UNIVERSITI PUTRA MALAYSIA

EFFECT OF EARLY SECRETED ANTIGEN TARGET-6 GENE OF *MYCOBACTERIUM TUBERCULOSIS* AS GENETIC ADJUVANT FOR AVIAN INFLUENZA VIRUS DNA VACCINE IN CHICKENS

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IB 2009 4

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By

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

May 2009

Abstract of dissertation presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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May 2009

Chairman: Professor Abdul Rahman Omar, PhD

Institute: Institute of Bioscience

Influenza virus, belongs to the family *Orthomyxoviridae* and genus *Influenza virus A*, causes major disease problems and serious economical losses in poultry industry. Highly pathogenic avian influenza H5N1 subtype which is associated with acute infection with high morbidity and mortality in susceptible birds, is still enzootic in poultry in Asia as well as European and African countries. The virus may also possess serious threat to the emergence of influenza pandemic in humans. Vaccination is one of the biosafety measures which has the greatest impact on improving global health and preventing morbidity and mortality due to avian influenza (AI) infection. The explosion of knowledge in molecular immunology has paved radical developments in vaccine technology. Immunization with DNA vaccines and genetic adjuvants as immunostimulators is an attractive approach in the development of future generations of vaccines and adjuvants. The viral envelope proteins, hemagglutinin (HA or H) and neuraminidase (NA or N), have been shown to play key roles in triggering protective

immune responses against AI infection. Meanwhile, nucleocapsid protein (NP) may play a central role in cross protection between AI virus serotypes. The *Mycobacterium tuberculosis* Early Secreted Antigenic Target-6 (ESAT-6) antigen has been shown to elicit both humoral and cellular immunity, thus it has an ability to act as a genetic adjuvant. This study examined the ability of ESAT-6 to modulate antibody response against H5 following vaccination with DNA vaccine in chickens. In order to study the immunological properties of AIV DNA vaccines, several recombinant plasmids pcDNA3.1/H5, pcDNA3.1/N1, pcDNA3.1/NP, pcDNA3.1/H5-ESAT6, pcDNA3.1/N1-ESAT6 and pcDNA3.1/NP-ESAT6 were constructed. The recombinant plasmid constructs were confirmed by restriction enzymes and sequence analyses. The expression of genes of interest in cell culture was confirmed by immunofluorescence test and Western blot analysis. The immunogenicity of the DNA vaccine pcDNA3.1./H5 with and without the presence of ESAT-6 in specific-pathogen-free (SPF) chicks was determined. Sera obtained from the chickens immunized with pcDNA3.1/H5 and pcDNA3.1/H5-ESAT6 demonstrated viral neutralizing activities based on haemagglutination inhibition (HI) test. The sera collected from chicks immunized with pcDNA3.1/H5-ESAT6 have higher HI titer compared to the group which was immunized with pcDNA3.1/H5. However, the increase in HI titer at different post immunization days between these groups was not statistically significant. When the tissue samples from the chest muscle of injection site and spleen from chickens immunized with the DNA vaccine were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR), all the samples were positive for H5 specific transcripts. In summary, the current study delineated that the constructed recombinant plasmids were

transcriptionally active in the *in vivo* chicken model and DNA immunization in SPF chicks with pcDNA3.1/H5 and pcDNA3.1/H5-ESAT6 produced humoral immune response. In conclusion, future studies are required to explore the role of ESAT-6 gene of *Mycobacterium tuberculosis* as an effective genetic adjuvant for H5 DNA vaccine in chickens.

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

KESAN GEN ESAT-6 *MIKROBAKTERIUM TUBERKULOSIS* SEBAGAI ADJUVAN GENETIK KE ATAS VAKSIN DNA VIRUS SELESEMA BURUNG DALAM AYAM

Oleh

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Virus selesema tergolong dalam famili *Orthomyxoviridae* dan genus *Influenza virus A*, adalah penyebab masalah penyakit major dan juga kerugian serius dalam industri ayam. Selesema burung yang amat patogenik subtip H5N1 yang menyebabkan jangkit akut dengan kadar jangkitan dan kematian yang tinggi bagi burung boleh dijangkiti, masih enzotik dalam ayam di Asia begitu juga di Eropah dan Afrika. Virus ini mempunyai ancaman yang besar dalam penjelmaan pandemik selesema dalam manusia. Vaksinasi adalah salah satu langkah biosekuriti yang mempunyai impak yang ketara dalam meningkatkan tahap kesihatan global dan pencegahan jangkitan dan kematian yang berkaitan dengan selesema burung (AI). Perkembangan dalam pengetahuan imunologi molekul telah mengorak pembangunan radikal dalam teknologi vaksin. Imunisasi menggunakan vaksin DNA dan adjuvan genetik sebagai perangsang keimunan merupakan satu pendekatan yang menarik dalam pembangunan ke depan generasi vaksin dan adjuvan.

