh

MAIZAN BT. HAJI MOHAMED

August 2000

Pengerusi : Profesor Madya Dr. Khatijah Mohd Yusoff

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Bronkitis berjangkit (IB) merupakan sejenis penyakit berkepentingan ekonomi dalam industri ayam di Malaysia. Semasa ini, wabak semulajadi IB dikawal melalui penggunaan vaksin. Walaubagaimanapun wabak ini masih berlaku di dalam kelompok ayam yang telah divaksinasi. Salah satu faktor penyebab yang dispekulasi adalah perlindungan separa yang diberikan oleh vaksin yang digunakan terhadap berbagai serotip dan varian virus IB (IBV) yang terdapat di negara ini. Oleh itu, kaedah yang cepat dan sensitif untuk pengesanan dan pencirian serotip yang menyebabkan wabak ini adalah penting untuk memahami dan mengesahkannya. Dalam pengkajian ini, tindak balas trankripsi berbalik rantai polimerase (RT-PCR) menggunakan primer rujukan (C2U/C3L, S1oligo3'/S1oligo5', S1oligo3'/S1New oligo5', IBP1+/IBRP2-, IBVN2+/IBVN1- dan UTR2+/UTR1-) telah digunakan dengan tujuan untuk mengesan kehadiran 14 isolat IBV tempatan. Hanya primer

UTR2+/UTR1- dan IBP1+/IBRP2- didapati boleh mengesan kehadiran kesemua 14 isolat ini. Oleh itu pasangan primer ini boleh dianggap sebagai primer universal untuk mengesan kehadiran isolat IBV tempatan.

Gen S1 virus IB isolat nepropatogenik tempatan (MH5365/95) dan bahagian yang berubah-ubah dalam gen tersebut untuk isolat virulen tempatan telah dijalankan tindak balas transripsi terbalik rantai polimerase dengan menggunakan primer S1oligo5-1/S1oligo3' dan TM897F/TM1328R, di klonkan, dibaca jujukan nukleotidanya dan dibuat perbandingan dengan jujukan nukleotida gen S1 dari strain-strain yang lain. Daripada perbandingan ini didapati MH5365/95 mempunyai jujukan nukleotida yang berbeza daripada lain-lain strain IBV rujukan. Tiga belas isolat IBV telah dikelaskan kepada 2 kumpulan am. Kumpulan pertama yang dinamakan kumpulan A, mengandungi 3 isolat IBV dan didapati berkait-rapat (lebih daripada 95% homologi) dengan strain Massachusetts (M41), iaitu strain vaksin yang paling banyak digunakan di negara ini. Kumpulan kedua yang mengandungi 10 isolat IBV mempunyai jujukan nukleotida yang berlainan (kurang dari 82% homologi) daripada strain IBV rujukan. Sepuluh isolat IBV tempatan ini telah di bahagikan lagi kepada Kumpulan B dan C, masing-masing terdiri daripada 7 dan 3 isolat IBV. Tiga isolat ini berada di bawah kumpulan yang sama dengan MH5365/95. Kumpulan B bagaimanapun mempunyai homologi jujukan nukleotida yang lebih rapat dengan M41 (lebih kurang 82%) berbanding dengan kumpulan C (kurang 80%). Homologi jujukan nukleotida antara kumpulan B dan C adalah lebih kurang 78%. Kesimpulannya, wabak yang berlaku di negara ini adalah disebabkan

oleh IBV strain baru yang berbeza dari strain vaksin, menyebabkan vaksin menjadi kurang berkesan untuk memberi perlindungan kepada ayam terhadap infeksi IBV tempatan ini.

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LIST OF ABBREVIATIONS

AF	:allantoic fluid
AGPT	:agar gel precipitin test
AGID	:agar gel immunodiffusion
B-ME	:β-mercapto-ethanol
°C	:degree Celcius
$CaCl_2$	:calcium chlorida
cDNA	:complementary DNA
CFT	:complement fixation test
$C_2H_3O_2K$	:potassium acetate
DNA	:deoxynucleotide acid
dNTP	:deoxynucleoside triphosphate
ECE	:embryonated chicken egg
ELISA	:enzyme-linked immunosorbent assay
g	:gravity
G+C	:Guanine+Cytosine
H	:hour
HA	:haemagglutination
HI	:haemagglutination inhibition
HVR	:hypervariable region
IB	:Infectious bronchitis
IBV	:Infectious bronchitis virus

kDa	:kilodalton
kb	:kilobase pair
KCl	:potassium chloride
KH_2PO_4	:di-potassium hydrogen phosphate
LB	:Luria Bertani
M	:membrane
Mabs	:monoclonal antibodies
$MgCl_2$	:magnesium chloride
MHV	:mouse hepatitis virus
min	:minute
M_w	:molecular weight
M41	:Massachusetts 41
µl	:microliter
N	:nucleocapsid
NaCl	:sodium chloride
NaOH	:sodium hydroxide
Na_2PO_4	:di-sodium hydrogen phosphate
ng	:nanogram
ORF	:open reading frame
PAGE	:polyacrylamide gel electrophoresis
PBS	:phosphate-buffered saline
PCR	:polymerase chain reaction
PCR-RFLP	PCR-Restriction Fragment Length Polymorphism

