UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF AN AVIAN INFLUENZA VIRUS H5N1 DNA VACCINE AND THE USE OF MDP-1 GENE OF *MYCOBACTERIUM BOVIS* AS GENETIC ADJUVANT

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By

BABAK JALILIAN

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

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SEPTEMBER 2008

CHAIRMAN: **PROFESSOR DR. ABDUL RAHMAN OMAR**

INSTITUTE: **INSTITUTE OF BIOSCIENCE**

Avian influenza (AI) virus subtype H5N1 is a highly pathogenic virus that causes acute infection with high mortality in susceptible birds. Additionally, the virus can also cause lethal infection in human. Effective control of AI requires various strategies which comprises of vaccination, biosecurity, education, diagnostics and surveillance. The crucial objectives of AI control strategies are to prevent introduction of AI, ease the losses and total eradication of AI. Immune response against the Hemagglutinin (HA) protein and the Neuraminidase (NA) protein by the immune components results in protection against AI. Currently, several conventional and genetically engineered AI vaccines using recombinant technology has been developed and tested in experimental trials. However, its application in commercial chickens has not been studied thoroughly except for conventional and fowlpox virus based vaccines. Vaccination using DNA vaccines is an attractive approach to induce vaccine-induced immunity. On the other hand, DNA vaccine is relatively less immunogenic. Furthermore, several inoculations are required to induce strong vaccine-induced immunity. Different approaches such as

adjuvants are available to enhance the immunogenicity of DNA vaccine. The objectives of this study were to construct and express the pcDNA3.1/H5, pcDNA3.1/N1 and pcDNA3.1/NP of H5N1 and to explore the adjuvancy role of Mycobacterial DNA binding Protein-1 (MDP1) in augmenting H5 DNA vaccine in inducing specific antibody response. Constructed pcDNA3.1/MDP1 plasmids encoding MDP1 was obtained from Osaka University, Japan. The complete genes of H5, N1 and NP gene of Malaysian H5N1 virus (A/Ck/Malaysia/5858/04) were cloned separately into pcDNA3.1+ vector. The orientation of the cloned fragments was verified by restriction mapping and DNA sequencing. The expression of protein of the cloned genes was evaluated by transfection of Vero cell lines followed by detection of bands of the expected sized using Western blotting analysis. The immunogenicity of the cloned H5 DNA vaccine was tested in SPF chickens. The chickens were divided into 5 groups namely H5, H5+MDP1, pcDNA3.1, PBS and negative control. The constructed plasmids were injected intramuscularly to 10 days old chickens followed by two booster injections at 14 and 28 days. Bleeding via wing vein was conducted every week post immunization and the collected sera were analyzed using HI test. The HI test showed successful antibody production second week after immunization with an increase in antibody titers during the course of experiment in group inoculated with H5 and H5+MDP1. The result showed that the constructed DNA vaccines were able to induce the production of detectable antibody titer. Furthermore, spleen and muscle samples from chickens inoculated with H5 and H5+MDP1 expressed H5 RNA transcripts. However, the higher antibody titers in chickens inoculated with H5+MDP1 was not

statistically significant when compared with chickens inoculated with H5 alone. The highest HI titers for both groups never exceeded 16 HI unit.

Abstrak tesis ini dipersembahkan kepada Senat Universiti Putra Malaysia untuk memenuhi syarat-syarat Ijazah Sarjana Sains

PEMBANGUNAN VAKSIN DNA BAGI VIRUS SELESEMA BURUNG H5N1 DAN PENGGUNAAN GEN MDP-1 *MYCOBACTERIUM BOVIS* SEBAGAI ADJUVAN GENETIK

