UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION AND PATHOGENICITY OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN IRAN

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By

MOHAMMAD ALI BAHMANINEJAD

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

September 2004

DEDICATION

DEDICATED WITH LOVE AND APPRETIATION TO

MY WIFE AND CHILDREN

FOR THEIR ETERNAL LOVE AND KINDNESS

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

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MOHAMMAD ALI BAHMANINEJAD

April 2004

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

Faculty: Veterinary Medicine

Infectious bursal disease (IBD) is one of the most important diseases among the poultry industries in Iran. Ten IBD virus (IBDV) isolates were obtained from IBD field outbreaks. These isolates were from the vaccinated and non IBD vaccinated broiler and layer flocks in Iran and were designated as IR197, IR297, IR198, IR298, IR398, IR199, IR299, IR399, IR499 and IR599.

These isolates showed clinical signs, mortality, gross and histological lesions in commercial chickens that were typical of IBD during field outbreaks. The Iranian IBDV isolates were isolated and identified by conventional methods including agar gel precipitation test (AGPT), egg inoculation, chicken embryo fibroblast (CEF) cell culture, transmission electron microscopy (TEM), and immunoperoxidase staining (IPS) as IBDV. These isolates could not propagate onto CEF cell culture except IR298 isolate, which produced cytopathic effect (CPE) in cell culture. IR298 and IR499 isolates were

inoculated into 28-day-old specific pathogen free (SPF) chicken; IR298 isolate did not produce any mortality and the gross lesions in SPF chickens but the bursa of Fabricius was atrophied, whereas IR499 isolate induced 70% mortality, gross and histological lesions which were typical of very virulent (vv) vvIBDV.

All isolates were further characterised by molecular techniques based on RT-PCR-RFLP and nested PCR. The hypervariable region of VP2 gene were amplified and sequenced. The phylogenetic tree was constructed based on aligned sequences of the Iranian isolates and published IBDV strains. All the isolates had the characteristics of vvIBDV strain similar to the earlier reports, except IR298 isolate showed the characteristics of vaccine strain. The phylogenetic tree of the isolates showed that all isolates except IR298 belongs to vvIBDV subgroups of serotype 1. Isolates IR197, IR299, IR399, IR398, IR499 and IR599 formed the distinct sub branch within the subgroup of vvIBDV strain. IR298 isolate was located within the subgroup of the vaccine strain. The origin of these isolates could be similar to the vvIBDV strains isolated in Europe, Japan and Hong Kong, whilst IR298 isolate could be similar to the Chinese and Egyptian classical as well as vaccine strains.

The IR499 isolate ($10^{6.7}$ EID_{50}) was inoculated in 28-day-old SPF chickens via oral route to determine the response of gut-associated lymphoid tissues (GALT) to vvIBDV isolated in Iran. The GALT were lymphoid cell aggregations at the oesophagus and proventriculus junction, proventriculus and gizzard junction, duodenum, Meckel's diverticulum, caecal tonsil, ileum and bursa of

Fabricius. Among the organs, the bursa of Fabricius showed the most severe lesions including degeneration, necrosis, inflammation, haemorrhage, follicular lymphoid cells depletion, and follicular cyst formation. The virus induced degeneration, necrosis and depletion in the lymphoid cells of the GALT in the rest of the organs at days 2, 3 and 4 post inoculation (pi). The finding in this study showed that the acidic (pH 2.6) of proventriculus may hamper the infectivity or population of the virus. Thus, the following oral inoculation of vvIBDV, the virus is primary multiplied in the upper part of the GALT, at the junction between the oesophagus and proventriculus within 6 to 12 hours pi, rather than the lower GALT leading to primary viraemia.

it was concluded that all the Iranian IBDV isolates were successfully isolated, identified and characterised as vvIBDV (except IR298 isolates) using conventional and molecular methods. They showed similar characteristic of vvIBDV reported from Europe, Japan and Hong Kong, except IR298 isolate showed the characteristics similar to the Chinese and Egyptian classical strains as well as vaccine strains. Inoculation of IR499 isolate (vvIBDV) in the SPF chickens produced the most severe lesions in the bursa of Fabricius at days 2, 3, 4 and 10 pi when compare to the GALT in the other organs.

