UNIVERSITI PUTRA MALAYSIA

DETECTION AND CHARACTERIZATION OF CHICKEN ANEMIA VIRUS ISOLATED FROM COMMERCIAL BROILER BREEDER FARMS IN MALAYSIA

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FPV 2008 8

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By

ZERIHUN HAILEMARIAM NEGASI

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

September 2008

DEDICATED TO

My beloved wife, Konjit Getachew

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

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September 2008

Chairman: Associate Professor Abdul Rahman Omar, PhD

Faculty: Veterinary Medicine

Chicken anemia virus (CAV) is the causative agent of chicken infectious anemia (CIA). It is an economically important pathogen with a world-wide distribution. Study on the type of CAV isolates present and their genetic diversity, transmission to their progeny and level of protection afforded in the breeder farms is lacking in Malaysia. Hence, the present study was aimed to detect CAV from commercial broiler breeder farms using molecular, serological and immunohistochemical methods and characterize CAV positive samples based on sequence and phylogenetic analysis of partial VP1 gene. In the present study CAV DNA was detected in all 60 commercial broiler breeder hens obtained from 12 farms in three states of Malaysia. Results from ELISA also showed that 96.15% of blood samples collected from the same farms were positive for antibody against CAV supporting the finding from the nested PCR assay. Both of these findings indicate that CAV is widespread in commercial

broiler breeder hens at least in the three states of Malaysia. Testing pooled embryonic tissue samples consisting of thymus, bursa of Fabricius and spleen together with egg shell membrane (ESM) showed positive embryos for CAV DNA in the range of 40% to 100% for different commercial broiler breeder farms despite the presence of neutralizing antibodies in majority of the hens (96.15%) tested for CAV antibodies. This shows high level of occurrence of vertical transmission of viral DNA to the progeny. The CAV antigen was also detected in the lymphocytes within the cortex of the thymus and in the hemocytoblasts of the bone marrow by indirect immunoperoxidase staining in some birds. The analysis of 165 amino acid portion of the VP1 protein of 12 isolates from commercial broiler breeder farms revealed unique amino acid residues proline (P) at amino acid position 22 and glutamine (G) at amino acid position 48 in isolates NF4A and PYT4, respectively. Generally, isolates from the commercial broiler breeder farms can be grouped into two based on their amino acid profile at positions 75, 97, 139 and 144. Seven of the isolates (NF4A, PPW4, P24A, P12B, M3B5, MF3C and MF1A) from the commercial broiler breeder farms had 75-I, 97-L, 139-Q and 144-Q and clustered together in cluster IIIa of the deduced amino acid phylogenetic tree whilst the remaining five isolates (M1B1, NF1D, NF2C, NF3A and PYT4) had similar 75-V, 97-M, 139-K and 144-E profile and found in cluster I and II of the deduced amino acid phylogenetic tree. When compared with previously published local field isolates, six isolates from the commercial broiler breeder farms (MF1A, MF3C, M3B5, NF4A, P12B and P24A) were found to have maximum homology with SMSC-1 isolate, four isolates

(M1B1, NF3A, PYT4 and PPW4) were found to have maximum homology with BL-5 isolate and the remaining two (NF1D and NF2C) have similar maximum homology both with isolates 3-1 and BL-5. The sequence and phylogenetic analysis further indicated high similarity of current isolates from the commercial broiler breeder farms with isolates in this part of the globe while still having limited variation with isolates from different geographical places. The importance of unique amino acid substitutions observed in this study requires further research in order to identify the detail characteristics of the isolates.