Protein envelop virus, Hemagglutinin (HA atau H) dan neuraminidase (NA atau N) telah memainkan peranan utama dalam mencetus gerak balas imun pelindung terhadap jangkitan AI. Sementara itu, protein nukleokapsid (NP) memainkan peranan sentral dalam perlindungan silang antara serotip virus AI. Antigen ESAT-6 *Mikrobakterium tuberkulosis* berupaya merangsang keimunan humor dan sel seterusnya berkebolehan bertindak sebagai adjuvan genetik. Kajian ini menerangkan kebolehan ESAT-6 untuk mengawal gerak balas antibodi terhadap H5 selepas vaksinasi dengan vaksin DNA dalam ayam. Bagi mengkaji ciri keimunan vaksin DNA AIV, plasmid rekombinan berikut telah dihasilkan, pcDNA3.1/H5, pcDNA3.1/N1, pcDNA3.1/NP, pcDNA3.1/H5-ESAT6, pcDNA3.1/N1-ESAT6 dan pcDNA3.1/NP-ESAT6. Pembinaan plasmid rekombinan disahkan dengan penghadaman oleh enzim pembatas dan analisis jujukan. Ekspresi gen pilihan dalam kultur sel telah disahkan dengan ujian antibodi imunopendarfluoran dan analisis penompokan Western. Tahap keimunan vaksin DNA pcDNA3.1./H5 sama ada dengan kehadiran ESAT-6 atau tidak dalam ayam bebas-patogen-spesifik adalah ditentukan. Serum yang diperoleh daripada ayam yang telah disuntik dengan pcDNA3.1/H5 dan pcDNA3.1/H5 telah menghasilkan aktiviti peneutralan virus menerusi ujian perencatan hemagglutinin (HI). Sera yang dikumpulkan dari ayam yang disuntik dengan pcDNA3.1/H5-ESAT6 menunjukkan dengan jelas titer HI yang tinggi dibandingkan dengan kumpulan yang disuntik dengan pcDNA3.1/H5. Namun, peningkatan pada titer HI tersebut adalah tidak signifikan secara statistik. Apabila sampel tisu daripada tapak suntikan pada otot dada dan limpa daripada ayam yang telah diimunkan dengan vaksin DNA dianalisis menggunakan tindak balas rantai polimerase transkriptase membalik (RT-PCR), kesemua sampel adalah positif bagi transkrip

spesifik H5. Sebagai rumusan, kajian ini menerangkan bahawa plasmid rekombinan yang dibina adalah aktif dalam menghasilkan transkrip secara *in vivo* dalam model ayam dan pengimunan ayam SPF dengan pcDNA3.1/H5 dan pcDNA3.1/H5-ESAT6 telah menunjukkan penghasilan tindak balas antibodi. Kesimpulannya, kajian lanjut perlu dilaksanakan bagi mengkaji peranan gen ESAT-6 *Mikrobakterium tuberkulosis* sebagai adjuvan genetik yang efektif bagi vaksin DNA H5 dalam ayam.

ACKNOWLEDGMENTS

All the glories and thanksgiving to God almighty in the highest

My deepest gratitude to my supervisors, Prof. Dr. Abdul Rahman Omar, Prof. Datin Dr. Khatijah Mohd Yusoff and Dr. Fatemeh Jahanshiri for their valuable guidance, help and patience throughout the project, and for the critical review in the completion of this thesis.

I appreciate the friendship and assistance from all my labmates: Erin, Kah Fai and Faried in Virology Laboratory of the Department of Biochemistry and Microbiology and also Zizu, Siti, Hid and Lim in Biologics Laboratory, Faculty of Veterinary Medicine. I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis.

The members of the Veterinary Research institute who collaborated with my project and have welcomed me sincerely. I would like to express my gratitude to Dr. Sharifah Syed Hassan, Dr. Maizan Mohamed and Madam Ong.

Also I should be thankful to the chicks which have been involved in my project, without them none of this would have been possible.

Last but not least I would like to express my sincere gratefulness to my dear Mommy, Daddy and Shaghayegh which without their endless love, moral support and encouragement I would have never been able to complete this important step of my life. Also I would like to specially thank my grandparents, uncle Ali and aunty Soheila for their endless love, prayers and blessings. Dear Kaveh, thank you for your entire well wishes, kindness and encouragement which help me to be more patient and strong.

Without all of you, it would not be possible for me to complete my project and thesis. I wish God bless you all.

I certify that an Examination Committee met on 15 May 2009 to conduct the final examination of Sara Oveissi on her Master of Science thesis entitled "Effect of Early Secreted Antigenic Target-6 Gene of *Mycobacterium tuberculosis* as Genetic Adjuvant for Avian Influenza Virus DNA Vaccine in Chickens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that 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 and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DEDICATION

With all the gratefulness, I would like to dedicate this dissertation and my Master work to my beloved Mommy, Daddy and Sister. Your unconditional, unabated love and support that I have received all through my life mean more to me than I can say. I am so blessed to have you in my life.

Also to all the birds that remain to be the unwilling hosts of avian influenza virus, I wish their pure soul shine more than ever.

