



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**

SARA OVEISSI

IB 2009 4

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

By

SARA OVEISSI

**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

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

By

Sara Oveissi

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

Sara Oveissi

Mei 2009

Pengerusi : Professor Abdul Rahman Omar, PhD

Institut : Institut Biosains

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 subtipe H5N1 yang menyebabkan jangkitan 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 tuberculosis* 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 penompakan 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 tuberculosis* 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:

Rasedee Adbullah, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Daud Ahmad Israf, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Syahrilnizam Abdullah, PhD

Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Ishak Mat, PhD

Associate Professor
Advanced Medical and Dental Institute
University Science Malaysia
(Member)

BUJANG KIM HUAT, PhD

Professor and Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia.

Date:



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:

Abdul Rahman Omar, PhD

Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Datin Khatijah Mohd. Yusoff, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Fatemeh Jahanshiri, PhD

Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD

Professor and Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia.
Date: 9 July 2009



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



TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	viii
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xx
CHAPTER	
1. INTRODUCTION	1
1.1 Overview	1
2. LITERATURE REVIEW	5
2.1 Avian influenza virus	5
2.1.1 Virus classification	5
2.1.2 Virus structure	6
2.1.3 Avian influenza virus proteins	9
2.1.4 Replication cycle	11
2.1.5 Epidemiology and pathogenesis in birds	14
2.1.6 Avian influenza viruses of high or low pathogenicity	14
2.2 Host immune response to avian influenza virus	15
2.3 Avian influenza vaccines	16
2.3.1 Conventional vaccines	17
2.3.2 Experimental and future vaccines	19
2.4 Genetic adjuvant	22
2.5 The Early Secreted Antigenic Target 6-kDa (ESAT-6)	22
3. MATERIALS AND METHODS	24
3.1 Molecular cloning methods	24
3.1.1 General procedures	24
3.1.2 Construction of recombinant plasmids	25
3.1.3 PCR amplification of H5, N1, NP and ESAT-6 genes	26
3.1.4 Directional ligation of H5, N1 and NP genes into pcDNA3.1 (+)	30
3.1.5 Fusion of the ESAT-6 gene with constructed recombinant plasmids	30
3.1.6 Transformation of <i>Escherichia coli</i>	31
3.1.7 Identification of recombinant plasmids	32
3.2 <i>In vitro</i> transcription and translation	38
3.2.1 General procedures	38



3.2.2	Overview of Vero cell line	38
3.2.3	Overview of CHO cell line	39
3.2.4	Transient transfection of adherent mammalian cells	39
3.3	Identification of recombinant proteins	42
3.3.1	Indirect Immunofluorescence	42
3.3.2	Western Blot	43
3.4	Immunization study	47
3.4.1	The procurement of Endotoxin-free plasmids	47
3.4.2	Immunization of the specific-pathogen-free (SPF) chicks	49
3.5	Haemagglutination Inhibition (HI) Assay	51
3.6	Reverse Transcription-Polymerase Chain Reaction (RT-PCR)	52
3.6.1	Extraction of mRNA from chicken tissue	52
3.6.2	mRNA-RT-PCR analysis	52
4.	RESULTS	56
4.1	Amplification and cloning of the H5, N1 and NP into the pcDNA3.1 (+) mammalian expression vector	56
4.2	Construction of the recombinant pcDNA3.1/H5-ESAT6, pcDNA3.1/NP-ESAT6 and pcDNA3.1/N1-ESAT6	61
4.3	Optimization of transient transfection of adherent mammalian cells	66
4.4	<i>In vitro</i> expression of the recombinant plasmids in Vero cell line with Lipofectamine 2000 TM	71
4.4.1	Western Blot analysis of the recombinant proteins	71
4.4.2	Indirect Immunofluorescence analysis of the recombinant proteins	79
4.5	Immunogenicity evaluation of DNA vaccine in SPF Chicks	86
4.5.1	Large scale plasmid extraction and purification	86
4.6	Haemagglutination Inhibition (HI) Assay	88
4.7	Reverse Transcription-Polymerase Chain Reaction (RT-PCR)	92
5.	DISCUSSION	98
5.1	Cloning of the H5, N1 and NP genes into the pcDNA3.1 (+) mammalian expression vector	100
5.2	Fusing of the ESAT-6 gene of <i>Mycobacterium tuberculosis</i> into the downstream of the constructed recombinant plasmids	101
5.3	<i>In vitro</i> cell culture studies	101
5.4	<i>In vivo</i> studies	102
6.	CONCLUSION	104
	REFERENCES	106
	APPENDICES	121
	BIODATA OF STUDENT	125