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

PENCIRIAN MOLEKUL DAN PATOGENESITI VIRUS PENYAKIT BURSAL BERJANGKIT DIASINGKAN DI IRAN

Oleh

MOHAMMAD ALI BAHMANINEJAD

April 2004

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

Fakulti: Perubatan Veterinar

Penyakit bursal berjangkit (IBD) adalah merupakan salah satu penyakit yang terpenting dalam industri ayam di Iran. Sepuluh virus IBD (IBDV) dari kes wabak di lapangan diperolehi. Isolat ini datang dari ayam pedaging dan penelur yang divaksin dan tidak divaksin dengan IBD di Iran dan dikenalpasti sebagai IR197, IR297, IR198, IR298, IR398, IR199, IR299, IR399, IR499 dan IR599.

Isolat ini menunjukkan tanda klinikal, kematian, lesi mata kasar dan histologi pada ayam komersil serupa dengan wabak IBD dilapangan. Isolat IBDV Iran ini diasingkan dan dikenalpasti melalui kaedah konvensional termasuklah AGPT, inokulasi telur, tisu didik CEF, transmisi mikroskop electron (TEM) dan pewarnaan immunoperoksidas sebagai IBDV. Isolate ini tidak membiak dan tidak menghasilkan CPE pada tisu didik CEF kecuali IR298.

IR298 dan IR499 telah diinokulasi ke atas ayam SPF berumur 28 hari; IR298 tidak menyebabkan kematian dan lesi mata kasar pada ayam SPF, walaubagaimanapun

atrofi berlaku ke atas bursa, manakala IR499 menyebabkan 70% kematian dan lesi matakasar dan histologi mempunyai lesi mirip vvIBDV.

Kesemua sepuluh isolat telah dicirikan melalui teknik molekul melalui RT-PCR-RFLP dan nested PCR. Kawasan hipervariabel gen VP2 dihasilkan dan jujukan serta pokok filogenetik dibentuk berdasarkan jujukan keseimbangan isolat Iran dan strain IBDV yang telah diterbitkan. Kesemua IBDV isolat mempunyai ciri vvBDV sama seperti laporan terdahulu, kecuali IR298 menunjukkan ciri strain vaksin. Analisis pokok filogenetik isolat ini menunjukkan ke semua isolate, kecuali IR 298 subkumpulan dengan vvIBDV serotip 1. Isolat IR197, IR299, IR399, IR398, IR499 dan IR599 menghasilkan cabang ketara dalam subkumpulan strain vvIBDV. IR298 berada dalam subkumpulan strain vaksin. Asal usul isolat ini mungkin sama seperti strain dari Eropah, Jepun dan Hong Kong manakala IR298 sama seperti strain klasikal China dan Mesir juga strain vaksin.

IR499 isolat ($10^{6.7}$ EID_{50}) telah diinokulat ke atas ayam SPF berumur 28 hari untuk mengenalpasti tindakbalas tisu limfoid di organ penghadaman (GALT) kepada isolat vvIBDV Iran. GALT adalah merupakan sekumpulan limfoid sel terdapat diantra esofagus dan proventriculus, persimpangan proventriculus dengan mempadal, duodenum, diverticulum Meckel's, tonsil sekum, ileum dan bursa Fabricius. Berbanding dengan tisu lain, organ bursa menunjukan lesi yang paling teruk termasuklah degenerasi, nekrosis, inflamasi, perdarahan, pengurangan sel limfoid folikular dan pembentukkan sist folikular.

Virus ini juga merangsang degenerasi, nekrosis dan pengurangan sel limfoid pada organ GALT lain pada hari ke 2, 3, dan 4 postinokulasi (pi).

Penemuan kajian ini menunjukkan proventrikulus yang asidik (pH 2.6) mungkin mengurangkan infektiviti atau populasi virus. Oleh itu, berikutan inokulasi vvIBDV secara oral, virus akan membiak terutamanya dibahagian atas GALT, pada persimpangan di antara oesopagus dan proventriculus dalam jangkamasa 6 hingga 12 jam p.i, berbanding dengan GALT bawah, dan seterusnya menyebabkan viremia primer.