SARA OVEISSI

Date: 21 May 2009

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AI	avian influenza
AIV	avian influenza virus
AP	alkaline phosphatase
APS	ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolylphosphate
bp	base pair
cDNA	complementary DNA, made by conversion of (viral) RNA into DNA by reverse transcription
CHO cell	chinese hamster ovary cell line
CMV	cytomegalovirus
ºC	degrees Celsius
dH_2O	distilled water
DIF	direct immunofluorescence
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESAT-6	early secreted antigenic target 6-kDa
FAO	Food and Agriculture Organization

FITC	fluorescein isothiocyanate
FPV	fowlpox virus
g	g (gravity) force
GFP	green fluorescent protein
H5, H7	examples of haemagglutinin subtypes of influenza A viruses
HA	haemagglutinin
HI	haemagglutination inhibition
HPAI	highly pathogenic avian influenza
hr	hour
HRP	horseradish peroxidase
ICTV	international committee on the taxonomy of viruses
IFN-γ	gamma interferon
IgA	immunoglobulins of isotype A
IgG	immunoglobulins of isotype G
IgM	immunoglobulins of isotype M
IgY	immunoglobulins of isotype Y
iIFA	indirect immunofluorescent antibody test
i.m.	intramuscular
kb	kilo base
kDa	kilo dalton
LB	Luria Bertani
LPAI	low pathogenic avian influenza
µg	microgram

µl	microliter
µM	micromolar
M	matrix (protein)
mA	miliampere
MCS	multiple cloning site
min	minute
MHC	major histocompatability complex
mRNA	messenger RNA
N1, N7	examples of neuraminidase subtypes of influenza A viruses
NA	neuraminidase
NBT	nitro blue tetrazolium
nm	nanometer
NP	nucleoprotein
NSP	non-structural protein
OD_x	optical density (or absorbance) at x nm
OIE	office of International des Epizooties
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PH	*puissance hydrogene*
PCR	polymerase chain reaction
RBC	red blood cell
RE	restriction enzyme
RNA	ribonucleic acid, viral genome substrate

RNase	ribonuclease
RNP	ribonucleoprotein
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyle sulphate
SPF	specific pathogen free
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
TEMED	tetramethyl ethylenediamine
U	unit
uv	ultraviolet
Vol	volume
WHO	World Health Organization
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1.1 Overview

Influenza is a highly contagious and re-emerging disease that has burdened humans and animals since ancient times. According to the influenza virus inherent ability to infect a broad range of animal hosts and also their vast avian reservoirs, influenza continues to represent one of the most serious health and economic threats to humans' worldwide (Dubovi *et al*, 2004; Webster *et al*, 1992). Avian influenza is widely monitored in domestic poultry including chickens, turkeys, quails, domestic ducks, ratites and commercially-raised birds (WHO, 2008). The disease can result in severe economic loss if not detected immediately. If detected, depopulation of the affected and exposed flock and neighboring farms is carried out to contain the virus and prevent the spread of the disease. Depopulating or culling is the most economical and straightforward method of controlling the virus but this kind of measure is not possible in a wildlife setting (review in ECLAC, 2005-2006). For this reason, prevention and control of avian influenza in poultry industry are the two most critical biosafety measures in combination with surveillance. Influenza A viruses infecting poultry flocks are divided into two groups based on their apparent pathogenicity: high pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). HPAI viruses are composed of H5 and H7 hemagglutinin subtypes and may result in flock mortality as high as 100%. LPAI viruses

belong to any hemagglutinin subtype and usually present as a much milder respiratory disease, causing depression and decrease in egg production, but in conjunction with secondary viral and/or bacterial infections or poor environmental conditions can cause severe disease with high mortality (Kahn 2005, Lee and Suarez, 2005).

In Peninsular Malaysia, the poultry industry has developed to a most scientifically advanced industry and contributes to more than 60% of the total value of livestock. In order to maintain and increase the productivity of poultry products, both vaccination and therapeutic or prophylactic use of drugs play an important role in animal disease control. Several LPAI viruses of the H4N3, H4N6, H3N6 and H9N2 subtypes in domestic duck (Jasbir *et al*., 1999; Aini and Ibrahim 1986) and in passerine birds (Ibrahim *et al*., 1990) have been reported in Malaysia. Recently, a LPAI virus, subtype H5N2, was identified in ducks exported from a farm in Perak State, Malaysia to Singapore (FAO, 2004). The first case of the HPAI virus, subtype H5N1 was reported in two free-range chickens in a flock of approximately 60 birds located in the state of Kelantan, Malaysia near the Thailand border, in 19 August 2004 (OIE Country Report). A fresh case of H5N1 was reported in a flock of free range poultry in 19 February 2006 and the most recent case of H5N1 in village chickens in January of 2007 (WHO, 2008). This frequent occurrence of AI infection in recent years has made vaccination a necessity.

Currently two types of vaccines are in use: killed and live virus vaccines. The killed vaccines can be divided into whole virus vaccines which were the first to be developed