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

PENGESANAN DAN PENCIRIAN VIRUS ANEMIA AYAM DARI LADANG TERNAKAN AYAM BAKA PEMBIAK PEDAGING KOMERSIL DI MALAYSIA

Oleh

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Virus anemia ayam (CAV) merupakan agen yang menyebabkan penyakit anemia berjangkit (CIA). CAV merupakan patogen yang penting dari segi ekonomi dan tersebar secara meluas. Penyelidikan dari aspek jenis isolat CAV dan kepelbagaian genetik, penyebaran kepada progeni serta tahap perlindungan dalam ladang ternakan ayam di Malaysia masih tidak mencukupi. Oleh itu, tujuan penyelidikan ini adalah untuk mengesan CAV dari ladang ayam baka pembiak pedaging komersil dengan menggunakan kaedah-kaedah molekul, serologi serta imunohistokimia, dan mencirikan sampel-sampel yang positif bagi CAV berdasarkan pada analisis jujukan dan filogenetik sebahagian daripada gen VP1. Dalam penyelidikan ini, DNA CAV telah dikesan pada kesemua 60 ayam betina baka pembiak pedaging komersil yang didapati dari 12 ladang yang terletak dalam tiga buah negeri di

Malaysia. Keputusan ELISA juga menunjukkan sebanyak 96.15% sampel darah yang dikumpulkan dari ladang tersebut adalah positif bagi antibodi terhadap CAV, dan ini menyokong keputusan esei PCR nested. Kedua-dua keputusan ini menunjukkan bahawa CAV tersebar secara meluas dalam ayam betina baka pembiak pedaging komersil sekurang-kurangnya dalam tiga buah negeri di Malaysia. Pemeriksaan ke atas sampel-sampel tisu embrio berkumpul yang mengandungi timus, bursa Fabricius dan limpa, bersama-sama dengan membran cangkerang telur menunjukkan embrio tersebut adalah positif terhadap DNA CAV dalam lingkungan 40% sehingga 100% untuk ladang ayam baka pembiak pedaging komersil yang berbeza walaupun kebanyakan ayam-ayam betina (96.15%) yang telah diperiksa mempunyai antibodi peneutralan. Ini menunjukkan bahawa pemindahan vertikal DNA virus kepada progeni berada pada tahap yang tinggi. Antigen CAV juga telah dikesan pada limfosit dalam korteks timus dan di dalam hemositoblas dalam sum-sum tulang sesetengah burung melalui kaedah imunoperoksida tidak langsung. Hasil analisis 165 jujukan asid amino protein VP1 pada isolat dari ladang ayam baka pembiak pedaging komersil menunjukkan terdapatnya residu asid amino prolin (P) yang unik pada kedudukan asid amino 22 dan glutamin (G) pada kedudukan asid amino 48 bagi isolat NF4A dan PYT4 masing-masing. Secara umumnya, isolat-isolat dari ladang ayam baka pembiak pedaging komersil ini boleh dibahagikan kepada dua kumpulan berdasarkan profil asid amino pada kedudukan 75, 97, 139 dan 144. Tujuh daripada isolat-isolat (NF4A, PPW4, P24A, P12B, M3B5,

MF3C dan MF1A) dari ladang ayam baka pembiak pedaging komersil mempunyai 75-I, 97-L, 139-Q serta 144-Q, dan telah dikumpulkan dalam kelompok IIIa, manakala lima isolat yang selebihnya (M1B1, NF1D, NF2C, NF3A dan PYT4) mempunyai profil 75-V, 97-M, 139-K dan 144-E didapati berada di dalam kelompok I dan II dari pokok filogenetik yang dihasilkan. Apabila dibandingkan dengan isolat-isolat tempatan yang telah diterbitkan sebelum ini, enam isolat (MF1A, MF3C, M3B5, NF4A, P12B dan P24A) dari ladang ayam baka pembiak pedaging komersil ini didapati mempunyai homologi yang maksimum dengan isolat SMSC-1, empat isolat (M1B1, NF3A, PYT4 dan PPW4) didapati mempunyai homologi yang maksimum dengan isolat BL-5, dan dua isolat yang selebihnya (NF1D dan NF2C) mempunyai homologi yang maksimum dengan isolat 3-1 dan BL-5. Analisis lanjutan jujukan amino asid dan filogenetik menunjukkan terdapatnya persamaan yang tinggi pada isolat terkini dari ladang ayam baka pembiak pedaging komersil dengan isolat lain dari seluruh dunia, di mana variasi isolatnya masih terhad walaupun isolat tersebut berasal dari kedudukan geografi yang berbeza. Penukargantian unik asid amino yang ditemui dalam kajian ini perlu dibuat penyelidikan lanjutan bagi mengenal pasti pencirian mendalam isolat tersebut.

