UNIVERSITI PUTRA MALAYSIA

ENHANCEMENT OF ANTIBODY RESPONSES IN CHICKENS VACCINATED WITH A PLASMID DNA CONSTRUCT OF AVIAN INFLUENZA VIRUS H5 GENE INFUSED WITH HSP70 OF *MYCOBACTERIUM TUBERCULOSIS*

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IB 2009 5

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By

MEHDI RASOLI PIROZYAN

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

May 2009

Dedicated to:

My Father and Mother,

Professor Ali Akbar Rasoli

Madam Naghdifar

My Beloved sister,

Raheleh

&

Haniyeh

Whoever has provided me with care

and compassion throughout my life

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

ENHANCEMENT OF ANTIBODY RESPONSES IN CHICKENS VACCINATED WITH A PLASMID DNA CONSTRUCT OF AVIAN INFLUENZA VIRUS H5 GENE INFUSED WITH HSP70 OF *MYCOBACTERIUM TUBERCULOSIS*

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May 2009

Chairman: Associate Professor Dr. Abdul Rahman Omar, PhD

Faculty: Institute of Bioscience

Currently, this region is battling against highly pathogenic avian influenza (HPAI) virus H5N1 and the virus has been isolated in non-poultry birds in various countries in Middle East as well as in the European and African continents. These developments have ignited global fears of an imminent influenza pandemic. The adoption of a vaccination policy, targeted either to control or to prevent infection in poultry, is generally discouraged. Nevertheless, the need to boost eradication efforts in order to limit further spread of infection and avoid heavy economic losses, and advances in modern vaccine technologies, have prompted a re-evaluation of the potential use of vaccination in poultry as an additional tool in comprehensive disease control strategies. Hence, several types of vaccines are available and some of them

have been tested experimentally and/or used in commercial farms. DNA vaccines have been shown to be an effective approach to induce antigen-specific cellular and humoral immunity. However, the low immune intensity in clinical trials limits the application of DNA vaccines. Heat shock proteins (HSPs) or stress proteins are highly conserved molecules that act as chaperons. Among the HSPs, HSP70 family is well characterized protein that showed potent adjuvant effects on the innate and adaptive immune responses. In this study, we developed DNA vaccine based on H5 gene, and enhanced the DNA vaccine potency with *Mycobacterium tuberculosis* heat shock proteins 70 (HSP70) as adjuvant. Hence, a series of DNA plasmids encoding H5 and NP from Malaysian H5N1 (A/Ck/Malaysia/5858/2004) were constructed and then fused with HSP70. The H5, NP, H5-HSP70 and NP-HSP70 recombinant proteins were expressed in Vero cells. We further investigated the ability of the pcDNA3.1/H5 and pcDNA3.1/H5-HSP70 constructs in inducing H5 specific antibody responses in SPF chickens. pcDNA3.1/H5 and pcDNA3.1/H5-HSP70 were administered to 10 days old SPF chickens in three doses of 100 µg by the intramuscular route, two weeks apart. Chickens were bled every week and H5 specific antibody was measured using hemagglutination inhibition (HI) test. The ability of the constructed plasmids in inducing the expression of H5 and H5-HSP70, respectively, in chickens was examined by RT-PCR. *In vivo* expression was confirmed based on detection of H5 RNA transcripts in muscle and spleen of chickens inoculated with the constructed DNA vaccines. The HI test was carried out using H5 antigen from a low pathogenic avian influenza virus (LPAIV), A/Duck/Malaysia/8443/2004 (H5N2). Sequence analysis of H5 genes of H5N1 and H5N2, respectively, that was used in this study showed nucleotide and amino acid identity of more than 87%. In addition, all the chickens immunized with

pcDNA3.1/H5 and pcDNA3.1/H5-HSP70 showed HI titer in week three after the first immunization. The HI titer was more prominent from first booster onwards in the chickens immunized with pcDNA3.1/H5-HSP70. This study demonstrated that chickens immunized with HSP70 based H5 DNA vaccine developed higher antibody titer compared to chickens immunized with H5 alone. However, the increase in HI antibody titer was not significantly different ($P > 0.05$). As expected, the control chickens inoculated with pcDNA3.1/HSP70 and pcDNA3.1 showed no evidence of HI antibody responses. In conclusion, we have demonstrated for the first time that HSP70-based H5 DNA can improve the induction of humoral immune response in chickens and is a promising candidate of DNA vaccine for AIV infection. Further studies are required to explore the role of HSP70 as genetic adjuvant for DNA vaccine in chickens.

