UNIVERSITI PUTRA MALAYSIA

ADAPTATION AND ATTENUATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS IN AVIAN AND MAMMALIAN CELL CULTURES FOR VACCINE DEVELOPMENT

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KHOR SOK FANG

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

June 2007

Dedicated with love and gratitude to

my family, teachers and friends

who

show me the fun of education.

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

ADAPTATION AND ATTENUATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS IN AVIAN AND MAMMALIAN CELL CULTURES FOR VACCINE DEVELOPMENT

By

KHOR SOK FANG

June 2007

Chairman : Mohd. Hair Bejo, PhD

Faculty : Veterinary Medicine

Infectious bursal disease virus (IBDV) is an immunosuppressive virus causing infectious bursal disease (IBD) outbreaks since 1957. The economic impact of IBD is influenced by strain of virus, route of infection, intercurrent primary and secondary pathogens, and environmental management factors. Adaptation of IBDV to replicate in cell culture is associated with attenuation. Repeated passages of classical IBDV (cv IBDV) in avian primary cell culture, results in highly attenuated virus, which is commonly used as a live vaccine for many years. However, this cvIBDV vaccine does not provide full protection against very virulent IBDV (vvIBDV) infection. Field isolates particularly vvIBDV normally do not grow in cell cultures. It requires extensive passages and adaptation in chicken embryonated eggs before it can propagate in cell cultures.

This study attempts to adapt and attenuate two local isolates of vvIBDV namely B0081 and UPM93273 in mammalian continuous cell line (Vero

cell cultures) and avian primary cell line (Chicken embryo fibroblast, (CEF)). A newly modified method of adaptation and attenuation is introduced in this study. Both isolates were successfully adapted and attenuated in Vero cell cultures of 22 passages throughout the proposed modified method. Furthermore, through the modified method, both isolates can be adapted and propagated faster and easier in mammalian continuous cell cultures when compared with the conventional method. Passage 10 of isolate B0081 in Vero cell cultures using modified method, called B0081T was tested for pathogenicity in specific pathogen free (SPF) chickens. This study showed that UPM0081T caused sudden onset of 100% morbidity with no mortality during infection and the infected bursal recovered from the infection.

Sequence comparison has also been carried out for nucleotides (nt) and deduced amino acids (aa) in hypervariable region (HVR) of VP2 of UPM0081T with the parental isolate B0081 in this study. As a result, the parental isolate B0081 revealed one mutation from G to A in UPM0081T at nucleotide position 850. This nucleotide substitution resulted in the substitution of Ala in the parental virus to Thr in the cell culture attenuated strains at residue 284. Amino acid residues Gln, Asp and Ala at position 253, 279 and 284 were conserved in most strains of high pathogenicity; and His, Asn and Thr were conserved in most of the cell culture attenuated strains of low pathogenicity in SPF chickens. The sequence of hypervariable region of VP2 of UPM0081T was deposited in GeneBank under accession number EF208038.

In conclusion, IBDV B0081 was successfully adapted and propagated in mammalian continuous cell cultures by the proposed modified method. The adapted virus was named UPM0081T. It may serve as a seed virus in the production of local vaccine against vvIBDV infections, for the prevention and control of IBD in Malaysia.

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

PENYESUAIAN DAN PELEMAHAN VIRUS PENYAKIT BURSA BERJANGKIT JENIS AMAT VIRULEN DALAM TITISAN SEL MAMALIA DAN UNGGAS UNTUK PERKEMBANGAN VAKSIN

Oleh

KHOR SOK FANG

Jun 2007

Pengerusi : Mohd Hair-Bejo, PhD

Fakulti : Perubatan Veterinar

Virus penyakit bursa berjangkit (IBDV) ialah virus yang boleh melemahkan imun dan menyebabkan wabak penyakit infeksi bursa (IBD) sejak tahun 1957. Kesan ekonomi oleh IBD dipengaruhi oleh jenis virus, cara infeksi, patogen asas dan kedua, dan juga faktor pengurusan alam sekitar. Penyesuain IBDV untuk membiak dalam sel kultur sering kali dikaitkan dengan pelemahan. Ulangan pemindahan IBDV jenis klasik (cvIBDV) dalam sel kultur ayam asas, dimana pelemahannya tinggi biasanya diguna sebagai vaksin hidup sejak lama lagi. Walaubagaimanapun, vaksin daripada cvIBDV tidak dapat menyediakan perlindungan ayam sepenuhnya daripada infeksi IBDV amat virulen (vvIBDV). Tambahan pula, asingan liar terutamanya vvIBDV biasanya tidak dapat tumbuh dalam sel kultur. Ia memerlukan pemindahan yang panjang dan penyesuaian dalam embrio ayam sebelum dapat membiak dalam sel kultur terutamanya sel kultur berurutan.

