MOLECULAR CHARACTERISATION AND PATHOGENICITY EVALUATION ON ATTENUATED MALAYSIAN STRAINS OF INFECTIOUS BRONCHITIS VIRUS

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By

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Chairperson: Professor Abdul Rahman Omar, PhD
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Recent molecular epidemiology study detected local Malaysian variant (MV) and QX-like IBV strains circulating in commercial farms in Malaysia. Subsequent pathogenicity study showed these two strains are pathogenic in specific-pathogen-free (SPF) chickens. However, the efficacy study of commercially available vaccines in conferring protection against these two IBV strains in chickens and their molecular characteristics following attenuation in embryonated chickens eggs (ECE) have not been well studied. Hence, two previous characterised IBV isolates, MV IBS037A/2014 and QX-like IBS130/2015 strains were used as challenged viruses in vaccine efficacy and in virus attenuation studies.

In the vaccine efficacy study, four commercially available IB vaccines strain Mass H120, K2, 1/96 and 4/91 were prepared as recommended by the respective manufacturers. SPF chickens were grouped according to the respective vaccine groups and vaccinated once via oculonasal route. At 21 day post-vaccination (dpv), the chickens were challenged with $10^{5.0}$ EID_{50}/0.1 ml of wildtype IBS037A/2014 or IBS130/2015. Results showed that all the vaccinated groups successfully induced antibody titre as detected by ELISA 3 weeks post vaccination. Following challenged, all the vaccinated groups showed different clinical signs and lesions of IB. Based on the protection results obtained, all the tested IBV vaccine strains were able to confer partial protection against IBS037A/2014 except Mass H120 vaccine that induced poor protection. However, all the tested vaccines were unable to provide protection against IBS130/2015 except for IBV vaccine strain 1/96 that able to confer partial protection. Virus neutralisation assay showed that the IBS037A/2014 has minor antigenic subtype difference with Mass H120 and 4/91 strains, whilst IBS130/2015 has minor antigenic subtype difference with K2 strain.

The S1 sequence analysis showed that passage 70 attenuated IBS037A/2014 virus has 9% nucleotide and amino acid variations, while the passage 70 attenuated IBS130/2015 virus has 7% and 11% nucleotide and amino acid variations, respectively, when compared to their respective wildtype viruses. The attenuated IBS037A/2014 virus has majority of the amino acid variations in the hypervariable region (HVR) 1, followed by HVR 2 and 3. Meanwhile, the attenuated IBS130/2015 has the highest amino acid variations in the HVR 2, followed by HVR 3 and 1.

The pathogenicity of the passage 70 attenuated viruses were compared with their respective wildtype viruses in SPF chickens. Significant differences in the microscopic lesions were detected in trachea ($p=0.001$) and kidney ($p=0.022$) of chickens inoculated with the attenuated IBS037A/2014, and trachea ($p=0.001$) of chickens inoculated with the attenuated IBS130/2015. The tracheal ciliary activity was significantly higher ($p=0.001$) in the attenuated IBS037A/2014 group, whilst no significant differences ($p=0.062$) in the attenuated IBS130/2015 group when compared to their respective wildtype viruses. Significant decreased in the virus copy numbers (VCN) were also detected in the trachea ($p=0.013$) and kidney ($p=0.001$) of chickens inoculated with the attenuated IBS037A/2014, and trachea of chickens inoculated with the attenuated IBS130/2015. However, a significantly increased in VCN were detected in the kidney ($p=0.001$) of chickens inoculated with the attenuated IBS130/2015 when compared to the wildtype viruses. In conclusion, the tested vaccine strains used in this study unable to induce complete protection against MV and QX-like. Meanwhile, the attenuated MV IBS037A/2014 showed reduced pathogenicity, whilst the attenuated QX-like IBS130/2015 is still pathogenic resembling the wildtype virus.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN MOLEKULAR DAN PENILAIAN PATOGENISITI VIRUS BRONKITIS BERJANGKIT STRAIN MALAYSIA YANG TELAH DI ATENUASI

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Kajian epidemiologi molekular terkini telah mengesan kehadiran virus IBV varian tempatan dan seumpama-QX Malaysia beredar di ladang komersial di Malaysia. Kajian patogenisiti telah menunjukkan kedua-dua strain ini adalah patogenik ke atas ayam bebas-patogen-spesifik (SPF). Walaupun begitu, belum ada kajian dilakukan terhadap efikasi vaksin IB komersial yang dapat memberikan perlindungan kepada ayam daripada kedua-dua strain dan juga kajian pencirian molekular IBV strain Malaysia yang telah diatenuasi daripada varian tempatan (MV) and seumpama-QX Malaysia menggunakan telur ayam berembrio (ECE). Dengan ini, dua isolat IBV yang telah dicirikan sebelum ini, iaitu strain MV IBS037A/2014 dan seumpama-QX IBS130/2015 telah digunakan sebagai virus cabaran dalam kajian efikasi vaksin dan atenuasi virus.