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

Peningkatan gerak balas antibodi dalam ayam yang divaksin dengan konstruk plasmid DNA gen H5 virus selesema burung yang dicantum dengan HSP70 *Mikobakterium tuberkulosis*

Oleh

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Mei 2009

Pengerusi: Profesor Madya Dr. Abdul Rahman Omar, PhD

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Pada masa ini, rantau ini masih sedang memerangi virus H5N1 iaitu virus selesema burung yang sangat patogenik (HPAI) dan virus tersebut telah diasingkan daripada burung bukan unggas di pelbagai negara timur tengah dan juga di benua Eropah mahupun Afrika. Perkembangan ini telah mencetuskan ketakutan global berkenaan penyakit pandemik selesema. Penerimaan polisi menggunakan vaksin sama ada untuk mengawal atau mencegah jangkitan di kalangan unggas, pada lazimnya tidak digalakkan. Namun demikian, adalah perlu untuk meningkatkan tahap pembasmian jangkitan agar dapat mengurangkan penyebaran yang lebih teruk dan mengelakkan kerugian ekonomi yang lebih besar, dan kemajuan dalam bidang teknologi vaksin

moden, telah membantu penilaian semula potensi penggunaan vaksinasi dalam unggas sebagai kaedah tambahan bagi strategi pengawalan penyakit yang komprehensif. Dengan ini, beberapa vaksin adalah tersedia dan sesetengahnya telah pun diuji secara eksperimen dan/atau telah digunakan dalam ladang-ladang komersial. Vaksin DNA merupakan satu pendekatan yang efektif untuk mencetuskan keimunan sel dan humor yang spesifik terhadap antigen. Walau bagaimanapun, keamatan imun yang rendah dalam percubaan klinikal telah membatas penggunaan vaksin DNA. Protein Heat Shock (HSPs) atau protein tekanan merupakan molekul terpelihara tinggi yang bertindak sebagai caperon. Antara HSPs, keluarga HSP70 merupakan protein yang telah dicirikan secara mendalam dan menunjukkan efek adjuvan yang kuat pada gerak balas imun inat dan terperoleh. Dalam kajian ini, kami telah membangunkan vaksin DNA berasaskan pada gen H5, dan meningkatkan potensi vaksin DNA dengan menggunakan gen HSP70 *Mikobakterium tuberkulosis* sebagai adjuvan. Dengan ini, beberapa plasmid DNA yang mengekodkan H5 dan NP daripada virus H5NI Malaysia (A/Ck/Malaysia/5858/2004) telah dibangunkan dan kemudian dicantumkan dengan HSP70. Plasmid rekombinan berasaskan H5, NP, H5-HSP70 dan NP-HSP70 diuji dalam sel Vero. Penyelidikan lanjut dilakukan ke atas kebolehan vaksin DNA yang dibangunkan untuk mencetuskan gerak balas antibodi khusus H5 dalam ayam SPF. pcDNA3.1/H5 dan pcDNA3.1/H5-HSP70 kemudiannya disuntik ke dalam ayam SPF berusia 10 hari dalam tiga dos 100 µg melalui cara intraotot, dua minggu berasingan. Darah ayam dikumpulkan setiap minggu dan antibodi spesifik H5 telah diukur menggunakan ujian rencatan hemagglutination (HI). Keupayaan plasmid yang telah dibangunkan untuk mengaruh ekspresi H5 dan HSP70 masing-masing, di dalam ayam telah diuji menggunakan RT-PCR. Ekspresi *in vitro* telah disahkan berdasarkan pada pengesanan transkrip

