UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN MALAYSIA FOR THE DEVELOPMENT OF DIAGNOSTIC TOOLS

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FPV 2002 4

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By

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

May 2002

Dedicated with love and gratitude
to:

My husband Wesley Voon, parents,
daughter (Wynnie) and son (Wingates)

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: Mohd Hair-Bejo, Ph.D

Faculty: Veterinary Medicine

The P97/302 field outbreak isolate was identified as a very virulent infectious bursal disease virus (vvIBDV) of serotype 1, based on the conventional and molecular characterization methods. The high mortality, gross and histopathological lesions observed during the outbreak and in the experimental infected SPF chickens induced by P97/302 IBDV isolate were characteristics of vvIBDV strain reported previously. The sequence of P97/302 isolate has amino acid substitutions at 222(A), 256(I), 294(I) and 299(S) similar to other reported vvIBDV. This isolate does not have 249(K) and 254(S) amino acid residues which have been reported to be present in variant strains. The amino acid residues of the P97/302 at the two hydrophilic regions and the serine-rich heptapeptide region are the same as reported for vv strains of UK661, HK46 and OKYM. The P97/302 IBDV isolate can be digested with *Taq*1, *Acc*1, *Sty*1, *Spe*l enzymes and not with

*Sac*1 as reported for other vv IBDV. This isolate is most homologous to the reported vv IBDV strains especially UK661. Phylogenetic analysis based on the nucleotide sequence of the hypervariable region revealed that P97/302 IBDV isolate can be clustered with the vvIBDV strains and is distinct from classical, variant and attenuated strains. Using this P97/302 IBDV local isolate, the study has successfully developed three diagnostic tools for antibody and antigen detection. They are the indirect, double antibody sandwich (DAS) and reverse-transcription (RT) nested polymerase chain reaction (PCR) enzyme-linked immunoassay assay (ELISA). The developed indirect and DAS ELISA are based on the use of regression equation line which generated from the standard curves to measure IBD antibody titre. They are highly significant correlated ($p< 0.01$) when compared with IDEXX commercial ELISA. They are more advantage than the commercial kits whereby the local isolate was used for the ELISA coating which was able to enhance the accuracy to predict the protection to IBD. The developed DAS ELISA for antigen detection is also highly specific. The developed RT nested PCR was highly specific and ten times more sensitive when compared to conventional RT/PCR. The RT nested PCR ELISA method was also highly specific and hundred times more sensitive when compared to conventional RT/PCR with agarose gel electrophoresis detection method. It was concluded that the P97/302 local isolate was vvIBDV strain and the developed indirect, DAS and RT nested PCR ELISA are potentially used for monitoring and screening large number of samples for antibody and antigen detection from chicken flocks.

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

PENCIRIAN VIRUS PENYAKIT BURSA BERJANGKIT YANG DIPEROLEHI DARI MALAYSIA UNTUK MEMBUAT ALAT-ALAT DIAGNOSA

Oleh

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Mei 2002

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P97/302 strain dari ladang ayam telah dikenalpasti sebagai serotip 1 virus penyakit bursa berjangkit yang amat virulen (vvIBDV) berdasarkan pencirian cara konvensional dan molekul. Kadar kematian yang tinggi, lesi-lesi patologi dan histopatologi semasa outbreak dan pada ayam bebas-patogen khusus (SPF) telah mencirikannya sebagai vvIBDV seperti yang pernah dilaporkan. Jujukan P97/302 mempunyai penggantian asid amino di kedudukan 222(A), 256(I), 294(I) dan 299(S) seperti yang dilaporkan pada vvIBDV yang lain. P97/302 tidak mempunyai asid amino residu di kedudukan 249(K) dan 254(S) seperti yang dilaporkan pada strain varian. Ia mempunyai asid amino residu yang sama dengan vvIBDV yang lain iaitu UK661, HK46 dan OKYM, di dua kawasan hidrofilik dan kawasan heptapeptid yang kaya dengan serine. Ia boleh dicernakan oleh enzim pembatas *Taq*1, *Acc*1, *Sty*1, *Spe*1 dan tidak pula oleh *Sac*1 sepertimana