Dalam kajian efikasi vaksin, empat vaksin IB komersial daripada strain Mass H120, K2, 1/96 dan 4/91 telah disediakan seperti yang telah disarankan oleh pengeluar vaksin masing-masing. Ayam SPF dibahagikan kepada beberapa kumpulan berdasarkan kepada kumpulan vaksin berbeza dan di vaksinasi sekali secara okulonasal. Pada 21 hari selepas vaksinasi (dpv), ayam tersebut telah dicabar dengan $10^{5.0}$ EID_{50}/0.1 ml virus lapangan IBS037A/2014 atau IBS130/2015. Hasil yang diperoleh menunjukkan kesemua kumpulan vaksinasi berjaya menghasilkan titer antibodi seperti yang telah dikesan oleh ELISA 3 minggu selepas vaksinasi. Dapatan daripada cabaran, kesemua kumpulan yang telah di vaksinasi menunjukkan perbezaan pada tanda klinikal dan lesi IB. Berdasarkan kepada keputusan perlindungan yang diperoleh, kesemua strain vaksin yang diuji berupaya memberikan perlindungan separa terhadap IBS037A/2014 kecuali vaksin Mass H120 yang menghasilkan perlindungan yang lemah. Namun begitu, kesemua strain vaksin yang diuji tidak berupaya menyediakan perlindungan terhadap IBS130/2015 kecuali strain vaksin 1/96

yang berupaya memberikan perlindungan separa. Asai neutralisasi virus menunjukkan IBS037A/2014 mempunyai perbezaan subtip antigenik minor dengan strain Mass H120 dan 4/91, manakala IBS130/2015 mempunyai perbezaan subtip minor dengan strain K2.

Analisis jujukan S1 menunjukkan virus IBS037A/2014 yang telah teratenuasi pada pasaj 70 mempunyai variasi nukleotida dan asid amino 9%, sementara virus IBS130/2015 yang telah teratenuasi pada pasaj 70 mempunyai variasi nukleotida 7% dan asid amino 11%, masing-masing, apabila dibandingkan dengan setiap virus lapangan. Virus IBS037A/2014 yang telah teratenuasi mempunyai majoriti variasi asid amino pada kawasan hipervariabel (HVR) 1, diikuti HVR 2 dan 3. Sementara, IBS130/2015 yang telah teratenuasi mempunyai variasi asid amino tertinggi pada HVR 2, diikuti HVR 3 dan 1.

Patogenisiti virus teratenuasi pada pasaj 70 dibandingkan dengan virus jenis lapangan masing-masing pada ayam SPF. Terdapat perbezaan signifikan pada lesi mikroskopik yang dikesan pada trakea ($p=0.001$) dan ginjal ($p=0.022$) ayam yang diinokulasi dengan IBS037A/2014 yang telah teratenuasi dan trakea ($p=0.001$) ayam yang diinokulasi dengan IBS130/2015 yang telah teratenuasi. Aktiviti silia trakea adalah signifikan lebih tinggi ($p=0.001$) pada kumpulan IBS037A/2014 yang telah teratenuasi, sementara tiada perbezaan signifikan ($p=0.062$) pada kumpulan IBS130/2015 yang telah teratenuasi apabila dibandingkan dengan virus jenis lapangan masing-masing. Penurunan yang signifikan pada bilangan salinan virus (VCN) juga dikesan pada trakea ($p=0.013$) dan ginjal ($p=0.001$) ayam yang diinokulasi dengan IBS037A/2014 yang telah teratenuasi dan trakea ayam yang diinokulasi dengan IBS130/2015 yang telah teratenuasi. Meskipun begitu, peningkatan yang signifikan ($p=0.001$) pada VCN dikesan pada ginjal ayam yang diinokulasi dengan IBS130/2015 yang telah teratenuasi apabila dibandingkan dengan virus lapangan. Kesimpulannya, strain vaksin yang diuji tidak berupaya untuk mengaruh perlindungan sepenuhnya terhadap MV dan seumpama-QX. Namun begitu, MV IBS037A/2014 yang teratenuasi mempunyai penurunan patogenisiti, sementara seumpama-QX IBS130/2015 yang teratenuasi masih patogenik yang menunjukkan persamaan dengan virus lapangan.