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Virus selesema burung jenis H5N1 adalah virus yang sangat patogenik yang menyebabkan jangkitan akut dan mengakibatkan kadar kematian yang tinggi terhadap spesis burung. Virus ini juga boleh mengakibatkan jangkitan yang membawa maut kepada manusia. Bagi mengawal penyakit selesema burung secara efektif, pelbagai strategi diperlukan yang melibatkan kesepakatan dari segi vaksinasi, keselamatan biologi, pendidikan, diagnostik dan pengawasan yang rapi. Beberapa objektif penting dalam strategi mengawal penyakit selesema burung ialah mengelak permulaan wabak penyakit, mengatasi kelemahan, dan membasmi penyakit ini sepenuhnya. Tindak balas imun terhadap protein Hemagglutinin (HA) dan protein Neuraminidase (NA) oleh komponen imun dapat memberi kan perlindungan daripada penyakit tersebut. Kini, terdapat beberapa vaksin konvensional dan vaksin rekombinan yang terhasil dengan menggunakan teknologi kejuruteraan genetik yang telah diuji secara ujian makmal namun pengaplikasiannya secara komersial terhadap ayam belum lagi dilakukan secara menyeluruh kecuali bagi vaksin konvensional dan vaksin rekombinan berasaskan virus

fowlpox. Vaksinasi yang menggunakan vaksin DNA merupakan satu pendekatan menarik untuk mengaruh keimunan. Namun, vaksin DNA ini agak kurang imunogenik. Selain itu, beberapa inokulasi diperlukan untuk mengaruh keimunan yang kuat. Beberapa pendekatan berbeza seperti adjuvan boleh didapati dalam meningkatkan sifat imunogenik vaksin DNA. Objektif kajian ini adalah untuk meneroka peranan keadjuvanan Protein Pengikat DNA – 1 Mikobakteria (MDP1) dalam meningkatkan keupayaan vaksin DNA H5 menghasilkan tindak balas antibodi, Plasmid pcDNA3.1/MDP1 yang mengkod MDP1 telah diperoleh dari Universiti Osaka, Jepun. Gen lengkap H5, N1 dan NP untuk virus H5N1 dari Malaysia (A/Ck/Malaysia/5858/04) telah diklonkan secara berasingan di dalam vektor pcDNA3.1+. Kedudukan fragmen yang diklonkan telah disahkan dengan pemetaan sekatan dan penjujukan DNA. Penghasilan protein bagi gen-gen yang telah diklonkan dinilai dengan transfeksi sel Vero, diikuti dengan pengesanan jalur yang telah ditentukan saiznya menggunakan analisis Blot Western. Sifat imunogenik vaksin DNA H5 yang diklonkan diuji dalam ayam SPF. Ayam dibahagikan kepada 5 kumpulan iaitu kumpulan H5, H5+MDP1, pcDNA3.1, PBS, dan kawalan. Plasmid yang dibangunkan telah disuntik secara intraotot pada ayam yang berumur 17 hari, diikuti dengan dua suntikan penggalak pada hari ke-14 dan ke-28 selepas imunisasi pertama. . Pendarahan pada vena kepak dilakukan setiap minggu dan serum yang dikumpulkan dianalisis menggunakan Ujian HI. Ujian HI menunjukkan kejayaan penghasilan antibodi pada minggu ke-2 imunisasi dengan peningkatan jumlah titer antibodi semasa ujian dijalankan terhadap kumpulan yang diinokulat dengan H5 dan H5+MDP1. Keputusan menunjukkan bahawa vaksin DNA yang dibangunkan berupaya menghasilkan titer antibodi yang dapat dikesan.

Selain itu, sampel limpa dan otot daripada ayam-ayam yang telah diinokulat dengan H5 and H5+MDP1 menunjukkan transkrip RNA H5. Namun, jumlah titer antibodi yang tinggi daripada ayam yang diinokulat dengan H5+MDP1 tidak signifikan secara statistik jika dibandingkan dengan ayam yang diinokulat dengan H5 sahaja. Titer HI yang tertinggi daripada kedua-dua kumpulan tidak melebihi 16.

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I certify that an examination committee has met on 3 September 2009 to conduct the final examination of Babak Jalilian on his Master of Science thesis entitled "Development of an Avian Influenza Virus H5N1 DNA vaccine and the use of MDP1 gene of *Mycobacterium bovis* as genetic adjuvant" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 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 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 University Putra Malaysia or at any other institution.

