UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION, PATHOGENICITY AND IMMUNOLOGICAL STUDIES OF CHICKEN ANAEMIA VIRUS ISOLATED IN MALAYSIA

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FPV 2001 11

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By

SHAH MD. ZIQRUL HAQ CHOWDHURY

Thesis Submitted in Fulfilment of the Requirement for the
Degree of Doctor of Philosophy in the
Faculty of Veterinary Medicine
Universiti Putra Malaysia

October 2001

DEDICATED TO

My Parents, ***SHAH MD. LUTFAR RAHMAN CHOWDHURY***
AND MRS RABEYA KHATUN CHOWDHURY

My Wife, ***FAUZIA YASMIN CHOWDHURY*** **and**
My Daughter, ***FARZANA YASMIN CHOWDHURY***

My Five Brothers, Three Sisters and One Late Sister

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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October 2001

Chairman: **Professor Dr. Aini Ideris**

Faculty: **Veterinary Medicine**

A comprehensive study was carried out to isolate, identify and characterise chicken anaemia virus (CAV) isolated in Malaysia. The study resulted in the isolation of five CAV isolates from broiler chickens, designated as BL-1, BL-2, BL-3, BL-4 and BL-5. These isolates together with three isolates (SMSC-1, SMSC-2 and 3-1) provided by Veterinary Research Institute (VRI), Malaysia and a reference Cux-1 isolate were analysed by different restriction endonuclease enzymes. The whole genome of each CAV isolate was amplified by PCR into four fragments: Fragments A, B, C and D. Fragment A was digested with *Sty*I, fragment B with *Sty*I, *Hp*aII and *Mbo*I, fragment C with *Hae*III, and fragment D with *EcoR*I. The overall analysis revealed that the four isolates, BL-1, BL-2, BL-4 and BL-5, exhibited the same restriction profiles in all enzymatic reactions and they are placed in one group, whereas, the other five

isolates (SMSC-1, SMSC-2, 3-1, BL-3 and Cux-1) were found to be different from each other and also from the group of four isolates mentioned above.

The pathogenicity studies in specific pathogen free (SPF) chickens inoculated with SMSC-1, 3-1 and BL-5 isolates at 1-day old showed that, the isolates produced clinical signs and characteristic lesions suggestive of CAV infection at 14-16 days post inoculation (p.i.). The histopathological lesions in infected chicks showed severe depletion of lymphocytes from thymus, bursa and spleen and aplastic changes in bone marrow. The repeated passages of two VRI isolates, SMSC-1 and 3-1, in MSB1 cell line until passage sixty (P60), and passage 123 (P123), produced attenuated viruses (SMSC-1/P60, 3-1/P60, SMSC-1/P123 and 3-1/P123) which showed significantly reduced level of pathogenicity in SPF chickens compared to the pathogenic parent isolates.

The whole genome of two non-attenuated isolates (SMSC-1 and 3-1) and two attenuated isolates (SMSC-1/P60 and 3-1/P60) were sequenced using the Perkin Elmer's BigDye Terminator Cycle Sequencing Kit. The high G:C regions of the CAV genome were sequenced using the same kit by the development of a modified method. The results showed that the complete genome of all isolates consisted of 2298 nucleotides. Three major ORFs of 1347 bp, 648 bp and 363 bp long were found in the plus DNA strand in all isolates, coding for putative proteins of about 52 kDa (VP1), 24 kDa (VP2) and 13 kDa (VP3), respectively. The alignment and antigenic index of VP1

sequence revealed the appearance of a hypervariable region from amino acid positions 139 to 157. The results showed that 76 nucleotide changes in SMSC-1/P60 and 43 nucleotide changes in 3-1/P60 isolates compared to their parent isolates, were thought to contribute to virus attenuation. Among these nucleotide changes, only one nucleotide difference (T→C) at position 816 resulted in changes of amino acid residues at positions 153 in VP2 from V to A, and 118 in VP3 from C to R. This single nucleotide change is probably important for the change in virus pathogenicity or attenuation. The phylogenetic analysis showed that the SMSC-1 isolate is close to the Australian 704 and Japanese TR20, the 3-1 isolate is close to the German Cux-1 isolate and the attenuated cloned isolate 10 (derived from the Cux-1). The attenuated SMSC-1/P60 and 3-1/P60 isolates were very close to the Japanese isolate A2.

The apoptosis study carried out with electron microscopy and DNA fragmentation analysis, detected apoptosis both in infected thymocytes and infected MSB1 cells. The immunological studies with P1, P60 and P123 isolates of SMSC-1 and 3-1, and also with BL-5 isolate, after inoculation into 1-day-old SPF chickens showed that each of the isolates elicited CAV antibody responses, both at 14-16 days and 30 days p.i. Based on the findings of antibody response and pathogenicity studies, the attenuated isolates of P60 and P123 are potential candidates for live vaccines.