ACKNOWLEDGEMENTS

I would like to express my heartiest gratitude and appreciation to Assoc. Prof. Dr. Abdul Rahman Omar, chairman of the supervisory committee for his excellent guidance, support and encouragement throughout my study. He was always willing to take time out of his hectic schedule to discuss the project; and without fail, gives invaluable comments and suggestions.

I wish to express my sincere thanks and appreciation to Prof. Dr. Mohd Hair Bejo, member of the supervisory committee for his constructive comments, suggestions, proper guidance and encouragement throughout my study.

A very special thanks goes to Dr. Tan Ching Giap and Wan Keng Fei and other fellow graduate students at the Biologics Laboratory of Faculty of Veterinary Medicine at UPM for their constant support, encouragement, friendship and sharing their knowledge. I am also very grateful to my Ethiopian colleagues here in UPM for their friendship during my study period.

I am highly indebted to Dr. Goh Yong Meng, for his statistical advice and feedback. I would like to thank also Siti Khatijah Muhamad, staff at the Biologics Laboratory for her great technical assistance.

I am also grateful for the Netherlands Organization for International Cooperation in Higher Education (Nuffic) for providing me the opportunity to pursue my masters program with the financial support.

I would like to express my deepest gratitude and sincere appreciation to my beloved wife Konjit Getachew and my parents for their endless encouragement, patience, understanding and sacrifices.

Finally, many more persons have participated in various ways to ensure I succeed in my study and I am thankful to them all.

I certify that an Examination Committee has met on September 23, 2008 to conduct the final examination of Zerihun Hailemariam Negasi on his Master of Science thesis entitled “Detection and Characterization of Chicken Anemia Virus Isolated from Commercial Broiler Breeder Farms in Malaysia” in accordance with Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the degree of Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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Zerihun Hailemariam Negasi

Date:

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LIST OF ABBREVIATION

A	Alanine
bp	Base pair
CAV	Chicken anemia virus
CIA	Chicken infectious anemia
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DAB	Diamino benzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
pi	post inoculation
DR	Direct repeats
ds	double-stranded
E	Glutamic acid
EDTA	Ethlenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESM	Egg shell membrane
FAV	Fowl adenovirus
G	Glycine
H	Histidine
HIER	Heat-induced epitope retrieval

HRP	Horse radish peroxidase
HVR	Hypervariable region
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFAT	Indirect immunofluorescence antibody test
IgY	Immunogloulin Y
IHC	Immunohistochemical
IPS	Indirect immunoperoxidase Staining
K	Lysine
Kb	Kilobase pair
kDa	Kilo Dalton
MD	Marek's disease
MDCC	Marek's disease chicken cell line
MDV	Marek's disease virus
MHC	Major histocompatability complex
mM	millimolar
mRNA	Messenger RNA
MSB1	Avian T cells transformed by Marek's disease virus
N	Asparagine
nt	Nucleotide
O.D.	Optical density
ORF	Open reading frame

P	Proline
PBFDV	Psittacine beak and feather disease virus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Porcine circovirus
pmol	Picomole
Q	Glutamine
REV	Reticuloendotheliosis virus
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse- transcriptase PCR
S/N	Sample to negative ratio
SPF	Specific-pathogen-free
T	Threonine
TAE	Tris-acetate-EDTA-buffer
TBS	Tris-bufferd saline
$TCID_{50}$	50% Tissue culture infective dose
TRIS-HCl	Trishydroxymethyleaminomethane-hydrogen chloride
v/v	Volume per volume
VN	Virus neutralization
VP	Viral protein
VRI	Veterinary Research Institute