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Babak Jalilian

Date: 21 December 2009

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LIST OF ABBREVIATIONS

%	Percentage
°C	Celsius temperature (centigrade temperature)
µg	Microgram
µl	Microliter
Ab	Antibody
AI	Avian influenza
AIV	Avian influenza virus
APS	Ammonium persulfate
bp	Base pair
dH_2O	Distilled water
ddH_2O	Deionized distilled water
DNA	Deoxyribonuclic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double-Stranded
EDTA	Ethlene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
G	Gram
H_20	Water
HA	Haemagglutinin
Ig	Immunoglobulin
IM	Intramuscular
Kb	Kilobase pair
kDa	Kilo Dalton
L	Liter
LB	Luria-bertani
LPS	Lipopolysaccharides
M	Molar
Mg	Milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatability complex
Ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NA	Neuraminidase
NaCl	Sodium chloride
NP	Nucleoprotein
O.D.	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Puissance hydrogen (Hydrogen-ion concentration)
RBC	Red blood cells
RNA	Ribonucleic acid

Rpm	Revolution per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	Specific-pathogen-free
Ss	single stranded
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
v/v	Volume per volume
VRI	Veterinary Research Institute
w/v	Weight per volume

Chapter 1

Introduction

Influenza virus can cause an acute, highly transmittable respiratory disease, which could result in high morbidity and mortality in both human and animals (Murphy & Webster 1996). In 1918, a pandemic of influenza H1N1 virus (Spanish flu) cause a loss of 100 million lives worldwide. More recently, the 1997 Hong Kong outbreak (H5N1) showed that avian influenza is still a potential threat to human, which is believed to be transmitted from infected birds. In that outbreak, 30% of the infected humans died, indicating that if avian influenza can be transmitted from human to human, there will be destructive consequences (Yuen *et al.* 1998, De Jong *et al.* 1997). The Hong Kong outbreak of avian influenza H5N1 was controlled by slaughtering 1.5 million chickens, which cost more than 245 million dollars (HKD) in one month. Therefore, antivirals and vaccines are prospective solutions to avoid future outbreaks of avian influenza virus. Nonetheless, logistical restrictions will prevent widespread usage of antiviral agents in pandemics regions globally.

Currently, inactivated vaccines containing HA as the main component, are the common vaccines to prevent avian influenza. However, it requires large numbers of specific-pathogen-free embryonated chicken eggs and some 6 months to propagate (De Jong *et al.* 1997). On the other hand, this is not an ideal method to produce inactivated vaccine for highly pathogenic strains, as they kill the embryo shortly after propagation and require a high level of biosecurity to handle (Voeten *et al.* 1999). Such vaccines had been successful in producing protective immunity against

infections using homologous virus but failed in preventing the outbreaks of heterologous virus (Couch & Kasel 1983, Meyer *et al.* 1978, Hoskins *et al.* 1976). Presently, various vaccines against avian influenza H5N1 virus with different level of protective immunity, such as DNA plasmid-based vaccine, baculovirus recombinant H5 vaccine, fowl pox based H5 vaccine and reverse genetic H5 vaccine have been examined experimentally and/or available commercially (Govorkova *et al.* 2006, Boyle *et al.* 2000, Kodihalli *et al.* 2000, Crawford *et al.* 1999, Ulmer *et al.* 1993).