yang dilaporkan pada vvIBDV yang lain. P97/302 sangat homologi dengan vvIBDV lain yang dilaporkan, terutamanya dengan UK661. Analisis filogenesis berdasarkan kawasan hiper boleh ubah jujukan nukleotid menunjukkan strain ini sekumpulan dengan vvIBDV dan berlainan kumpulan dengan strain-strain klasik, varian dan attenuated. Dengan menggunakan P97/302 IBDV, kajian ini telah berjaya membuat tiga jenis alat diagnosa untuk mengesan antibodi dan antigen. Alat-alat ini ialah indirect, double antibodi sandwich (DAS) dan reverse-transkripsi (RT) nested reaksi rantaian polimerasi (PCR) asai immunoerap terangkai enzim (ELISA). Indirect dan DAS ELISA ini adalah berdasarkan penggunaan formula regressi yang dibentuk dari garis standard untuk mengukur antibodi IBD titer. Alat-alat ini menunjukkan kolerasi yang sangat ketara ($p< 0.01$) apabila dibandingkan dengan komersial ELISA IDEXX. Alat-alat ini lebih baik daripada ELISA komersial kerana strain tempatan digunakan untuk melapis ELISA yang akan meningkatkan ketepatan meramal perlindungan daripada IBD. DAS ELISA juga sangat spesifik untuk mengesan antigen. RT nested PCR juga didapati sangat specifik dan menunjukkan sepuluh kali lebih sensitif apabila dibandingkan dengan konvensional RT/PCR. RT nested PCR ELISA pula didapati sangat spesifik dan telah menunjukkan seratus kali lebih sensitif apabila dibandingkan dengan konvensional RT/PCR yang menggunakan agarose gel electroforesi. Kesimpulannya, P97/302 strain adalah vvIBDV dan indirect, DAS dan RT nested PCR ELISA ini adalah berpotensi untuk mengkaji dan menyiasat sampel antibodi dan antigen dari kelompok ayam.

ACKNOWLEDGEMENTS

I would like to express my heartiest gratitude and appreciation to Associate Professor Dr. Mohd Hair Bejo, chairman of the supervisory committee for providing invaluable advice and untiring assistance in this Ph.D research. His constant guidance, support and encouragement were the motivation that enabled me to accomplish my work.

I would like to express my sincere thanks and appreciation to Professor Dr. Aini Ideris, a member of the supervisory committee for her constructive suggestion, proper guidance and encouragement throughout my study period. My sincere thanks and appreciation are also extended to Dr. Abdul Rahman Omar, for his participation in laboratory works, constant support and mostly for sharing knowledge on molecular technique and guidance.

I am grateful to Dr. Nadzri Salim and Dr. Goh Yong Meng for helping and guiding me in SPSS statistical analysis. Special thanks also go to the staffs in Biologics Lab., Puan Rodiah Husin and En. Adam Ahmad and Virology Lab., Assoc. Prof. Dr. Mohd Azmi Mohd Lila and En. Mohd Kamarudin; and friends, Kit Yee, Sandy, Zeenatul, Lee Kim, Do Yew, Fang, May Ling, Lih Ling, Sheaw Wei, Siti, Sheila and everybody for always being so willing to render assistance throughout the course of my study.

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-di(3-ethyl)benzthiazoline sulpnonic acid
AGPT	Agar gel precipitin test
ASA	5-Aminosalicyclic acid
BGM	Baby grivet monkey kidney
BHK	Baby hamster kidney
bp	Basepair
BSA	Bovine serum albumin
Ca	Calcium
CAM	Chorioallantoic membrane
CAV	Chicken aneamia virus
cDNA	Complementary deoxyribonucleic acid
CEB	Chicken embryo bursal cell
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
CEP	Cytopathic effect
°C	Degree Celsius
d-	Deoxy
DAS	Double antibody sandwich
dd	Dideoxy
DEPC	Diethyl pyrocarbonate
DH_2O	Distilled water
DIG	Digoxigenin
DMSO	Dimethysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double strand
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme-immuno-assay
EID_{50}	Embryo infective dose fifty
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HCl	Hydrochloric acid
HIS	Hyperimmune serum
HSV	Herpes simplex virus
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
hv	Hypervariable
IB	Infectious bronchitis

IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFA	Immunofluorescent test
Ig G	Immunoglobulin G
IPA	Immunoperoxidase test
kb	Kilobase
KCl	Potasium chloride
kDa	Kilodalton
kV	Kilovolt
LB	Luria-Bertani
M	Molar
MA	Rhesus monkey kidney
Mab	Monoclonal antibody
Mg	Magnesium
ml	Millilitre
SPF	Specific-pathogen-free
Mg_2Cl	Magnesium chloride
Mg_2SO_4	Magnesium sulphate
mM	Millimolar
MTP	Microtiter plate
uM	Micromolar
ug	Microgram
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDV	Newcastle disease virus
ng	Nanogram
nm	Nanometer
OD	Optical density
OPD	*O*-Phenylenediamine dihydrochloride
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
PD	Primer dimer
pi	Post inoculation
pmol	Picamol
PNT	Positive negative threshold
QGDPT	Quantitative gel diffusion precipitin test
RAPD	Random amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
RK	Rabbit kidney
RNA	Ribonucleic acid
RT	Reverse-transcriptase
SD	Standard deviation

SDS	Sodium dodecyl sulphate
S/P	Sample-to-positive ratio
SNT	Serum neutralization test
SPSS	Statistical program for social science
STC	Standard challenge strain
TAE	Tris-acetate-EDTA
TCVN	Tissue culture virus neutralization
TBE	Tris-borate-EDTA
TEM	Transmission electron microscopy
Tm	Melting temperature
TMB	Tetramethylbenzidine
Tris	2-amino-2-(hydroxymethy)-1, 3 propandiol
UPGMA	Unweighted pair group with arithmetric mean
UPM	Universiti Putra Malaysia
UV	Ultraviolet
Vero	Green monkey kidney
VN	Virus neutralization
vv	Very virulent
VP	Virus protein
(w/v)	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-ß -D-galactopyranoside

	Single/Three Letter Amino Acid Code	
Alanine	A	Ala
Asparagine	N	Asp
Aspartic Acid/Aspartate	D	Asp
Glutamine	Q	Glu
Glutamic acid/Glutamate	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val

CHAPTER 1

INTRODUCTION

Infectious bursal disease (IBD) is an acute highly contagious viral disease in chickens which is caused by IBD virus (IBDV). Lymphoid tissue is the primary target organ of IBDV with the special predilection for the bursa of Fabricius. The disease was first recognized as a clinical entity in 1957 in USA by Cosgrove (1962) and was referred to as 'avian nephrosis' because of the extreme kidney damage found in birds that succumbed to infections. Since the first outbreaks of IBD in the area of Gumboro, Delaware, "Gumboro disease" was a synonym for this disease and is still frequently used. The disease becomes important in poultry industry worldwide due to significant economic losses resulting from high mortality, impaired growth, excessive carcass condemnation and profound immunosuppression which lead to increase susceptibility to other pathogens. The disease can interfere the effectiveness of the vaccination programmes against other highly virulent disease (Allan, *et al.,* 1972; Okoye, 1984).

Two distinct serotypes of IBDV, designated as serotype 1 and serotype 2 have been identified. The serotype 1 strains are pathogenic to chickens and vary in their virulence, whereas serotype 2 strains, isolated from turkey are apathogenic to both turkey and chicken (Lukert and Saif, 1991). According to antigenic variation and virulence, serotype 1 strains can be divided into several groups: classical

virulent, attenuated, antigenic variant and very virulent (vv) strains (Cao, *et al.*, 1998).

Since the first outbreak of IBD in 1957, the disease was relatively under control due to proper immunization programmes in both the parent stock and their progeny. In the mid-80's, antigenic and pathogenic serotype 1, variants of IBDV were isolated by Saif and Rosenberger (Lukert, 1995) from the Delmarva area. Subsequently, the variants were recognized in other areas of the United States. The variant strains were significantly different antigenically to those of classical virulent IBDV and they could overcome higher levels of chicken maternal antibody within the first week to 10 days (Lukert, 1995). These variant strains were also different pathogenically which did not cause clinical disease in older chickens (4-6 weeks of age) when compared to the classical strains, but they could induce severe bursal atrophy and immunosuppression (Lukert, 1995). Recently, Giambrone (2001) described that the latest IBDV variants to evolve are strains that can contribute to infectious proventriculus. However, the evidence of the presence of the variant strains of IBD has never been presented in Europe or Malaysia.

In the late 1980's, outbreaks of clinical IBD with high mortality, up to 90% due to highly virulent strains of serotype 1 IBDV occurred throughout Europe (Chettle, *et al.*, 1989; Stuart, 1989; Van Den Berg, *et al.*, 1991). The disease had spread worldwide and was described in Asia in 1990's (Nunoya *et al.*,