PCI	:phenol chloroform isoamyl
RNA	:ribonucleic acid
RT	:reverse transcription
RT-PCR	: reverse transcription- polymerase chain reaction
S	:spike
s	:second
SDS	:sodium dodecyl sulphate
Sec	:section
sM	:small membrane
SPF	:specific pathogen free
TBE	:Tris borate EDTA
TE	:Tris EDTA
TGEV	transmissible gastroenteritis virus
TOC	:tracheal organ culture
TSR	:template suspension reagent
U	:unit
UPM	:Universiti Putra Malaysia
UTR	:untranslated region
UV	:ultraviolet
VN	:virus neutralisation
VRI	:Veterinary Research Institute
v/v	:volume/volume
w/v	:weight/volume

CHAPTER 1

INTRODUCTION

1.1 INFECTIOUS BRONCHITIS

Infectious bronchitis (IB) is an acute highly contagious and infectious disease of the respiratory system in young chickens. Mortality in young chicks is usually 25-30% but in some outbreaks, it can be as high as 75%. In older birds, the disease often goes unnoticed, but in laying hens, there is a marked drop in egg production and quality (King and Cavanagh, 1991). Nephrosis is also observed in chicken infected with certain IB virus (IBV) strains. The severity of respiratory infections with IBV can be greatly enhanced by the presence of other pathogens of the respiratory tract such as mycoplasma and *Escherichia coli*. The disease was first reported in 1931 in the U.S.A (Beach and Schalm, 1936). IB is of economic importance because it is a cause of poor weight gain and feed efficiency, also a component of mixed infection that produce air-sacculitis resulting in condemnations of broilers at processing (King and Cavanagh, 1991).

In Malaysia, IBV was first reported as early as 1967 where the disease was mild and vaccination unwarranted (Chong and Omar, 1967). However, the situation changed dramatically ten years later when the IB cases increased in parallel with intensification of poultry farming. It was commonly believed for many years that most of the IBV isolates were of the Massachusetts (Mass) serotype since vaccine of

only this serotype was used. The increase in the incidence of IB occurred in flocks vaccinated with M41 vaccine. Nine out of 20 Malaysian isolates characterised by virus neutralisation test (VNT) at the Poultry Research Laboratory in Ethens, Greece were found to be related to the Mass serotype and the other 11 isolates were considered as "local" strains (Opitz *et al.*, 1979). In 1995, a local nephropathogenic strain (MH5365/95) which caused more than 20% mortality in chickens was isolated and it was found not to be related to the Australian T and M41 strains by VNT (Aziz *et al,* 1996). During the same time, an IBV Dutch strain was also present in this country (Azri *et al.*, 1997). Despite the widespread use of both live attenuated and inactivated IB vaccines, IB disease continues to be a major problem in Malaysia. Vaccine breakdown, lack of cross-protection between IBV vaccinal and field viruses, and the emergence of variant strains are some of the factors that resulted in the continued occurrence of IB outbreaks (Azri *et al.*, 1996). Variant strains may arise from the emergence of new antigenic variants and also to the inadvertent introduction of geographically exotic serotypes of the virus, of which protection is not covered by the vaccine currently used (Wang *et al.*, 1993). Other possibilities include mutations of the viruses or recombinations between strains due to genetic reassortments (Kusters *et al.*, 1990). Recovered birds may become carriers and shed the virus for variable periods of time, some are suspected of cycling recurrent infections within the flocks.

IBV infection in chickens is currently diagnosed by using embryonated chicken eggs (ECE) and tracheal organ cultures (TOC). Virus isolation can be a lengthy process,

because most field strains require adaptations and usually two or three passages are required before typical lesions are produced in the embryonated chicken eggs (ECE) and in the TOC. Other methods to identify the virus includes VNT test, haemagglutination (HA) test, enzyme-linked immunosorbent assay (ELISA) and electron microscopy (Lukert, 1980). These methods are expensive, labour intensive and often give inconclusive results, hence the development of an alternative method which is more sensitive, specific and rapid is needed.

1.2 OBJECTIVES OF THIS STUDY

The objectives of this study were :

i. to identify universal primers that can detect all the IBV strains isolated in Malaysia.

ii. to sequence the complete S1 gene of the prototype Malaysian nephropathogenic strain of IBV (MH5365/95).

iii. to characterise 13 Malaysian IBV isolates by phylogenetic tree analysis using the variable region of the S1 gene sequence.

CHAPTER 2

LITERATURE REVIEW

2.1 Incidence and distribution

The first isolate of IBV is of the Beaudette strain (Beaudette and Hudson, 1937). Later, the M41 strain was isolated in the North Dakota and was found to be serologically related to Beaudette strain (Bracewell, 1975). Strains of this serotype were among the first to be made into live vaccines, e.g. H120 (derived from a Dutch isolate of 1955). Strain M41 and its prototype; i.e. the Connecticut (Jungherr *et al.*, 1956), Holte and Gray strains have been widely used as a basis for comparison with other isolates mostly by the virus neutralisation (VN) test and these strains can cause problems and pathological changes to the kidney (Winterfield and Hitchner, 1962).

In Europe, only 30% of the isolates were serologically related to the known North American serotypes in the 1970s. The majority of the remainder strains were found to be related to four Dutch strains; D207, D212, D3128 and D3896 (Davelaar *et al.*, 1984) by VN test. Many of the British IBV strains isolated between 1981 to 1983 were closely related to the Dutch Strains; D207, D3128 and D3896 (Cook, 1984). Although these viruses caused respiratory disease and aberrant drop in egg production in layer flocks, they are not associated with poor egg quality (Cook, 1986).

The Australian 'T' strain was the first IBV strain isolated in Australia in 1962. It caused both nephritic and respiratory forms of IB with 10 to 15% mortality in