Kajian ini cuba untuk menyesuaikan dan melemahkan dua asingan tempatan vvIBDV yang dinamakan UPM93273 dan B0081 dalam sel jenis berurutan

mamalia monyet hijau Afrika (Vero sel kultur) dan sel jenis fibroblas embrio (embrio fibroblas ayam, CEF). Satu cara ubahsuai untuk penyesuaian dan melemahkan virus telah dicadangkan dalam kajian ini. Kedua-dua asingan berjaya disesuai dan dilemahkan dalam Vero sel kultur selama 22 pasage dengan menggunakan cara ubahsuai. Tambahan pula, dengan cara ubahsuai ini, kedua-dua asingan boleh sesuai dan tumbuh dengan lebih cepat dan senang dalam titisan sel berurutan mamalia bila dibanding dengan cara rutin. Pasage 10 untuk B0081 dalam Vero sel kultur menggunakan cara ubahsuai dinamakan UPM0081T telah diuji patogenisinya dalam ayam spesifik pathogen bebas (SPF). Keputusannya menunjukkan bahawa UPM0081T menyebabkan 100% morbiditi tanpa kematian semasa infeksi dalam ayam, dan bursa yang berinfeksi sembuh daripada jangkitan. Selain daripada ini, perbandingan rangkaian untuk nukleotida (nt) dan asid amino (aa) dalam bahagian variabel tinggi (HVR) protein virus 2 (VP2) UPM0081T dengan virus induk B0081 telah dijalankan. Keputusannya, virus induk telah menunjukkan satu penggantian dari G ke A dalam UPM0081T pada kedudukan 850. Penukaran nt ini menyebabkan penggantian aa Ala dalam virus induk kepada Thr dalam virus penyesuaian sel kultur, UPM0081T. Asid amino Gln, Asp dan Ala pada kedudukan 253, 279 dan 284 sememangnya kekal dalam kebanyakan strain penyesuaian sel kultur yang patogenesinya rendah pada ayam SPF. Rangkaian nukleotida dan asid amino dalam bahagian melampau berubah protein virus 2 untuk UPM0081T telah disimpan dan dipamerkan dalam GeneBank dengan nombor perolehan EF208038.

Kesimpulannya, IBDV B0081 telah berjaya disesuaikan dan membiak sebagai strain lemah UPM0081T dalam sel jenis berurutan mamalia dengan menggunakan cara ubah suai. Strain lemah ini mungkin boleh digunakan sebagai benih vaksin tempatan menentang infeksi vvIBDV di Malaysia pada masa depan.

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I am grateful to all my friends and staff in the laboratory for always being willing to render assistance throughout the course of my study. Special thanks also extended to my family for their understanding and support.

I certify that an Examination Committee met on 25th June 2007 to conduct the final examination of Khor Sok Fang on her Master of Science thesis entitled "Adaptation and Attenuation of Very Virulent Infectious Bursal Disease Virus in Avian and Mammalian Cell Lines for Vaccine Development" 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:

Hassan Hj. Mohd. Daud, Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Adbul Rani Bahaman, Ph.D.
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Fauziah Othman, Ph.D.
Associate Professor
Faculty of Medicine
Universiti Putra Malaysia
(Member)

Zulkeflie Zamrod, Ph.D.
Associate Professor

Universiti Kebangsaan Malaysia
(Independent Examiner)

_____

ZAKARIAH ABD. RASHID, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

APPROVAL SHEET

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 Supervisory Committee were as follows:

Mohd Hair Bejo, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Aini Ideris, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 November 2007

DECLARATION

I hereby 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.

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KHOR SOK FANG

Date: 13 August 2007

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LIST OF ABBREVIATIONS

(v/v)	Volume/Volume
(w/v)	Weight /Volume
µg	Microgram
aa	Amino Acid
AGPT	Agar Gel Diffusion Precipitation Test
at	Attenuated
ATV	Antibiotic-Trypsin-Versine
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CAM	Chorio-Allantoic Membrane
cDNA	Complementary Deoxyribonucleic Acid
CEB	Chicken Embryo Bursa Cell
CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo Kidney
CMI	Cell-Mediated Immunity
CPE	Cytopathic Effect
CT	Threshold Cycle
cv	Classical Virulent
DEPC	Diethyl Pyrocarbonate
DH_2O	Distilled Water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
ds	Double Stranded
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
HIS	Hyperimmune Serum
HRP	Horseradish Peroxidase
hv	Hypervariable
IBD	Infectious Bursa Disease
IBDV	Infectious Bursa Disease Virus
IFT	Immunofluorescent Test
Ig G	Immunoglobulin G
IIPS	Indirect immunoperoxidase Staining
KCl	Kalium Chloride

kDa	Kilodalton
ml	Milliliter
mM	Milimolar
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NJ	Neighbour-Joining
nt	Nucleotide
ºC	Degree Celcius
OD	Optical Density
OK	Ovine Kidney
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pi	Post Inoculation
pmol	Picomole
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolution Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
$TCID_{50}$	Tissue Culture Infectivity Dose
UPGMA	Un-Weighted Pair Group Method with Arithmetic Mean
UPM	Universiti Putra Malaysia
UV	Ultra-Violet
va	Variant
vac	Vaccine
Vero	Green Monkey Kidney
VN	Virus Neutralization
VP	Viral Protein
vv	Very Virulent
UPM 0081T	Passage 10 of isolate UPM0081 in cell culture
UPM 93273T	Passages of isolate UPM93273 in cell culture