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

PENCIRIAN MOLEKUL, KAJIAN KEPATOGENAN DAN IMUNOLOGI VIRUS ANEMIA AYAM YANG DIPENCILKAN DI MALAYSIA

Oleh

SHAH MD. ZIQRUL HAQ CHOWDHURY

Oktober 2001

Pengerusi: Profesor Dr. Aini Ideris

Fakulti: Perubatan Veterinar

ABSTRAK

Satu kajian yang komprehensif telah dijalankan untuk memencil, mengenalpasti dan mencirikan virus anemia ayam (CAV) yang di isolat di Malaysia. Kajian ini menghasilkan pemencilan lima isolat CAV daripada ayam pedaging, dinamakan sebagai BL-1, BL-2, BL-3, BL-4 dan BL-5. Isolat-isolat tersebut bersama tiga isolat (SMSC-1, SMSC-2 dan 3-1) diberikan oleh Institut Penyelidikan Veterinar (VRI), Malaysia, dan isolat rujukan Cux-1 telah dianalisis melalui enzim endonukleas pembatas. Keseluruhan genom bagi setiap isolat CAV diamplifikasi melalui PCR kepada empat fragmen: Fragmen A, B, C dan D. Fragmen A telah dipotong dengan *Sty*I, Fragmen B dengan *Sty*I, *Hpa*II dan *Mbo*I, Fragmen C dengan *Hae*III dan fragmen D dengan *EcoR*I. Analisis keseluruhan menunjukkan bahawa empat isolat, BL-1, BL-2, BL-4 dan BL-5 menghasilkan profil pembatas yang sama di dalam kesemua tindak balas enzim dan isolat berkenaan diletakkan dalam satu kumpulan, manakala lima isolat

(SMSC-1, SMSC-2, 3-1, BL-3 dan Cux-1) didapati berbeza antara satu sama lain dan juga daripada kumpulan empat isolat yang disebut di atas.

Kajian kepatogenan ke atas ayam bebas patogen spesifik (SPF) yang diinokulat dengan SMSC-1, 3-1 dan BL-5 menunjukkan tanda-tanda klinikal dan ciri-ciri lesi CAV. Lesi histopatologi dalam ayam terjangkit menunjukkan pengurangan limfosit daripada timus, bursa dan limpa dan perubahan aplastik dalam sum-sum tulang. Pengulangan inokulasi bagi dua isolat VRI, SMSC-1 dan 3-1, dalam sel MSBI hingga ke inokulasi 60 (P60) dan inokulasi 123 (P123) menghasilkan virus atenuat (SMSC-1/P60, 3-1/P60, SMSC-1/P123 dan 3-1/P123) yang mana menunjukkan pengurangan tahap kepatogenan yang signifikan dalam ayam SPF berbanding dengan isolat patogenik asal.

Keseluruhan genom bagi dua isolat yang tidak diatenuat (SMSC-1 dan 3-1) dan dua isolat yang diatenuat (SMSC-1/P60 dan 3-1/P60) telah dijujukkan menggunakan Perkin Elmer's BigDye Terminator Cycler Sequencing Kit. Kawasan G:C yang tinggi bagi genom CAV dijujukkan dengan menggunakan kit sama dengan ubahsuaian. Keputusan menunjukkan bahawa genom yang lengkap bagi kesemua isolat terdiri daripada 2298 nukleotid. Tiga ORF major daripada 1347 bp, 648 bp dan 363 bp telah dijumpai pada bebenang DNA tambahan dalam kesemua isolat, dengan mengekod protein putatif pada anggaran 52kDa (VP1), 24 kDa (VP2) dan 13 kDa (VP3), masing-masing. Jujukan VP1 yang disusun memperlihatkan kemunculan satu kawasan

hiper boleh-ubah daripada asid amino berkedudukan 139 hingga 157. Indeks antigenik VP1 juga menunjukkan kawasan hiper boleh-ubah di antara isolat-isolat, dalam kawasan asid amino 122 hingga 165. Keputusan tersebut menunjukkan bahawa 76 nukleotid terubah dalam SMSC-1/P60 dan 43 nukleotid terubah dalam 3-1/P60 isolat berbanding dengan isolat asal. Perubahan ini dianggapkan menyumbang kepada pengakenuatan virus. Di kalangan perubahan nukleotid ini, hanya perbezaan nukleotid (T→C) pada kedudukan 816, yang menghasilkan perubahan dalam asid amino residu pada kedudukan 153 dalam VP2 daripada V kepada A, dan 118 dalam VP3 daripada C kepada R. Perubahan satu nukleotid adalah penting untuk merubah kepatogenan atau pengakenuatan virus. Analisis filogenetik menunjukkan bahawa isolat SMSC-1 adalah hampir kepada isolat Australia 704 dan Jepun TR20, isolat 3-1 adalah hampir kepada isolat Jerman Cux-1 dan klon isolat 10 yang diatenuat. Isolat SMSC-1/P60 dan 3-1/P60 yang diatenuat adalah sangat hampir kepada isolat Jepun A2.

