UNIVERSITI PUTRA MALAYSIA

PRODUCTION, CHARACTERIZATION AND SCALING UP OF MONOCLONAL ANTIBODIES AGAINST NEURAMINIDASE GLYCOPROTEIN OF AVIAN INFLUENZA VIRUS SUBTYPE H9N2

SANA AWAD ABDELAZIZ FADUL

FBSB 2011 44

PRODUCTION, CHARACTERIZATION AND SCALING UP OF MONOCLONAL ANTIBODIES AGAINST NEURAMINIDASE GLYCOPROTEIN OF AVIAN INFLUENZA VIRUS SUBTYPE H9N2

By

SANA AWAD ABDELAZIZ FADUL

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

December 2011

DEDICATION

This work is dedicated to my father who passed away before completing my PhD journey. I love and miss him so much; he will always be in my heart

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

PRODUCTION, CHARACTERIZATION AND SCALING UP OF MONOCLONAL ANTIBODIES AGAINST NEURAMINIDASE GLYCOPROTEIN OF AVIAN INFLUENZA VIRUS SUBTYPE H9N2

By

SANA AWAD ABDELAZIZ FADUL

December 2011

Chairman: Muhajir Hamid, PhD

Faculty: Biotechnology and Biomolecular Sciences

Avian influenza viruses (AIVs) are a group of pathogens responsible for many respiratory illnesses, infected human and animals worldwide. Although early detection of influenza diseases relied on conventional techniques, monoclonal antibodies (mAbs) have been reported to be useful in rapid detection, identification and treatment of influenza infections. In this study, monoclonal antibodies against neuraminidase (NA) protein of avian influenza virus A/Chicken/Iran/16/2000/H9N2 were produced to contribute to the enhancement of detection, diagnosis and control of avian influenza virus infections.

Avian influenza virus (AIV) subtype H_9N_2 was propagated in embryonated chicken eggs. Propagated virus was purified and used as immunizing agent for Balb/c mice. Hybridoma clones were produced by fusing spleen cells from AIV-immunized mouse with SP2/O (myeloma cells) using polyethylene glycol (PEG) as fusing agent. Supernatant of constructed hybridomas was screened by ELISA for the detection of the antibodies. The highly positive clones were subjected

to a series of limiting dilutions in order to ensure their monoclonality. Subclasses of the selected mAbs were determined; as well immunoblotting was performed to identify the specificity of produced mAbs. Clones which exhibited specificity towards NA protein were selected and characterized by neuraminidase inhibition test, elution inhibition test, and virus yield reduction in embryonated chicken eggs allantoic fluids. Characterization was extended by assessing the effect of anti-neuraminidase mAbs in neutralizing the virus in MDCK cells. Most importantly, cross reactivity was done to determine whether the produced mAbs exhibit cross-reaction to H_5N_1, H_1N_1, H_3N_2 and H_3N_8 influenza strains. The *in vivo* protection efficacy of anti-neuraminidase mAbs was evaluated in Balb/c mice, which were challenged with lethal dose of H_9N_2 viruses 24h before or after treatment with the antibodies. In order to study the large productivity of hybridoma culture, cells were grown in fed batch culture mode using cost effective spinner flask system.

Seven hybrid cell lines that produced mAbs against H_9N_2 were developed and designated as 2H5, 4H2, 2A5, 2A12, 3C7, 5F3, and 3D7. Isotyping showed that 2H5 was IgG_1; 3C7 was IgG_{2b}; 4H2, 2A5, 2A12 and 5F3 were IgM and 3D7 was IgA. Kappa (κ) light chains were found in all mAbs. Immunoblot analysis of the clones revealed on only five clones' that recognized H_9N_2 proteins. Among the five clones, mAbs 2H5 and 4H2 recognized NA protein, mAb 2A12 recognized HA protein and both 2A5 and 3C7 recognized M protein, whereas mAbs 5F3 and 3D7 did not match any epitope. The clones that produced mAbs against neuraminidase (NA) protein showed inhibitory effect on NA enzyme activity, strongly inhibited elution of the virus from RBCs, and reduced the virus yield in the infected allantoic fluids of embryonated chicken eggs. Furthermore, mAbs against NA inhibited virus replication in MDCK cells which led to

viability percentages of 78.8 % and 75.2 % at 1000 µg/mL mAbs concentration for 2H5 and 4H2 clones, respectively. The reactivity of each mAb with several subtypes of influenza virus revealed that clones 2H5 and 4H2 react strongly with H_5N_1, H_1N_1 and H_3N_2 strains. All clones showed moderate to weak reactivity towards H_3N_8. It was observed that pre treatment with anti-neuraminidase mAbs protect mice totally (100 %) from the virus induced mortality, with remarkable reduction in severity of the clinical symptoms and reduction of lung virus shedding to $2^{2.8}$ and 2^3 HAUs at dose 10 mg/kg of body weight for 2H5 and 4H2, respectively. Whereas, protection values of the mAbs administered 24 h post infection, did not show complete protection even when they were administrated at high doses (62.5% protection).