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

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
µl	Microlitre
µm	Micrometre
Ab	Antibody
AGID	Agar gel immunodiffusion
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CPI	Cross Protection Index
CV	Coefficient of variation
CXCL8	C-X-C motif ligand 8
Da	Dalton
DNA	Deoxyribonucleic acid
do	Day old
dpc	Day post-challenge
dpi	Day post-inoculation
dpv	Day post-vaccination
E	Small membrane envelope protein
EID_{50}	Egg infective dose 50 percent
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
g	Gram
h	Hour
H&E	Hematoxylin-eosin staining
HA	Haemagglutination
HI	Haemagglutination inhibition
HVR	Hypervariable regions
IACUC	Institutional Animal Care and Use Committee
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
IBDV	Infectious bursal disease virus
ICTV	International Committee for Taxonomy of Viruses
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharides
M	Membrane glycoprotein
ml	Millilitre
MV	Malaysian variant
mm	Millimetre
mRNA	Messenger ribonucleic acid
N	Nucleocapsid protein
NC	Nucleocapsid
NCBI	National Center for Biotechnology Information
ND	Newcastle disease
NI	Neutralization index
nm	Nanometer

nsp	Non-structural protein
OIE	World Organization of Animal Health
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PRR	Germline-encoded pattern-recognition receptor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RBC	Red blood cell
RBD	Receptor binding domain
RdRp	RNA-dependent RNA-polymerase
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse-transcriptase PCR
RT-qPCR	Real time quantitative reverse-transcriptase PCR
RTC	Replication-transcription complex
S	Spike glycoprotein
S1	S1 subunit of spike glycoprotein
S2	S2 subunit of spike glycoprotein
SD	Standard deviation
SEM	Standard error mean
sgRNA	Sub-genomic ribonucleic acid
SPF	Specific-pathogen-free
ssRNA	Single stranded ribonucleic acid
TAE	Tris acetate EDTA
TLR	Toll-like receptor
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United State of America
UTR	Untranslated region
UV	Ultraviolet
VCN	Virus copy number
VI	Vaccination index
VN	Virus neutralization

CHAPTER 1

INTRODUCTION

1.1 General background

Infectious bronchitis virus (IBV) is recognised as the causative agent of infectious bronchitis (IB) disease affecting chickens (Abdel-Moneim, 2017). Based on International Committee on Taxonomy of Viruses (ICTV) classification, IBV is classified as *Gammacoronavirus* under subfamily *Orthocoronavirinae*, family *Coronaviridae*, suborder *Cornidovirineae*, order *Nidovirales* (ICTV, 2018). The virus is enveloped with a round to pleomorphic shape with about 120 nm in diameter and 20 nm club-shaped projected on its surface (Lunge *et al*., 2016). The genome of IBV is single-stranded positive-sense RNA and contains 27.6 kb in length with several open reading frames (ORFs) that encode for structural and non-structural proteins (Cavanagh, 2007). These include structural proteins [spike glycoprotein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N)], non-structural proteins (1a, 1b, 3a, 3b, 5a and 5b) and accessory proteins (Cook *et al*., 2012; Xu *et al*., 2016a).

IBV was first recognised in North Dakota, USA by Schalk and Hawn (1931). Since then, various IBV strains were detected worldwide and reviewed by numerous authors (Bande *et al*., 2017; de Wit *et al*., 2011; Jackwood, 2011; Lin and Chen, 2017). Currently, more than 50 strains of IBV have been identified (de Wit, 2000; Jackwood *et al*., 2005). The reason of this very wide diversity of IBV is because of mutation or recombination events occurring during viral replication which led to the emergence of novel IBV in non-controlled or vaccine-controlled population of chickens (Jackwood and Lee, 2017; Toro *et al*., 2012a, 2012b). Hence, IBVs can be classified into two major groups based on their serotype and genotype analysis; classical and variant groups (Jackwood, 2012).

