UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY AND MOLECULAR CHARACTERISTICS OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATES OF NEPAL

KARUNA SHARMA

FPV 2004 12

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By

KARUNA SHARMA

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

August 2004

DEDICATION

I dedicate this thesis with love and gratitude to my late father Bhoj Raj Aryal and beloved mother Saraswati Aryal for their continuous encouragement and support which has brought me this far in my life and carrier.

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

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Chairman: Associate Professor Mohd Hair-Bejo, Ph.D.

Faculty: Veterinary Medicine

Infectious bursal disease (IBD) outbreak in chickens was first reported in Nepal in 1991. The objective of this study was to identify, characterise and determine the pathogenicity of IBD virus (IBDV) isolates of Nepal. The IBDV isolates, NP2K and NP1SSH were obtained from severe IBD outbreaks in Nepal in 2002. The IBDV isolates were inoculated in specific pathogen free (SPF) chickens and embryonated chicken eggs and identified as IBDV based on conventional methods. The hypervariable region of VP2 gene of NP2K and NP1SSH was amplified by revere transcriptase polymerase chain reaction (RT-PCR). The sequences were aligned, analysed to determine the molecular characteristic of the virus. A phylogenetic tree was constructed and the nucleotide sequences of the isolates were subjected to restriction fragment length polymorphism (RFLP) using computer-generated programme. The NP2K isolate was inoculated into

28-day-old SPF chickens to determine the pathogenicity and response of the bursa of Fabricius, caecal tonsil, thymus and kidney to the IBDV.

The NP2K and NP1SSH isolates induced 100% mortality in SPF eggs in passage 1, 2, 3 and 4 post inoculation (pi). In 28-day-old SPF chickens, NP2K isolate produced lesions similar to those of field IBD outbreaks which were more pronounced at day 3 to 5 pi with 55% mortality. A significant decreased ($p<0.05$) in body weight of IBD group was recorded at day 3 pi. The bursa to body weight ratio was significantly different ($p<0.05$) at day 10 pi, between the control and IBD groups.

The gross and histological lesions in the bursa of Fabricius were more pronounced than those of the thymus, caecal tonsil and kidney. In the bursa of Fabricius severe lesions (score of 5) were recorded at day 2, 3, 4 and 10 pi. The lesions in the thymus and caecal tonsil were moderate to severe (score of 3 to 5) at day 2, 3 and 4 pi, but remained normal or mild at the rest of observation days. In the kidney, only mild lesions were observed at day 3 and 4 pi. The viral antigen was detected only in the bursa of Fabricius and caecal tonsil at day 2, 3 and 4 pi, and was absent in the thymus and kidney using immunoperoxidase technique.

The 1326 bp nucleotide and 446 amino acid sequences of NP2K and NP1SSH isolates were compared with other very virulent IBDV (vvIBDV), variant, vaccine, and classical strains of IBDV. The deduced amino acid sequences of NP2K and NP1SSH isolates were very similar to the vvIBDV of Japan (OKYM), Europe (UK661), China (HK61),

Israel (IBDVKS), Malaysia (UPM97/61) and Belgium (849VB). Amino acid substitution at four positions were 300 (E to A), 308 (I to F), 334 (A to P) and 438 (I to S) for NP2K and at positions thirteen for NP1SSH at 27 (S to T), 28 (I to T), 31 (D to A), 36 (H to Y), 135 (E to G), 223 (G to S) 225 (V to I), 300 (E to A), 308 (I to F), 334 (A to P), 351 (L to I), 352 (V to E) and 339 (K to N) compare to published vvIBDV strains. Based on RFLP analysis with *Ssp*I, *Sty*I, *Taq*I, *Stu*I, *Acc*I *Bmst*I, *Mbo*I and *Sac*1 and the sequence analysis of NP2K and NP1SSH isolates showed similar characteristics to vvIBDV isolated from Japan, China, Europe and South East Asia. Absence of *Ssp*I site was likely due to the change in nucleotide at position 765 from T to C. The NP2K and NP1SSH isolates have *BspM*I, *Taq*I, *Mol*I and *Stu*1 sites in the nucleotide sequence considered as a marker of vvIBDV. The amino acid substitution in different position indicates that a new strain of IBDV is still evolving.