CHAPTER 1

GENERAL INTRODUCTION

Chicken anemia virus (CAV) is a circovirus that was first isolated in specific pathogen free (SPF) chicks in Japan by Yuasa *et al*. (1979). It is the causative agent of chicken infectious anemia (CIA) and has recently been classified in the family Circoviridae, genus Gyrovirus (Pringle, 1999). It is small, non- enveloped virus that has spherical or hexagonal shape, ranging from 23 to 25 nm in diameter, containing circular single-strand negative sense DNA genome of 2.3kb (Adair, 2000; Gelderblom *et al*., 1989; Todd *et al*., 1991). The genome of CAV consists of a 5' nontranscribed region that has promoter/enhancer activity (Noteborn *et al*., 1994; Phenix *et al*., 1994) and three partially overlapping functional open reading frames (ORF) coding for proteins 52 (VP1), 24 (VP2) and 14 (VP3) kDa (Cleassens *et al*., 1991; Meehan *et al*., 1992; Noteborn *et al*., 1991) that are transcribed as a single, unspliced mRNA (Noteborn *et al*., 1992, Phenix *et al*., 1994). VP1 encode for capsid protein that plays an important role in virus spread and cell tropism (Renshaw *et al*., 1996), VP2 is a non-structural protein that acts as a scaffold protein in virion assembly (Noteborn *et al*., 1998a) and recently has been shown to have dual protein phosphatase activity (Peters *et al*., 2002). VP3 (14 kDa) involves with the induction of apoptosis (Noteborn *et al*., 1991).

Chicken anemia virus is an economically important pathogen and has been found in many countries with poultry industry (Von Bülow and Schat, 1997; Schat, 2003). CAV can be transmitted vertically from parent to the chick (McNulty, 1991; McNulty *et al*., 1991) and horizontally from chicken to chicken, resulting in clinical and subclinical infections. It infects and depletes hemoblastocysts in the bone marrow and precursor T cells in the thymus, resulting in severe anemia, hemorrhage, and immunosuppression, leading to death, increased susceptibility to secondary infections, and decreased responsiveness to vaccines. These problems occur when virus is transmitted vertically by infected hens, when chicks without CAV maternal antibody are infected early in life, or when CAV–antibody-free chicks are experimentally infected at 1 day of age (Adair, 2000). Thus, vertical transmission of the virus from non-immune hens to their progeny is regarded as a major determinant of disease outbreaks in commercial flocks.

Characteristic symptoms are aplastic anemia paired with hemorrhagic lesions. Other lesions include watery blood, pale bone marrow, atrophy of thymus and bursa, and swollen and discolored liver. Direct mortality caused by CAV is usually relatively low. However, economic losses from CAV stem from increased mortality, the cost of antibiotics used to control secondary bacterial infections, poor growth and poor weight gain (McNulty, 1991; McIlroy *et al*., 1992).

Maternal antibodies protect against infection of young chicks when hens are infected well before the onset of lay (Otaki *et al*., 1992; Yuasa *et al*., 1980a). Infections after the decay of maternal antibody, when the chicks are 2–3 weeks of age only lead to subclinical infection (Toro *et al*., 1997). Therefore, it used to be a common practice to infect breeder flocks by exposing them to contaminated litter or inoculating them with a live, non-attenuated virus before the onset of lay (Fussell, 1998; Steenhuisen *et al*., 1994; Vielitz *et al*., 1987; Vielitz and Voß, 1994). However, infection of chickens older than 2 weeks, although considered subclinical, it has immunosuppressive effects (McConnell *et al*., 1993b; Toro *et al*., 1997). The immunosuppression caused by CAV infection result in increased susceptibility to disease caused by other infectious agents.

A tentative diagnosis can be made with the support of clinical signs and gross pathological lesions. However, to confirm presence of CAV infection, laboratory diagnosis has to be carried out. This consist of isolation of virus using MSB1 cell line which consists of Mareks disease virus (MDV) transformed chicken lymphocytes derived from Mareks disease (MD) lymphoma.

Detection of CAV is commonly done through serology and immunological tests such as virus neutralizing, immunofluorescence and ELISA (Brewer *et al*., 1994). However, immunofluorescence and virus neutralization test require continuous passage of the virus, which makes them cumbersome for use in