IB is a highly contagious disease causing high morbidity and mortality, especially among young chickens (evidence shows that morbidity may up to 100% and reaching 50% mortality in some strains) and high indirect economic losses (Jackwood, 2012; OIE, 2013). The virus is transmitted mainly by aerosol. However, the virus is also reported able to spread by ingestion of contaminated feed and water or closed contact with contaminated equipment or clothing (Dhama *et al*., 2014; Jahantigh *et al*., 2013). The virus affects the respiratory tract and cause respiratory tract disease, poor body weight gain in broilers (Cavanagh and Naqi, 1997), and cause reduction of egg quality and quantity in laying hens because of the impairment of their oviduct (Chousalkar and Roberts, 2007; Samiullah *et al*., 2016).

1.2 Statement of research problem

Vaccination is the main strategy in the control and prevention of IB in commercial farms. In addition, vaccination is considered as the most cost effective approach (Britton *et al*., 2012). However, some of the IB vaccinations are unsuccessful because of the continual emergence of different antigenic IBV variants which differ from IBV vaccine serotypes used (Cook *et al*., 2012; de Wit *et al*., 2011). The IBV variant which exists in the form of diverse antigenic or genotypic types is the challenges toward the current IB vaccines protection (Umar *et al*., 2016). Furthermore, the IBV show poor cross protection among different strains (de Wit, 2000). Therefore, proper selection of IBV strain as vaccine antigen will determine the successful vaccination programmes used against IB. On the other hand, the introduction of new vaccine strains may facilitate the emergence of new IBV strains.

1.3 Justification

To date, most IBV vaccines are based on live attenuated or killed vaccines derived from classical or variant serotypes. These vaccines are developed from strains originating from the USA such as M41, Ma5, Ark, and Conn and from the Netherlands, H52 and H120, as well as European strains such as 793/B, CR88, and D274. However, studies have shown that vaccines against these strains often lead to poor immune response especially against local strains belonging to certain regions (Shirzad *et al*., 2012; Sun *et al*., 2011). Furthermore, progress in new IB vaccine technology development is very slow compared to other avian viral diseases (Jordan, 2017). Therefore, development of local vaccine strain may overcome this problem.

The characterisation of IBV is a crucial step in the development of diagnostics and vaccines against IB (Bande *et al*., 2015). Recent studies have shown the predominant IBV strains circulating in Malaysia besides the Mass strains are the Malaysian variant (MV) and QX-like despite the use of vaccines in the commercial farms (Khanh *et al*., 2017; Leow *et al*., 2018). Among these viruses, the MV strain designated IBS037A/2014 and QX-like strain designated IBS130/2015 strains which were isolated from broiler and layer chickens, respectively, have been well characterised (Khanh *et al*., 2017). Pathogenicity studies have shown that both viruses have high tropism in both respiratory and kidney systems in both young and older chickens (Khanh *et al*., 2018). However, the antigenic relatedness of these two viruses compared to some of the commercially available vaccine strains are poorly studied. Furthermore, the ability of some of the vaccine strains in conferring protection against the MV and QX-like strains is not well studied. By continuously passaging the virus in embryonated eggs, it is possible to attenuate the virus without tempering with its unique tissue tropism characteristics.

1.4 Research hypothesis

Conventional commercially available live attenuated IB vaccines unable to provide full protection in chickens following challenge with MV and QX-like IBV strain. There were nucleotide and amino acid variation at S1 gene during virus attenuation in SPF embryonated chicken eggs and the attenuated MV and QX-like viruses induced reduced pathogenicity index in chickens as compared to its wildtype counterpart.

1.5 General objective

The main objective of this study is to develop live attenuated IB vaccine from local isolate namely MV and QX-like strains IBV.

1.6 Specific objectives

1. To evaluate the efficacy of several commercially available IB vaccines following challenged with MV IBV strain IBS037A/2014 and QX-like IBV strain IBS130/2015 in SPF chickens;
2. To determine the molecular characteristics of S1 gene of MV IBV strain IBS037A/2014 and QX-like IBV strain IBS130/2015 following serial passage in SPF embryonated chicken eggs;
3. To compare the pathogenicity of the attenuated MV IBV strain IBS037A/2014 and QX-like IBV strain IBS130/2015 with their respective wildtype IBV strains in SPF chickens.

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