The NP2K and NP1SSH isolates were successfully isolated, identified and characterised using both conventional and molecular techniques as vvIBDV strain of serotype 1. The NP2K isolate produced severe lesions in the bursa of Fabricius and followed by caecal tonsil, thymus and kidney. The origin of the virus could be from China, Europe, Japan, or South East Asia. The accession numbers of the NP2K and NP1SSH isolates are AY 367560 and AY 605264 as provided from the gene bank, respectively.

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

PATOGENESITI DAN CIRI MOLEKUL VIRUS PENYAKIT BURSA BERJANGKIT DARI NEPAL

Oleh

KARUNA SHARMA

Ogos 2004

Pengerusi: Profesor Madya Mohd Hair-Bejo, Ph.D.

Fakulti: Perubatan Veterinar

Penyakit bursa berjangkit (IBD) dilaporkan menjangkiti ayam di Nepal sejak 1991. Objektif kajian ini adalah untuk mengesan, menciri dan mengenalpasti patogenesiti isolate IBDV dari Nepal. Isolat virus penyakit bursa berjangkit (IBDV), NP2K dan NP1SSH diperolehi dari wabak IBD di Nepal pada 2002. Virus tersebut diinokulat dalam ayam dan telur ayam berembryo bebas patogen khusus (SPF) dan dikenalpasti sebagai IBDV berdasarkan kaedah konvensional. Kawasan pemboleubah-hiper dalam gen VP2 NP2K dan NP1SSH diamplifikasi dengan reaksi rantai polimerase-transkriptasi berbalik (RT-PCR). Jujukan gen dianalisis untuk menentukan ciri-ciri molekul virus. Pokok pilogenetik dibentuk dan jujukan nukleotida juga dianalisa dengan poliforma fragman enzim pembatasan (RFLP). Isolat NP2K dijangkitkan ke dalam ayam SPF berumur 28 hari untuk menentukan patogenisiti dan gerakbalas bursa, tonsil sekum, kelenjar timus dan ginjal ke atas IBDV.

Semua embrio telur ayam SPF mati pada pasage 1, 2, 3, dan 4 setelah diinokulat dengan isolat virus NP2K and NP1SSH. Isolat virus NP2K menghasilkan lesi yang serupa dengan yang terdapat pada wabak IBD di lapangan dan lesi menjadi lebih ketara pada hari 3 dan 4 selepas inokulasi (pi) dengan 55% kematian. Perbezaan signifikan ($p<0.05$) dengan penurunan berat badan ayam pada kumpulan IBD pada hari ke 3 pi. Nisbah bursa kepada berat badan ayam adalah berbeza dengan signifikan ($p<0.05$) pada hari 10 pi di antara kawalan dan IBD ($p<0.05$).

Lesi matakasar dan histologi di bursa Fabricius adalah lebih ketara dibandingkan dengan timus, tonsil sekum dan ginjal. Lesi yang teruk (skor 5) direkodkan pada bursa Fabricius pada hari ke 2, 3, 4 dan 10 pi. Lesi pada timus dan tonsil sekum adalah sederhana hingga teruk pada hari ke 2, 3 dan 4 pi, tetapi tetap normal atau sedikit pada hari yang selainnya. Hanya lesi yang sedikit kelihatan di ginjal pada hari ke 3 dan 4 pi, Antigen virus dikesan pada bursa Fabricius dan tonsil sekum pada hari ke 2, 3 dan 4 pi, dan tidak dapat dikesan pada timus dan ginjal degan penggunaan teknik immunoperosida.