Concurrent studies have revealed that DNA vaccines encoding HA, N1 and NP of influenza A virus can result in the development of protective immune response against influenza virus challenge in animals (Chen *et al.* 2005, Vecino *et al.* 2004, Chen *et al.* 2000, Johnson *et al.* 2000, Chen *et al.* 1999, Lunn *et al.* 1999, Kodihalli *et al.* 1999, Chen *et al.* 1998, Deck *et al.* 1997, Webster *et al.* 1994). In most cases, two or three doses of naked plasmid DNA can induce immune response to the pathogen (Davis 1997, Hinkula *et al.* 1997, Leitner *et al.* 1997, Wang *et al.* 1993). Nevertheless, other studies had shown that a single dose of DNA vaccine can trigger protective immunity, which demonstrated the high potential of DNA vaccines as an alternative to inactivated vaccines (Lodmell *et al.* 2003, Lima *et al.* 2003, Sullivan *et al.* 2003, Moraes *et al.* 2002, Konishi *et al.* 1998, Robinson *et al.* 1997).

In order to improve release of the vaccine, the delivery and presentation to the host immune system, vaccines are frequently formulated with adjuvants. Adjuvants can augment both humoral and cellular responses of the host immune system (Vogel *et al.* 2000). In general, common adjuvants in use are aluminium and calcium salts, oil

emulsions, saponin, liposomes, microparticles, cytokines, polysaccharides, immune stimulating complexes (ISCOMS) and genetic adjuvants (Aucouturier *et al.* 2001). Genetic adjuvants comprised of plasmid vectors encoding specific cytokines, stimulatory molecules or ligands that improve the host immune response to the antigen via encoding cytokines or plasmid-encoded protein-activated cytokines (Lillehoj *et al.* 2005).

Mycobacterial DNA binding protein 1 (MDP1) is a main cellular protein produced by *Mycobacterium*. It has nucleic acid binding activity and up-regulates the stationary and dormant state of *Mycobacterium bovis.* On the other hand, the protein also prevents macro-molecular bio-synthesis and therefore suppresses bacterial growth (Matsumoto *et al.* 2000). MDP1 can bind to glycosaminoglycans and laminin (Aoki *et al.* 2004). Glycosaminoglycans is an important element in the extracellular matrix as it plays a vital role in attaching mycobacteria to nonphagocytic cells, such as fibroblasts and epithelial cells (Menozzi *et al.* 1996), the latter being the main reservoirs of *M. tuberculosis* in healthy humans (Hernández-Pando *et al.* 2000). Prabhakar *et al.,* in 1998, suggested that MDP1 is useful as an immunodominant Ag with a vital influence in host defense. MDP1 can augment the production of proinflammatory cytokines through a TLR9 dependent pathway, resulting in the stimulation of cellular and humoral responses (Hemmi *et al.* 2000, Krieg *et al.* 1995, Yamamoto *et al.* 1992) and significantly influence the immune response by inducing the secretion of IFN-γ (Cooper *et al.* 1993, Flynn *et al.* 1993). Prior to this study, MDP1 had been shown to be a potential DNA vaccine adjuvant in BCG, which has a unique ability in blocking DNase activity, and consequently decreasing the amount of DNA necessary for vaccination (Matsumoto *et al.* 2005, Krieg 2002).

Immunologists have gained a large interest in the application of DNA vaccines in regulating the immune response of the host due to their numerous advantages. Hence, MDP1 may play an important role as a potential adjuvant to boost the immunotherapeutic effects of these vaccines (Klinman 2004, Halperin *et al.* 2003, Jahrsdo□rfer & Weiner 2003, Krieg 2002).

The objective of this current study is to construct eukaryotic expression vector as DNA vaccines that are able to induce immune responses against H5N1 avian influenza virus in chicken and to determine whether the response can be augmented by co-administration of MDP1 gene as a genetic adjuvant. Thus, specific objectives of this study were:

1. to construct eukaryotic DNA plasmids expressing H5, N1 and NP of Malaysian H5N1 (A/chicken/H5N1/5858/2004)
2. to express the constructed DNA plasmids in cell culture system and
3. to determine the ability of MDP1 in augmenting antibody responses in chickens following vaccination with the H5 based DNA vaccine .