RNA H5 di dalam otot dan limpa ayam yang telah disuntik dengan menggunakan vaksin DNA yang telah dibangunkan. Ujian HI telah dijalankan menggunakan antigen H5 daripada AIV patogenik rendah, A/Duck/Malaysia/8443/2004 (H5N2). Analisis jujukan bagi gen H5 untuk H5NI dan H5N2, masing-masing, yang mana telah digunakan dalam kajian ini telah menunjukkan identiti nukleotide dan asid amino lebih daripada 87%. Tambahan pula, ke semua ayam yang telah disuntik dengan pcDNA3.1/H5 dan pcDNA3.1/H5-HSP70 telah menunjukkan titer HI dalam masa tiga minggu selepas imunisasi pertama. Titer HI adalah lebih ketara selepas suntikan penggalak pertama dalam ayam yang diimunkan dengan pcDNA3.1/H5-HSP70. Kajian ini menunjukkan ayam yang telah diimunkan dengan vaksin DNA H5 berasaskan HSP70 menghasilkan titer antibodi yang lebih tinggi berbanding dengan ayam yang diimunkan dengan H5 sahaja. Walau bagaimanapun, peningkatan dalam titer antibodi HI tidak menunjukkan perubahan yang signifikan. Seperti yang telah dijangkakan, ayam-ayam kawalan yang telah disuntik dengan pcDNA3.1/HSP70 dan pcDNA3.1 tidak menunjukkan gerak balas antibodi HI. Kesimpulannya, kajian ini telah menunjukkan buat pertama kalinya bahawa HSP70 berasaskan DNA H5 boleh meningkatkan aruhan gerak balas imun humor di dalam ayam dan merupakan calon vaksin DNA yang berpotensi untuk jangkitan AIV. Kajian yang lebih lanjut diperlukan untuk mengetahui peranan HSP70 sebagai adjuvan genetik untuk DNA vaksin di dalam ayam.

ACKNOWLEDGMENTS

I deeply appreciate all the wonderful people who have contributed significantly throughout the whole course of my study. I am also indebted to all of them for their help and support.

First and foremost, I would like to sincerely acknowledge my supervisors: **Assoc. Prof. Dr. Abdul Rahman Omar,** for his valuable guidance, suggestion, helpful advice and never-ending patience throughout my studies and giving me an opportunity to learn in his laboratory; **Prof. Datin Paduka Dr. Aini Ideris** for her thoughtful comments and helpful hints.

I am also grateful to all the colleagues and staff in the Biologic Laboratory and Molecular Biomedicine Laboratory. Jia Ling Koh, Babak Jalilian, Siti Khadijah Bt. Mohamad, Roszarina Rahmad, Farid Jalilian, Tan Ching Giap, Hassan Moein, Norhaszalina Md. Isa, Nancy Lew Woan Charn, Norhafiza Azwa Ghozali, Madam Ong, Tan Sheau Wei, Lim Kian Lam for their help in completion of my experiment and making my time in laboratory as joyable and pleasant.

I certify that an Examination Committee has met on 7th of May 2009 to conduct the final examination of Mehdi Rasoli Pirozyan on his Master of Science thesis entitled "Enhancement of Antibody Responses in Chickens Vaccinated with DNA Plasmid Constructs of Avian Influenza H5 Gene Fused with HSP70 Gene of *Mycobacterium Tuberculosis*" in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I here 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.

_____

Mehdi Rasoli Pirozyan

Date:

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
Ab	Antibody
AIV	Avian Influenza Virus
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CaCl	Calcium chloride
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon dioxide
°C	Degree Celsius
DNA	Deoxyribonucleic Acid
ddH_2O	Double Distilled Water
dNTP	Deoxynucleotide Triphosphate
FBS	Fetal bovine serum
g	Gram
H	Hour
HCl	Hydrochloric Acid
HSP	Heat Shock Protein
i.e.	In example
Kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria-Bertani
M	Molar