Sebanyak 1326 nukleotid dan 446 asid amino daripada NP2K dan NP1SSH diamplifikasi dan dibanding dengan isolat amat virulen IBDV (vvIBDV), varian, attenuat dan strain klasik IBDV. Jujukan asid amino dalam isolat NP2K dan NP1SSH adalah lebih kurang sama dengan isolat vvIBDV dari Jepun (OKYM), Eropah, China (HK46), Israel (IBDVKS), Malaysia (UPM97/61) dan Belgium (849VB). NP2K berubah pada empat posisi asid amino di posisi 300 (E kepada A), 308 (I kepada F), 334 (A kepada P) dan 438 (I kepada S) manakala NP1SSH berubah di tiga belas posisi iaitu

di posisi 27 (S kepada T), 28 (I kepada T), 31 (D kepada A), 36 (H kepada Y), 135 (E kepada G), 223 (G kepada S), 225 (V kepada I), 300 (E kepada A), 300 (I kepada F), 334 (A kepada P), 351 (L kepada I), 352 (V kepada E) dan 399 (K kepada N). Berdasarkan analisis RFLP dengan *Ssp*I, *Sty*I, *Taq*I, *Stu*I, *Acc*I, *Bmst*I, *Mbo*I and *Sac*I dan analisis jujukan gen serta asid amino, isolat NP2K dan NP1SSH mempunyai ciri yang sama dengan vvIBDV dari Jepun, China, Eropah dan Asia Tenggara, kecuali ketidakhadiran *Ssp*I yang mungkin disebabkan oleh perubahan nukleotid di posisi 765 daripada T kepada C. Isolat NP2K dan NP1SSH mempunyai *BspM*I, *Taq*i, *Stu*I dan *Mbo*I di dalam jujukan nukleotid yang merupakan ciri untuk vvIBDV. Perubahan asid amino di posisi berlainan menandakan bahawa kedua-dua isolat IBDV baru ini sedang berevolusi.

Isolat NP2K dan NP1SSH telah berjaya diasingkan, dikenalpasti dan dicirikan berdasarkan teknik konvensional dan molekular sebagai strain vvIBDV serotip 1. Isolat NP2K menghasilkan lesi yang teruk pada bursa Fabricius, diikuti dengan tosil sekum timus dan ginjal. Isolat NP2K dan NP1SSH mungkin berasal dari Eropah, Jepun, China atau Asia Tenggara. Nombor akses AY367560 bagi NP2K dan AY 605264 bagi NP1SSH telah diberi oleh bank gen.

ACKNOWLEDGEMENTS

All praises are due to almighty GOD for HIS guidance and blessings over me throughout the period of this study. I am sincerely grateful to Chaiman of supervisory committee; Associate Professor Dr. Mohd Hair-Bejo, for providing me valuable guidance, consistent encouragement and understanding. Although having hectic schedule and many responsibilities the most important thing I found on him is very patience and available whenever needed and always given priority to students works. All these affords made me towards the completion of my study and successful compilation of this thesis. I wish to express my thanks and appreciation to my co-supervisors; Professor Dr. Aini Ideris and Associate Professor Dr. Abdul Rahman Omar for their constructive guidance and encouragement throughout my study period.

I am also grateful to His Majesty's Government of Nepal, Ministry of Agriculture and Co-operative, Department of Livestock Service, for providing me the opportunity to pursue my Master of Veterinary Science programme in Malaysia with the financial support under European Community (SVSLDC) Project in Nepal.

I would also like wish to express my deepest thanks to my mother, my brothers; Vijay Kumar Aryal and his family, Bhagwan Aryal, and Parameswor Aryal, for their moral support and endurance during my study. I would like to take this opportunity to extend my thanks to other members of my family, Bhattarai and Aryal family, my family friend Shyam Ghimire and my colleagues in Nepal; Firoz Khan, who directly or indirectly

supported me during my study. My sincere acknowledgement to my husband Major Resmi Raj Bhattrai and my children; Stuti and Swastik for their patience, continuous support, sacrifice and affection throughout my study period.