In fed batch culture with the continuous addition of nutrients, antibodies reached concentrations of 550 mg/L and maximum cell density and viability of about 6.8×10^6 cells/mL and 96.9%, respectively. Compared with the results obtained from the conventional batch culture, the antibodies concentration was about 320 mg/L, and cell density and viability were lasted at around 1.2 $x10^6$ cells/mL and 51.3%, respectively. The addition of nutrient medium in fed batch process, maintained the continuous supply of nutrients specially glucose and glutamine and kept their by-products lactate and ammonia at low levels, which led to the enhancement of productivity in terms of antibodies concentration, cell density and cell viabilities.

In conclusion, this study had constructed and characterized monoclonal antibodies against NA protein of H_9N_2 virus which act effectively in inhibiting neuraminidase enzyme *in vitro* and *in vivo*. These monoclonal antibodies may have the potential to be used for detection, diagnosis and therapy of avian influenza virus infections.

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

PENGHASILAN, PENCIRIAN DAN PENINGKATAN SKALA ANTIBODI MONOKLON TERHADAP GLYCOPROTEIN NEURAMINIDASE VIRUS SELSEMA BURUNG SUBTIP H9N2

Oleh

SANA AWAD ABDEAZIZ FADUL

Disember 2011

Pengerusi : Muhajir Hamid, PhD

Fakulti: Bioteknologi Dan Sains Biomolekul

Virus selsema burung (AIV) dianggap sebagai punca utama bagi penyakit respiratori yang menjangkiti manusia dan haiwan di seluruh dunia. Walaupun pengesanan awal bagi penyakit selsema ini bergantung kepada kaedah konvensional, antibodi monoklonal (mAb) telah dilaporkan penggunaannya dalam diagnosis secara pantas, rawatan dan pengecaman agen penyebab selsema ini. Untuk peningkatan dalam pengesanan yang pantas, diagnosis dan kawalan penyebaran jangkitan virus selsema burung, antibodi monoklon terhadap protein neuraminidase (NA) pada virus selsema burung penyisihan A/Chicken/Iran/16/2000/H9N2 telah dihasilkan.

Virus selsema burung (AIV) sub-jenis H_9N_2 telah dibiakan dalam telur ayam berembrio. Virus yang dituai telah ditulenkan dan digunakan sebagai agen imunisasi terhadap mencit Balb/c. Sel hibridoma telah dihasilkan melalui pelakuran sel limfa dari mencit yang diimunisasi dengan AIV dan Sp2/0 (sel mieloma) menggunakan Poliethylena Glikol (PEG) sebagai agen pelakuran.

Supernatan bagi sel hibridoma telah digunakan untuk pengecaman antibodi melalui penyaringan ELISA. Klon positif yang berpotensi telah menjalani beberapa siri pencairan terhad bagi mengesahkan klon mereka. Subkelas untuk antibodi klon terpilih turut ditentukan. Imunoblotting dilakukan untuk menentukan kekhususan mAb yang dihasilkan. Klon terhadap protein NA telah dicirikan melalui ujian perencatan neuraminidase (NI), ujian perencatan elusi dan pengurangan hasilan virus pada cecair allantoic dalam telur. Pencirian dilanjutkan melalui kajian tentang kesan oleh mAb untuk meneutralkan virus dalam sel MDCK. Yang paling penting, silang kereaktifan juga dilakukan untuk menentukan sama ada antibodi mAb menunjukkan silang kereaktifan terhadap strain virus selsema H_5N_1, H_1N_1, H_3N_2 dan H_3N_8. Dalam ujian keberkesanan perlindungan secara in vivo, antibodi monoklon diuji pada mencit Balb/c. Mencit Balb/c disuntik dengan virus H_9N_2 pada dos kematian 24 jam sebelum atau selepas rawatan dengan antibodi. Untuk kajian produktiviti untuk kultur hibridoma, sel hibridoma yang menghasilkan antibodi monoklon IgM terhadap protein NA pada H_9N_2 virus telah ditumbuhkan dengan cara kultur suapan kelompok menggunakan sistem kelalang ‘spinner”.

Tujuh jenis klon sel hibrid yang menghasilkan antibodi monoklon (mAbs) telah dihasilkan dan dinamakan 2H5, 4H2, 2A5, 2A12, 3C7, 5F3, dan 3D7. Isotiping menunjukkan bahawa 2H5 adalah IgG_1, 3C7 adalah IgG_{2b}, 4H2, 2A5, 2A12, 5F3 adalah IgM dan 3D7 adalah IgA. Rantai ringan Kappa (κ) telah ditemui di semua mAb. Analisa imunoblotting pada klon mendedahkan bahawa hanya lima klon dapat mengenali protein H9N2. Antara lima klon, 2H5 dan 4H2 dapat mengenali protein NA, 2A12 mengenali protein HA dan kedua-dua 2A5, 3C7 mengenali protein M sedangkan 5F3 dan 3D7 tidak dapat mengecam sebarang epitop. Klon yang menghasil mAb terhadap protein NA menunjukkan kesan perencatan terhadap aktiviti enzim NA, perencatan

