UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF CHICKEN ANEMIA VIRUS (CAV) AND EXPRESSION OF THE CAV VP3 PROTEIN

SITI HASMAH MOHTAR

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By

SITI HASMAH MOHTAR

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

January 2003

UPM

DEDICATED TO

My Parents,

EN. MOHTAR IBRAN and PN. SAMSIAH OTSMAN

My Brothers and Sister,

MOHD SHUPIAN MOHTAR
MOHD NAJIB MOHTAR
SITI HAIRIAH MOHTAR

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

MOLECULAR CHARACTERIZATION OF CHICKEN ANEMIA VIRUS (CAV) AND EXPRESSION OF THE CAV VP3 PROTEIN

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January 2003

Chairman: Associate Professor Dr. Abdul Rahman Omar

Faculty: Veterinary Medicine

Chicken anemia virus (CAV) from non-attenuated and attenuated isolates were characterized based on sequence and phylogenetic analysis. The CAV BL-5 isolate, isolated from UPM was propagated and attenuated in MSB-1 cells until passage 90. The whole genome of non-attenuated isolate, BL-5P5 and attenuated isolate, BL-5P90 were amplified, cloned and subjected for sequencing. The sequences were analyzed and compared with other 25 isolates from local and foreign countries. Sequence analysis of VP1, VP2 and VP3 coding regions revealed that most of the variations were at the VP1 region. Sequence analysis of VP1 revealed that the BL-5P5 isolate was closely related to BL-5P90, CAF475 (China), AF313 (USA), C140 and A2 (Japan) and 3-1/P60 (Malaysia) isolates between 98% to 99% homology and distantly related to CAU269/7 (Australia) and SMSC-1 (Malaysia) isolates with 95% homology. However, analysis based on amino acid sequence indicated that the BL-5P5 isolate was closely related (98% to 99%) to all the above isolates, including the CAU269/7

isolate. It was found that the CAU269/7 has a very unusual low nonsynonymous/synonymous (NS/S) ratio of 0.188 when compared to the BL-5P5. Similarly, phylogenetic analysis based on the VP1 nucleotide sequences revealed that the BL-5P5 was closely related to BL-5P90, CAF475 (China) and AF313 (USA) and distantly related to CAU269/7 (Australia) and SMSC-1 (Malaysia) isolates. Analysis based on amino acid sequences revealed that the BL-5P5 was closely related to BL-5P90, CAU269/7 (Australia), CAF475 (China) and AF313 (USA) and distantly related to ConnB (USA), SMSC-1 (Malaysia) and P3102A9-resist isolates. The BL-5P90 showed only 15 nucleotide differences compared to BL-5P5 isolates. However, these differences associated with 11 amino acid changes which were found mainly in the hypervariable region of VP1. Thus, the NS/S ratio (2.75) is significantly higher than the S/NS ratio (0.36). The BL-5P90 isolate has an amino acid substitution at position 144 from glutamic acid (E) to lysine (K) in VP1 hypervariable region. This amino acid substitution might play an important role in viral attenuation. The CAV VP3 gene from non-attenuated BL-5P5 isolate was expressed as a fusion protein in prokaryotic system. The SDS-PAGE and Western blot analysis indicated that the expressed VP3 protein of approximately 18 kDa was observed from the cell lysate sample after 4 hours post induction with isopropyl-β-D-thiogalactosidase (IPTG). However, the protein was expressed in insoluble form and was relatively non-immunogenic since hyperimmune serum against the expressed protein showed non-specific reactions following Western blot and indirect immunofluorescence antibody test (IFAT) assay. Thus, the expressed VP3 protein in the present form

is not suitable for use as antigen in production of antibody for the development of VP3 protein as diagnostic marker. Further studies on the application of the VP3 as diagnostic protein of CAV remains to be confirmed.