Amino Acid	**Single/Three Letter Amino Acid Code**	
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp

Glutamine	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
Thyptophan	W	Trp
Valine	V	Val

CHAPTER 1

INTRODUCTION

Infectious bursal disease (IBD) is caused by a birnavirus, infectious bursal disease virus (IBDV). IBD was first described by Cosgrove (1962). The first outbreak of the disease in 1957 was occurred around Gumboro, Delaware. Since then, the name Gumboro disease was used extensively in the past. However, clinical outbreak of the disease due to very virulent IBDV (vvIBDV) was only reported in Malaysia in 1991 (Hair-Bejo, 1992a; Loganathan *et al*., 1992). Infectious bursal disease is endemic in most poultry producing areas of the world, widespread in commercial chicken. The acute stage of the disease, the immunosuppression that follows, and the widespread distribution of the disease, are the major factors contributing to the economic significance. The virus is ubiquitous and under natural conditions, chickens acquire infection by the oral route.

The bursa of Fabricius is the primary site of virus replication, but lesions are also detected in the spleen, thymus, cecal tonsils and Harderian gland. The outcome of infection depends on the age of the bird and the virulence of the infecting strain. Infectious bursal disease can cause high mortality in young, susceptible chicks but in older birds effects are usually mild unless complicated by secondary bacterial or other viral infections. The acute phase of the disease lasts for about 7-10 days.

Within this phase, bursa follicles are depleted of B cells and the bursa becomes atrophic.

Infection with IBDV often results in immunosuppression. Allan *et al*. (1972) first reported the immunosuppressive nature of IBDV. The immunosuppressive effects appear to be more pronounced if virus exposure occurs within the first 2-3 weeks after hatched. The degree of immunosuppression varies depending on the virulence of the virus and, when the infection occurs. Immunosuppression may accompany overt clinical or subclinical outbreaks of IBD. The humoral immune response is clearly depressed, but a transient depression occurs in the cellular immune response (Confer *et al*., 1981).

IBDV is a member of the genus *Avibirnavirus* in the family *Birnaviridae*, member of this family contain a genome consists of two segments of double-stranded RNA (dsRNA), designated A and B (Dobos *et al*., 1979b; Muller *et* al., 1979b), with icosahedral symmetry and a diameter of about 50 to 55nm (Leong *et al*., 2000). The virus has five proteins recognized as VP1, VP2, VP3, VP4 and VP5. The smaller RNA segment known as segment B of the genome, with a length of about 2.8kb, encodes for VP1, which is a 90-kD multifunctional protein with polymerase and capping enzyme activities (Spies *et al*., 1987; Kibenge and Dhama, 1997). The larger segment A with a length of about 3.2kb, encodes for VP2, VP3, VP4 and VP5. The VP2 and VP3 are the major proteins of the virion constituting 51% and 40%, respectively of the total

proteins and contain the major neutralizing epitopes. The VP2 has the serotype specific epitope and VP3 has a group specific antigen. VP4 is a minor protein involved in the processing of the precursor polyprotein (Birghan *et al*., 2000).

There are two recognized serotypes of IBDV, designated 1 and 2. Viruses of both serotypes naturally infect chickens and turkeys, but the disease is recognized only in chickens and only serotype 1 viruses are pathogenic. Chicken is the only avian species to be susceptible to clinical disease and characteristic lesions caused by IBDV. It occurs as clinical or subclinical IBDV infection. According to antigenic variation and virulence, serotype 1 strains can be divided into several groups: classical, variant and very virulent strains (Winterfield and Thacker, 1978). Classical strains can cause mortality (<20%) and bursal lesions. It is able to break through a moderate level of maternal derived antibody. Variant strains do not express certain virus (neutralisation) epitopes typical for classical strains. It is able to break through higher levels of maternal derived antibody than classical strains causing an early IBDV infection with severe bursal damage (atrophy), resulting in immunosuppression, and, the mortality rate is less than 5%. Very virulent strains of IBDV emerged in Europe in the late eighties and spread throughout the world causing substantial economic losses. Very virulent strains are characterized by their ability to break through high level of maternally derived antibodies and to induce mortality more than 20%.