Kesimpulannya, kesemua isolate IBDV Iran telah berjaya diasingkan, dikenalpasti dan dicirikan sebagai vvIBDV (kecuali isolat IR298) dengan menggunakan teknik kenventional dan molekul.

Kesemua isolat menunjukkan ciri yang sama seperti vvIBDV yang telah dilaporkan di Eropah, Jepun dan Hong Kong kecuali IR298 isolat menunjukkan ciri strain sama seperti China dan Mesir dan juga strain vaksin. Inokulasi IR499 vvIBDV isolat ke atas ayam SPF menghasilkan lesi yang paling teruk pada bursa jika dibandingkan lesi pada GALT di organ yang lain.

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I certify that an Examination Committee met on ……..2004 to conduct the final examination of Mohammad Ali Bahmaninejad on his Doctor of Philosophy thesis entitled “Molecular Characterisation and Pathogenicity of Infectious Bursal Disease Virus Isolated in Iran” 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:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the supervisory committee are as follows:

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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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MOHAMMAD ALI BAHMANINEJAD

Date:

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LIST OF ABBREVIATION

ABTS	2,2-Azino-Bis (3-Ethylbenzthiazoline-6-Sulfunic acid)
AC-ELISA	Antigen captured-ELISA
AGPT	Agar gel precipitin test
ANOVA	Analysis of variance
APS	Ammonium persulfate
BF	Bursa of Fabricius
BGM	Baby grivet monkey kidney
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
cDNA	Complementary deoxyribonucleic acid
CEB	Chicken embryo bursa
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
CER	Chicken embryo rough
CMI	Cell mediated immunity
CPE	Cytopathic effect
CsCl	Caesium chloride
DAB	Diaminobenzadine HCl
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSA-ELISA	Double sandwich antibody-ELISA
dH_2O	Distilled water
dsRNA	Double stranded RNA
DXV	Drosophilae X virus
EDTA	Ethylene diamine tetra acetic acid
EM	Electron microscopy
EID_{50}	Embryo infective dose fifty
ELISA	Enzyme link immunosorbent assay
FCS	Foetal calf serum
FRET	Fluorescence resonance energy transfer
GALT	Gut associated lymphoid tissue
HRP	Horse radish peroxidase
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IBDV-BDA	IBDV-bursal disease antibody
IF	Immunofluorecent
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPNV	Infectious pancreatic necrosis virus
IPS	Immunoperoxidase staining
kDa	Kilo dalton
kV	Kilovolt
LE	lymphoepithelium
Mab	Monoclonal antibody

MDV	Marek’s disease virus
MHC	Major histocompatibility complex
NCBI	National Centre for Biotechnology Information
NCR	Non coding region
NJ	Neighbour joining
nm	Nanometer
OD	Optical density
ORF	Open reading frame
OV	Oyster virus
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHYLIP	Phylogenetic interference package
pi	Post inoculation
pmol	Picamol
PTA	phosphotungstic acid
QAGPT	Quantitative agar gel precipitin test
RdRp	RNA dependent RNA polymerase
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
RT-PCR	Reverse transcriptase-PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SPF	Specific pathogen free
SPSS	Statistical package for social science
STC	Standard challenge strain
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEM	Transmission electron microscopy
TEMED	N,N,N´,N´-tetramethyllenediamine
TNE	Tris-NaCl-EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3 propandiol
TV	Telina virus
UPGMA	Unweighted pair group with arithmetic mean
UV	Ultraviolet
VN	Virus neutralization
VNF	Virus neutralizing factor
VP	Viral protein
v/v	Volume per volume
vv	Very virulent
w/v	Weight per volume

Amino Acids Code

Amino acid	3 letter designation	1 letter designation
Alanine	Ala	A
Arginine	Arg	R
Asparagines	Asn	N
Aspartic acid/ Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid/ Glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V