yang kuat terhadap elusi virus daripada sel darah merah dan mengurangkan penghasilan virus dalam cecair allantoik telur berembrio yang dijangkiti. Selanjutnya, mAb terhadap NA merencatkan replikasi virus dalam sel MDCK yang menyebabkan peratus kehidupan 78.8% dan 75.2% telah dicapai pada kepekatan mAb 1000 μg/mL untuk klon 2H5 dan 4H2 masing-masing. Kereaktifan daripada setiap mAb dengan beberapa subjenis virus selsema mendedahkan bahawa klon 2H5 dan 4H2 bertindak secara ketara terhadap H_5N_1, H_1N_1 dan H_3N_2 yang sangat patogenik. Semua klon menunjukkan tindakan yang sederhana atau lemah terhadap strain H_3N_8. La adalah diperhatikan bahawa rawatan pra dengan mAbs anti-neuraminidase melindungi tikus sepenuhnya(100%) dari kematian virus yang berpunca daripada,dengan penguran yang luar biasa dalam ketetukan gejala-gejala klinikal dan pengurangan virus paru-paru menumpahkan to $2^{2.8}$ dan 2^3 HAUs pada dos 10 mg /Kg berat badan masing –masing untuk 2H5 dan 4H2. Manakala, nilai perlindungan mAbs yang ditadbir 24 jam selapas jankitan, tidak menujukkan perlindugan yang lengkap walaupun mereka telah ditadbir pada dos yang tinggi (62.5% pelindungan).

Dalam sistem kultur suapan sekelompok dengan penambahan zat makanan yang berterusan, antibodi dapat mencapai kepekatan 550 mg/L dan maksimum kepadatan sel dan kehidupan sel sekitar 6.8 x 10^6 sel/mL dan 96.9% masing-masing. Berbanding dengan keputusan yang diperolehi daripada kultura kelompok konvensional, kepekatan antibody adalah sekitar 0.32 mg/L dengan kepadatan sel maksimum dan kehidupan sel agak 1.2 x10^6 sel/ml dan 51.3% masing-masing. Penambahan zat media dalam proses suapan kelompok memelihara bekalan zat nutrien yang berterusan terutamanya glukosa dan glutamine serta menjaga produk sampingan laktat dan ammonia pada paras yang rendah, justerunya meningkatkan produktiviti seperti kepekatan antibodi, kepadatan sel dan kehidupan sel.

Ringkasnya, kajian ini telah membina dan menandakan dua klon yang produktif dan stabil dalam penghasilan antibodi monoklon terhadap virus H_9N_2 dimana ianya dapat merencatkan enzim Neuraminidase secara berkesan pada in ovo dan in vitro. Antibodi monoklon ini berpotensi untuk mengesan dan mengdiagnosis jangkitan virus selsema burung.

ACKNOWLEDGEMENTS

In the name of Allah the most gracious and merciful

My greatest deeply appreciation specially dedicated to all member of my supervisory committee; Associate Professor Dr. Muhajir Hamid, Professor Aini Ideris and Professor Dr. Abdul Manaf Ali for their supportive advice, especially Dr. Muhajir, throughout my thesis-writing period, he provided encouragement, sound advice, good teaching, good company, lots of good ideas, and most of all for his patience.

My deepest and sincere thank also goes to Prof. Dr. Abdul Rahman Omar from Institute of Bioscience for his assistance by providing all the viruses and MDCK cells those has been used in this study, and allowing me to use the lab facilities as well as providing valuable suggestions that improved the quality of this work.

I indebted to my many student colleagues for providing a stimulating and fun environment in which to learn and grow. I am especially grateful to my lab mate Dr. Yeap, for his assistance, helping me handling and inoculating the mice throughout experimentation periods.

I would like to acknowledge honorary group members of the animal and plant cell culture lab, University Darul Iman (UDM), Kuala Terengganu, Terengganu where I carry out the scaling up production of monoclonal antibodies.

I wish to thank my best friends (Randa and Manal) for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring they provided.

I wish to thank my entire extended family for providing a loving environment for me; my husband (Gamal), my son (Mohamed), my sisters (Sawsan, Suhir and salma), my brothers (Abdel aziz, Ashraf and Mohamed), and my nephews and niece as well.

Most importantly, I wish to thank my mother: she taught me, loved me and supported spiritually throughout my life.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of **Doctor of Philosophy**. The members of the Supervisory Committee were as follows:

Muhajir Hamid, PhD

Associate Professor

Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia

(Chairman)

Aini Ideris, PhD

Professor

Faculty of Veterinary Medicine
Universiti Putra Malaysia

(Member)

Abdul Manaf Ali, PhD

Professor

Faculty of Agriculture and Biotechnology
Universiti Darul Iman

(Member)

BUJANG BIN KIM HUAT, PhD

Professor and Dean

School of Graduate Studies

University Putra Malaysia

Date:

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 institutions.

_____

SANA AWAD ABDELAZIZ

Date: 30 December 2011