Kajian apoptosis dengan menggunakan mikroskop elektron dan analisis serpihan DNA, telah mengesan apoptosis dalam timosit terjangkit dan sel MSBI terjangkit. Kajian imunologi ke atas P1, P60 dan P123 bagi isolat SMSC-1 dan 3-1 dan juga dengan isolat BL-5, menunjukkan bahawa setiap isolat menghasilkan tindakbalas antibodi CAV. Berdasarkan penemuan tindakbalas antibodi dan kajian kepatogenan, isolat-isolat yang dilemahkan bagi P60 dan P123 mempunyai potensi sebagai calon vaksin hidup.

ACKNOWLEDGEMENTS

Bismillahir Rahmanir Rahim

All praises and credits are due to Almighty Allah who has given me the ability and strength to complete this challenging task.

I would like to express my deepest sense of appreciation and gratitude to Professor Dr. Aini Ideris, Dean of Graduate School, UPM and Chairman of Supervisory Committee, for her invaluable guidance, advice and suggestions throughout the study. I am also grateful to her for sending me to UKM, to complete two short training courses on molecular works.

My sincere gratitude and appreciation is due to Dr. Abdul Rahman Omar, a member of the supervisory committee for his continuous guidance, suggestions and cooperation throughout the study.

I am expressing my sincere thanks and gratitude to Assoc. Prof. Dr. Mohd. Hair Bejo, a member of the supervisory committee for his guidance, suggestions and encouragement throughout the study.

My sincere appreciation and gratitude is due to Dr. Abdul Aziz Jamaluddin, Director, Veterinary Research Institute (VRI), Ipoh, Perak, Malaysia, and a member of the supervisory committee, for his invaluable guidance, suggestions and cooperation throughout the study. I am really

grateful to him for arranging a training programme for me in VRI, for the isolation and identification of chicken anaemia virus (CAV) from field samples. My cordial thanks are also due to him for providing three VRI CAV isolates for this study.

I would like to express my sincere gratefulness to Dr. Y. Kono, a JICA Specialist at VRI, for his invaluable technical training, suggestions and cooperation during the study. I would like to appreciate the contribution of Dr. Nadzri Salim, Lecturer, Faculty of Veterinary Medicine, UPM, for helping me to analyse my research data statistically.

I would like to express my sincere thank to Assoc. Prof. Dr. Rahmah Mohamed and Dr. Wan Kiew Lian of UKM, Dr. Tan Siang Hee and Dr. Hairkrishna of Genetic Lab., UPM for providing the facilities for sequencing.

My sincere thanks are due to Dr. Badrul Munir Md. Zain of UKM, for his help and cooperation during phylogenetic analysis of the CAV sequences. Thanks are also due to Mr. Mohd. Noor Mat Isa, MTDC, UKM, and Mr. Lee, Genetic Lab., UPM, for their help and cooperation during sequence analysis.

I really appreciate the help and cooperation of Dr. Azizah Darus of VRI, Perak, Malaysia, and Dr. Haas Md Yatim of MTDC, Malaysia, for their help, cooperation and valuable suggestions during collection of CAV samples from

different broiler farms. Thanks are due to Dr. Reuben Sharma of Parasitology Lab., Ms Siti Hasmah Mohtar, Kong Lih Ling and Tan Sheau Wei of Biologics Lab., UPM, for their help and cooperation during the study. I would also like to thank the staffs at Biologics Lab., Histopathology Lab., Haematology Lab., Electron microscopy unit, Genetic Lab. at UPM, and the staffs at VRI, Perak, Malaysia for their help, cooperation and suggestions during the study.

I would like to express my sincere thanks to the World Bank for providing financial support (IDA Credit 2815-BD) for the scholarship under ARMP-BLRI part. I am also grateful to the Government of Bangladesh and Government of Malaysia for providing other financial support to ensure completion of my Ph.D. studies.

My cordial thanks are to the Ministry of Livestock and Fisheries, Bangladesh Secretariate, Dhaka, and Director General, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh, for their continuous help during the study, extension of my scholarship and deputation.

My heartfelt appreciation to my parents, brothers and sisters for their help and encouragement during the study. Finally, I am expressing my utmost gratitude and appreciation to my wife, Fauzia Yasmin Chowdhury and my only daughter, Farzana Yasmin Chowdhury, for their patience, continuous help, cooperation and encouragement throughout the study period.

I certify that an Examination Committee has met on 12th October 2001 to conduct the final examination of Shah Md. Ziqrul Haq Chowdhury on his Doctor of Philosophy thesis entitled "Molecular Characterisation, Pathogenicity and Immunological Studies of Chicken Anaemia Virus Isolated in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommended 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 fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date: 10 JAN 2002

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.

SHAH MD. ZIQRUL HAQ CHOWDHURY

Date: November 21, 2001

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