I would not forget to acknowledge all the friends and colleagues I worked and related with at Universiti Putra Malaysia; Mohammed Ali Bahmaninejad, Broomand Chaharaein, Dr Karim Alwan, Mar Mar Kyi, Tan da Min, Biliyamin (President, International Students' Association – UPMISA), Najim (Secretary – UPMISA). My sincere thanks to my entire laboratory mate especially to Tan Do Yew, Kenny Voon Gah Leong, Nurulfiza, Roosevien Farida and Khor Sok Fang Wan Keng Fei, Yap May Ling, Aini Hirul and Sheau Wei and to my country mates Rabindra Thakur, Khadak Singh Bista, Parsu Ram Bhusal, Bala Ram Thapa and friends Thomas, Nwe Nwe Htin, Saw Po Po, Lat Lat Hin, Hezel Monica, Prabhat Helena Khatoon as well as my housemates Yogeetha, Daljeet Kaur and Thawa Malar, for their lovely supports and understanding during my stay in Malaysia.

My special thanks to Dr. Myint Thein and Khin Hnin Swe for their assistance during the initial and analytical stages of my study and to Seyed Davood Hosseini who helped me with the molecular part of my study. Last but not least I would like to extend my sincere appreciation and thanks to Mr Krishnan, Mr Rajan, Mr Fauzi and Mr Saipuzaman Ali in their support for computer application, photography and others and to everyone whoever willingly render their assistance throughout the course of my study.

I certify that an Examination Committee met on 4 August 2004 to conduct the final examination of Karuna Sharma on her Master of Veterinary Science thesis entitled "Pathogenicity and Molecular Characteristics of Infectious Bursal Disease Virus Isolates of Nepal" 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:

SALEHA ABDUL AZIZ, Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

SHEIKH OMAR ABDUL RAHAMAN
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

SITI SURI ARSHAD, Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

DHANIRAT SANTIVATR, Ph.D.
Associate Professor
Dean
Faculty of Veterinary Medicine
Kasetsart University
Thailand
(Independent Examiner)

GULAM RASUL RAHMAT ALI, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee are as follows:

MOHD HAIR BEJO, Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

AINI IDERIS, Ph.D.
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ABDUL RAHMAN OMAR, Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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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KARUNA SHARMA

Date:

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LIST OF ABBREVIATIONS

ABTS	2, 2'-Azino-di (3-ethyl) benzthiazoline sulphonic acid
AGPT	Agar gel precipitin test
BGM	Baby grivet monkey kidney
BHK	Baby hamster kidney
bp	Base pair
BSA	Bovine serum albumin
Ca	Calcium
CAM	Chorioallantoic membrane
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
dd	Dideoxy
DEPC	Diethyl pyrocarbonate
DH_2O	Distilled water
DIG	Digoxigenin
DMSO	Dimethysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double stranded
DTT	Dithiothreitol
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
Hcl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFA	immunoflourescent test
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPA	Immunoperoxidase test
Kb	Kilobase
Kcal	Potassium chloride
kDa	Kilodalton
KV	Kilovolt
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
NP	Nepal
uM	Micromolar
ug	Microgram
NaCl	Sodium iodine
Nai	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
PCR	Polymerase chain reaction
PD	Primer dimer
pi	Post inoculation
pmol	Picamol
RFLP	Restriction fragment length polymorphism
RK	Rabbit kidney
RNA	Ribonucleic acid
RT	Reverse-transcriptase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNT	Serum neutralization test
SPSS	Statistical program for social science
STC	Standard challenge strain
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
Tm	Melting temperature
TMB	Tetramethylbenzidine
Tris	2-amino-2-(hydroxymethy)-1, 3 propandiol
UPGMA	Unweighted pair group with arithmetic mean
UPM	Universiti Putra Malaysia
UPMISA	Universiti Putra Malaysia International Students Association
UV	Ultraviolet
Vero	Green monkey kidney