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

PENCIRIAN MOLEKUL VIRUS ANEMIA AYAM (CAV) DAN PENGEKSPRESAN PROTEIN VP3 CAV

Oleh

SITI HASMAH MOHTAR

Januari 2003

Pengerusi: Profesor Madya Dr. Abdul Rahman Omar

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Virus anemia ayam (CAV) daripada isolat tidak diakenuat dan diakenuat telah dicirikan berdasarkan kepada analisis jujukan dan filogenetik. CAV isolat BL-5, yang dipencilkan dari UPM telah dibiakkan dan diakenuat dalam sel MSB-1 sehingga turutan 90. Keseluruhan genom bagi isolat tidak diakenuat, BL-5P5 dan strain diakenuat, BL-5P90 telah diamplifikasi, diklon dan didedahkan kepada penjujukan. Jujukan-jujukan dianalisis dan dibandingkan dengan 25 isolat tempatan dan luar negara. Analisis jujukan bagi bahagian berkod VP1, VP2 dan VP3 menunjukkan bahawa kebanyakan variasi adalah pada kawasan VP1. Analisis jujukan bagi VP1 menunjukkan bahawa isolat BL-5P5 berhubung rapat dengan isolat BL-5P90, CAF475 (China), AF313 (USA), C140 dan A2 (Jepun) dan 3-1/P60 (Malaysia) di antara 98% hingga 99% persamaan dan berhubung jauh dengan isolat CAU269/7 (Australia) dan SMSC-1 (Malaysia) dengan 95% persamaan. Walau bagaimanapun, analisis berdasarkan jujukan asid amino menunjukkan bahawa isolat BL-5P5 berhubung rapat (98% hingga 99%) dengan

kesemua isolat di atas, termasuk isolat CAU269/7. Didapati bahawa isolat CAU269/7 mempunyai nisbah tak sinonim/sinonim (NS/S) rendah yang luar biasa iaitu 0.188 apabila dibandingkan dengan BL-5P5. Analisis filogenetik berdasarkan kepada jujukan nukleotid VP1 menunjukkan bahawa BL-5P5 berhubung rapat dengan isolat BL-5P90, CAF475 (China) dan AF313 (USA) dan berhubung jauh dengan isolat CAU269/7 (Australia) dan SMSC-1 (Malaysia). Analisis berdasarkan jujukan asid amino menunjukkan bahawa BL-5P5 berhubung rapat dengan BL-5P90, CAU269/7 (Australia) dan AF313 (USA) dan berhubung jauh dengan isolat SMSC-1 (Malaysia) dan ConnB (USA). BL-5P90 menunjukkan hanya 15 perbezaan nukleotid berbanding dengan isolat BL-5P5. Walau bagaimanapun, perbezaan ini berhubung kait dengan perubahan 11 asid amino yang mana dijumpai terutama dalam bahagian hiperbolehubah VP1. Maka, nisbah NS/S (2.75) adalah secara signifikan lebih tinggi daripada nisbah S/NS (0.36). Isolat BL-5P90 mempunyai satu perubahan asid amino pada kedudukan 144 daripada asid glutamik (E) kepada laisin (K) dalam bahagian hiperbolehubah VP1. Penggantian asid amino ini berkemungkinan memainkan peranan yang penting dalam pengakenuatan virus. Gen VP3 CAV daripada isolat BL-5 tidak diakenuat telah diekspreskan sebagai protein gabungan dalam sistem prokariotik. SDS-PAGE dan analisis sap Western menunjukkan bahawa protein VP3 yang diekspres kira-kira 18 kDa telah dikenalpasti daripada sampel sel selepas 4 jam diinduksi dengan IPTG. Walau bagaimanapun, protein tersebut telah diekspres dalam bentuk tak terlarut dan secara relatif tidak imunogenik memandangkan serum hiperimun terhadap protein yang diekspres menunjukkan

tindak balas tak spesifik selepas ujian sap Western dan IFAT. Oleh yang demikian, VP3 yang diekspres dalam bentuk ini adalah tidak sesuai digunakan sebagai antigen dalam penghasilan antibodi bagi pembangunan protein VP3 sebagai penanda diagnostik. Kajian lanjut terhadap aplikasi VP3 sebagai protein diagnostik bagi CAV masih perlu dipastikan.

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I certify that an Examination Committee met on 31st January 2003 to conduct the final examination of Siti Hasmah Mohtar on her Master of Science thesis entitled "Molecular Characterization of Chicken Anemia Virus (CAV) and Expression of the CAV VP3 Protein" 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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Date: 5 FEB 2003

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

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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.