CHAPTER 2

LITERATURE REVIEW

2.1 Influenza virus

2.1.1 Structure of influenza virus

The standard shape for influenza viruses is spherical and they are about 100nm in diameter but there are reports of observing them in different shapes and sizes (De jong 2000). Influenza viruses are enveloped single stranded RNA with negative polarity (ssRNA) (Geider *et al*. 1981). Influenza virus consists of Haemagglutinin (HA), Neuraminidase (NA), Nucleoprotein (NP), RNA polymerase subunits (PB1, PB2, PA), Matrix proteins (M1, M2) and Non-structural proteins (NS1, NS2) (Geider *et al.* 1981).

HA and NA are distinct surface glycoproteins, there is about 450-500 of them on each virion and they protrude about 10-14 nm out of the virions surface. For each NA there is about 4-8 HA on the surface and therefore about 25-40% of the virion's protein mass is due to HA and 5% to NA protein (De jong 2000, Geider *et al.* 1981).

PB1, PB2 and PA are three large proteins that bind to the nucleoprotein and their main role is to assist the RNA of virus to transcript and replicate. Matrix protein1 (M1) has an important role in the morphology of virus as it covers the lipid envelope

of the virus from underneath (Geider *et al.* 1981). Meanwhile, M2 is expressed in less abundance on the virion's envelope as an ion channel (Geider *et al.* 1981).

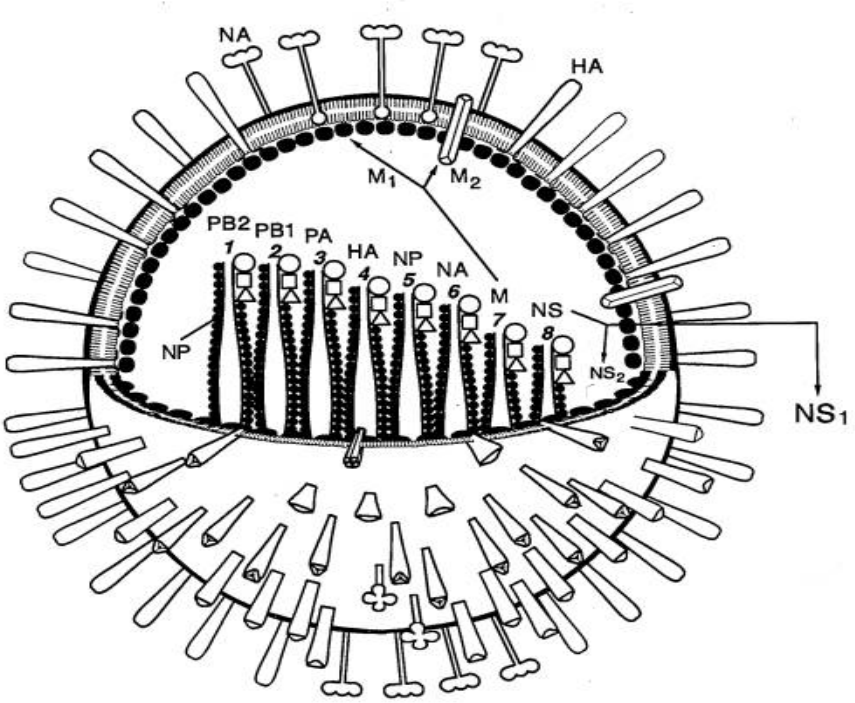

Figure 2.1: Structure of the influenza virus. The phospholipid surface has three different proteins, hemagglutinin (HA), neuraminidase (NA) and matrix protein (M2). The double layer phospholipid is from the infected host and makes the virions outer layer. The RNA segments and the rest of structural and non-structural proteins are enveloped in the bilayer lipid surface (Knipe *et al*. 2001)

2.1.2 Taxonomic classification

Influenza viruses are from the *Orthomyxoviridae* family (Wright 2002). They can be divided into three different genera called A, B and C based on two internal proteins named Nucleocapsid (NP) and Matrix proteins (M1 and M2). The different genera of influenza viruses have no cross-reactivity among themselves (Geider *et al*. 1981, Wright 2002). Sub-typing of influenza viruses is based on their surface