VN	Virus neutralization
vv	Very virulent
(w/v)	Weight/volume
(v/v)	volume /volume

Single/Three Letter Amino Acid Code		
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid/Aspartate	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic	E	Glu
acid/Glutamate	G	Gly
Glycine	H	His
Histidine	I	Ile
Isoleucine	L	Leu
Leucine	K	Lys
Lycine	M	Met
Methionine	F	Phe
Phenylamine	P	Pro
Proline	S	Ser
Serine	T	Thr
Threonine	W	Trp
Tryptophan	Y	Tyr
Tyrosine	V	Val
Valine		

CHAPTER I

INTRODUCTION

Infectious bursal disease (IBD) was first reported in 1957 in Southern Delaware town of Gumboro, USA in commercial broiler farm and was termed as "Gumboro Disease" (Cosgrove, 1962). IBD is an acute highly contagious immunosuppressive disease in chickens which has an affinity towards developing B lymphocytes located in the bursa of Fabricius (Hirai and Calnek, 1979; Lukert and Saif, 1991), caused by IBD virus (IBDV). IBDV was previously classified under the family picornavirus (Cho and Edgar, 1969) and reovirus (Lukert and Davis, 1974).

IBDV is a bisegmented double stranded (ds) RNA with icosahedral symmetry and is classified as a member of genus *Arbirnavirus* of *Birnaviridae* family (Dobos, 1979, Ismail *et al*., 1988; Murphy *et al*., 1995). Two distinct serotypes of IBDV are serotype 1 and 2. Serotype 1 strain is pathogenic to chickens and varies in its virulence. Whereas serotype 2 strain isolated from turkeys is avirulent for both turkeys and chickens (McFerran *et al.,* 1980; Jackwood and Saif, 1987; Becht *et al*., 1988). Serotype 1 is further classified into classical virulent, antigenic variant, very virulent (vv) and attenuated strains based on the virulence and antigenic variation (Snyder; 1990; Brown, *et al.,* 1994a; van den Berg, 2000; Zierenberg *et al.,* 2000). IBDV genome consists of two segments, segments A approximately 3.3 kb and B approximately 2.7 kb in length

and are packed inside a single shelled capsid of 60 nm in diameter (Dobos *et al.*, 1979; Muller *et al*., 1979b,).

IBDV genome consists of five viral proteins VP1, VP2, VP3, VP4 and VP5. Segment A (3.2 kDa) encodes two major structural proteins VP2 and VP3 and two non-structural proteins VP4 and VP5 (Sharma *et al*., 2000). Segment B (2.8 kDa) encodes non structural protein VP1, which is a viral transcriptase, putative RNA dependent RNA polymerase and has enzyme capping activities (Azad *et al*., 1985; Spies and Muller, 1990. Bayliss *et al*., 1991; Vakharia *et al*., 1992).

VP2 is the major structural protein of IBDV responsible for producing neutralising antibodies by the host (Schnitzler *et al*., 1993: Sharma, *et al*., 2000). The amino acid changes at VP2 gene are used to differentiate classical, variant, very virulent and attenuated strains of IBDV. A sequence analysis of VP2 indicates that it is highly conserved except at the hypervariable region located at *Acc*1-*Spe*1 restriction fragment site (Azad *et al*., 1985; Bayliss *et al*., 1990). There are two hydrophilic regions, peak A (214 to 222) and B (324 to 334) in amino acid sequence of hypervariable region (HVR). Any amino acids substitution at HVR (206 to 350) particularly peak A and B of VP2 gene which can differentiate the virulence and antigenic variations of different strains of IBDV (Azad *et al*., 1987; Heine *et al*., 1991; Schnitzler *et al*., 1993; Eterradossi *et al*., 1999). The amino acid changes at VP2 gene resulted in different pathotypes of IBDV including classical, variant, very virulent and attenuated strains of IBDV (Heine *et al*., 1991).