SITI HASMAH MOHTAR

Date: 5.2 2003

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LIST OF ABBREVIATIONS

bp	Base pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CAV	Chicken anemia virus
cm	Centimeter
CO_2	Carbon dioxide
$CaCl_2$	Calcium chloride
CI	Chloroform isoamylalcohol
CPE	Cytopathic effect
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double-stranded
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
IFAT	Indirect immunofluorescence antibody test
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactosidase
Kb	Kilobase pair
KDa	Kilo Dalton
LB	Luria-Bertani
Mab	Monoclonal antibody
MD	Marek's disease
MDCC	Marek's disease chicken cell line
MDV	Marek's disease virus
Min	Minute
mg	Milligram
ml	Millilitre
mm^2	Millimeter square
mM	Millimolar
MSB-1	Avian T cells transformed by Marek's Disease Virus
M W	Molecular Weight
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
ND	Newcastle disease
NDV	Newcastle disease virus
ng	Nanogram
NS/S	Nonsynonymous/Synonymous

nt	Nucleotide
O.D.	Optical density
ORF	Open reading frame
P	Passage
PBS	Phosphate buffered saline
PBFDV	Psittcine beak and feather disease virus
PCI	Phenol:chloroform:isoamylalcohol
PCR	Polymerase chain reaction
PCV	Packed cell volume
PCV	Porcine circovirus
pmole	Picomole
poly(A)	Polyadenylation
PVDF	Polyvinylidene Difluoride
RF	Replicative form
RE	Restriction endonuclease
RNA	Ribonucleic acid
REV	Reticuloendotheliosis virus
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPF	Specific-pathogen-free
ss	Single-stranded
TAE	Tris-acetate-EDTA-buffer
TEMED	Tetramethylethylenediamine
TLMV	TTV-like mini virus
TTV	TT virus
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Volt
V/V	Volume/Volume
VN	Virus neutralization
VP	Viral protein
VRI	Veterinary Research Institute
µg	Microgram
µl	Microlitre
µm	Micrometer

CHAPTER I

GENERAL INTRODUCTION

Chicken anemia virus (CAV) has recently been classified in a newly recognized animal virus family, the *Circoviridae* (Lukert *et al.*, 1995). CAV was first isolated in Japan by Yuasa *et al*. (1979). It has been isolated in many countries and is considered to have worldwide distribution, not only in commercial domestic fowl but also in specific pathogen free (SPF) chickens (O' Rourke *et al*., 1994). Two other members of the virus family *Circoviridae* are porcine circovirus (PCV) (Tisher *et al*., 1982) and psittacine beak and feather disease virus (PBFDV) (Ritchie *et al*., 1989). Although these viruses are grouped together on the basis of a common genome form, no similarities in amino acid composition, open reading frame (ORF) arrangement, or transcriptional machinery have been identified (Noteborn and Koch, 1995).

The virus is small, non-enveloped, spherical, 18 to 23 nm in diameter, containing a circular single-stranded DNA genome of 2.3 kb (Gelderblom *et al*., 1989; Todd *et al*., 1990; McNulty *et al*., 1991; Noteborn *et al*., 1991). The CAV genome has three partially overlapping major open reading frames coding for proteins of 52 (VP1), 24 (VP2) and 14 (VP3) kDa (Claessens *et al*., 1991; Meehan *et al*., 1992; Noteborn *et al*., 1991). VP1 is the capsid protein that plays important role in virus spread and cell tropism (Renshaw *et al*., 1996). The function of VP2 is not known, whereas VP3 (14 kDa) is involves with the

induction of apoptosis and responsible for the pathogenicity of CAV (Noteborn *et al*., 1991).

CAV can be transmitted vertically, horizontally and by injection of contaminated vaccines (Pope, 1991). The virus causes clinical and subclinical disease in chickens and is recognized as an important avian pathogen worldwide (McNulty, 1991; McIlroy *et al*., 1992). Vertical transmission of the virus through egg from infected breeder flocks can result in increased mortality in 10 to 14 days old chicks associated with anemia, hemorrhages and lymphoid depletion (McNulty, 1991). Subclinical disease in commercial broiler chicks resulting from infection with horizontally acquired virus can adversely affects growth and profitability (McNulty *et al*., 1991).

The virus replicates in lymphoid cells and is cytopathic (Yuasa and Imai, 1986; Noteborn and Koch, 1995). CAV causes severe anemia due to destruction of erythroblastoid cells in the bone marrow, immunodeficiency due to depletion of certain lymphoid cells and hemorrhages of subcutaneous and intramuscular in young chickens (Yuasa *et al*., 1986 and Von Bulow, 1991). Lymphocyte depletion results in immunosuppression and increased susceptibility to various viral and bacterial pathogens (Von Bulow, 1991). Older chickens are susceptible to virus replication but do not develop clinical signs (Jeurissen *et al*., 1989; McNulty, 1991). Currently, the diagnosis of the disease was through serology, polymerase chain reaction (PCR) and isolation of the virus in MSB1 cells (Yuasa *et al*., 1990).