LIST OF TABLES

Table		Page
3.1	Oligonucleotide primers used in amplification of full length H5, N1 and NP genes.	28
3.2	Oligonucleotide primers used in amplification of ESAT-6 gene for cloning into recombinant vectors; pcDNA3.1/H5, pcDNA3.1/N1 and pcDNA3.1/NP.	29
3.3	Restriction enzymes and reaction buffers.	35
3.4	Oligonucleotide primers used in sequencing of the H5, N1, NP and ESAT-6 genes in pcDNA3.1/H5, pcDNA3.1/H5-Esat6, pcDNA3.1/N1, pcDNA3.1/N1-Esat6, pcDNA3.1/NP and pcDNA3.1/NP-Esat6 recombinant plasmids.	37
3.5	Plasmid DNAs used for immunization in SPF chickens.	50
3.6	Oligonucleotides used to amplify the H5 gene.	53
3.7	RT-PCR reaction mix used in the amplification of H5 gene	54
4.1	Haemagglutination Inhibition (HI) test on the control groups.	89
4.2	Haemagglutination Inhibition (HI) test on the vaccinated SPF chicken sera using pcDNA3.1/H5 and pcDNA3.1/H5-Esat6 recombinant plasmids.	90

LIST OF FIGURES

Figure		Page
1.1	Structure of the influenza A virus particle.	8
1.2	Illustration of the influenza A virus replication cycle.	13
4.1	Agarose gel electrophoresis of PCR products of AIV strain A/chicken/Malaysia/5744/2004(H5N1) H5, NP and N1 genes.	57
4.2	Agarose gel electrophoresis of single and double digested pcDNA3.1/H5 and PCR products of H5 gene.	58
4.3	Agarose gel electrophoresis of digested pcDNA3.1/N1 recombinant plasmid and PCR products of N1 gene.	59
4.4	Agarose gel electrophoresis of single and double digested of pcDNA3.1/NP5 and PCR products of NP gene.	60
4.5	Agarose gel electrophoresis of PCR products of <i>Mycobacterium tuberculosis</i> ESAT-6 gene.	62
4.6	Agarose gel electrophoresis of digested products of pcDNA3.1/H5/ESAT6 recombinant plasmid and PCR products of H5 and ESAT-6 genes.	63
4.7	Agarose gel electrophoresis of digested products of pcDNA3.1/N1/ESAT6 recombinant plasmid and PCR products of N1 and ESAT-6 genes.	64
4.8	Agarose gel electrophoresis of single digested of pcDNA3.1/NP/ESAT6 and PCR products of NP and ESAT-6 genes.	65
4.9	Linearization of the PEGFP-N2 plasmids.	68
4.10	Investigation of the efficiency of different transfection parameters on the expression of Green Fluorescent Protein (GFP).	69
4.11	SDS-PAGE and Western blot analysis of Vero and CHO cells transfected with pEGFP-N2 through FuGENE® HD and Lipofectamine 2000™ transfection reagents.	70
4.12	Western blot analysis of the H5 protein with anti-hemagglutinin polyclonal antibody.	73

4.13	Western blot analysis of the N1 protein with anti-neuraminidase polyclonal antibody.	74
4.14	Western blot analysis of the NP protein with anti-nucleocapsid polyclonal antibody.	75
4.15	Western blot analysis of the H5-ESAT6 protein with anti-hemagglutinin polyclonal antibody.	76
4.16	Western blot analysis of the N1-ESAT6 recombinant protein with anti-neuraminidase polyclonal antibody.	77
4.17	Western blot analysis of the NP-ESAT6 protein with anti-nucleocapsid polyclonal antibody.	78
4.18	Transient transfection of Vero cells with pcDNA3.1/H5 recombinant plasmid.	80
4.19	Detection of recombinant H5-ESAT6 protein by fluorescent microscopy.	81
4.20	Transient transfection of Vero cells with pcDNA3.1/N1 recombinant plasmid.	82
4.21	Transient transfection of Vero cells with pcDNA3.1/N1-ESAT6 recombinant plasmid.	83
4.22	Transient transfection of Vero cells with pcDNA3.1/NP recombinant plasmid.	84
4.23	Transient transfection of Vero Cells with pcDNA3.1/NP-ESAT6 recombinant plasmid.	85
4.24	Agarose gel analysis of the extracted and purified recombinant plasmids with EndoFree® Plasmid Purification Maxi Kit(QIAGEN, Germany) before injection.	87
4.25	Comparison of log ₂ HI titer for chicks immunized with pcDNA3.1/H5 and pcDNA3.1/H5-ESAT6.	91
4.26	RT-PCR analysis of mRNA extracted from spleen and muscle of immunized chicks with pcDNA/H5 recombinant vector.	93



4.27	RT-PCR analysis of mRNA extracted from spleen and muscle of immunized chicks with pcDNA/H5-ESAT6 recombinant vector.	94
4.28	PCR analysis of mRNA extracted from spleen and muscle of immunized chicks with pcDNA/H5 recombinant vector.	96
4.29	PCR analysis of mRNA extracted from spleen and muscle of immunized chicks with pcDNA/H5-ESAT6 recombinant vector.	97



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
°C	degrees Celsius
dH ₂ O	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
μ g	microgram

μl	microliter
μM	micromolar
M	matrix (protein)
mA	miliampere
MCS	multiple cloning site
min	minute
MHC	major histocompatibility 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	<i>puissance hydrogene</i>
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 dodecyl sulphate
SPF	specific pathogen free
TAE	Tris-acetate-EDTA buffer
<i>Taq</i>	<i>Thermus aquaticus</i>
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