MA	Monoclonal Antibody
mg	Milligram
Mg_2Cl	Magnesium Chloride
min	Minute
mins	Minutes
ml	Milliliter
mM	Millimolar
NaCl	Sodium Chloride
NCBI	National Center of Biotechnology Information
µg	Microgram
µM	Micromolar
ng	Nanogram
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Puissance hydrogen (Hydrogen-ion concentration)
ρg	Picogram
ρmole	Picomole
RE	Restriction Endonuclease
RNA	Ribonucleic Acid
rpm	Rotation per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse Transcriptase
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis
Secs	Seconds
SPF	Specific-Pathogen-Free

T	Temperature
TAE	Tris-Acetate-EDTA
Taq	Thermus aquaticus
Tm	Melting Temperature
TAE	Tris-Acetate-EDTA Buffer
TEMED	*N,N,N,'N*-tetramethylethlenediamine
Tris	2-amino-2(hydroxymethy)-1,3 propandiol
ul	Microlitre
UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
w/v	Weight/Volume
v/v	Volume/Volume

CHAPTER 1

INTRODUCTION

Global influenza pandemics have appeared throughout history. During the 1918 pandemic, H1N1 influenza A virus killed 100 million people worldwide which was referred to as Spanish flu. Less destructive pandemics have occurred in 1957 (H2N2) and 1968 (H3N2) and more recently in 1997, Hong Kong residents were infected with an avian influenza A viruses (H5N1). This occurrence of 'bird flu' has reminded the scientists of the continuous threat of emerging influenza virus. Avian influenza viruses are the key to the emergence of human influenza pandemics. Epizootics of avian influenza A (H5N1) virus which is highly pathogenic for poultry and wild birds has crossed the species barrier to infect human in Southeast Asia and several other countries in Middle East region and African continent thus represents an increase in the threat of pandemic (Chen, 2002; Chotpitayasunondh *et al.,* 2005).

The ideal way to fight against new influenza viruses in human is to inhibit or reduce the probability of interspecies transfer. The best strategy is to eradicate all the flocks diagnosed with Avian Influenza (AI). This approach was successful in Hong Kong in 1997 and in Netherlands in 2003. The culling of infected birds will decrease the viral load and chance of transmission to human. This strategy to eliminate all the infected birds sometime is not possible and vaccination of the poultry is an alternative option. Routinely the egg grown inactivated influenza vaccine is used for vaccination of human but the problem arises in production of highly pathogenic H5 and H7 subtypes. For handling these viruses, high level biosecurity facilities are needed. Furthermore, propagation of this virus failed to obtain high yields of virus in

embryonated chickens eggs (Rowe, 2000; Wood, 2001; Zambon, 1998). With recent advances in plasmid based reverse genetic technology, now scientists are able to manipulate H5 and H7 viruses (Kodihalli *et al.,* 2000). Currently various vaccines against H5N1 such as fowlpox based vaccine (Boyle *et al.,* 2000), recombinant H5 vaccine from baculovirus (Johansson 1999; Crawford *et al.*, 1999)), DNA plasmid based vaccine (Ulmer *et al.,* 1993) and reverse genetic H5N1 vaccine (Govorkova *et al.,* 2006) are available for commercial application and/or experimental testing in chickens. These vaccines are able to induce various degree of protection against challenge with lethal H5N1. However, the protective immunity was not complete. Hence, continuous studies are needed to develop better vaccines.

The primary goal of vaccination is to generate immunity in the host against invading pathogens or other pathological processes. Presentation of antigens to the host's immune system induces both humoral and cellular responses which leads to critical memory control cells. The type of response is often enhanced by including adjuvants in the immunization protocol. Adjuvants play an important role in boosting the immune response, and also in directing it toward a specific type of response. The most commonly used adjuvant includes aluminium hydroxide, Freund's adjuvants, mineral oil or components of mycobacterial cell wall. Some of the existing vaccines do not induce complete protection. Therefore, the development of effective vaccine against influenza, as well as improvement of efficacy and safety of existing vaccine is required (Marx *et al.,* 1993; Douse *et al.,* 1995). The use of DNA vaccines has been a novel option in recent years as a safe and effective means of controlling a number of infectious diseases. Compared with traditional vaccine, DNA vaccines have advantages of being inexpensive and simple to produce and they do not need