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

BABAK JALILIAN

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

By

BABAK JALILIAN

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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 PENGUNAAN GEN MDP-1 MYCOBACTERIUM BOVIS SEBAGAI ADJUVAN GENETIK

OLEH
BABAK JALILIAN
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PENGERUSI: PROFESOR DR. ABDUL RAHMAN OMAR
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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 pengaplikasianya secara komersial terhadap ayam belum lagi dilakukan secara menyeluruh kecuali bagi vaksin konvensional dan vaksin rekombinan berasaskan virus...
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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Last but not least, my deepest appreciation will always go to my family, my parent and my brother: Mr. Rasoul Jalilian, Mrs. Roughayeh Farahbakhshian and Dr. Hadi Jalilian for their support and sacrifices.
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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Date: 16 November 2009
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.

______________________
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
dH₂O  Distilled water
ddH₂O  Deionized distilled water
DNA  Deoxyribonucleic acid
dNTP  Deoxyribonucleotide triphosphate
ds  Double-Stranded
EDTA  Ethylene diamine tetra acetic acid
ELISA  Enzyme linked immunosorbent assay
G  Gram
H₂O  Water
HA  Haemagglutinin
Ig  Immunoglobulin
IM  Intramuscular
Kb  Kilobase pair
kDa  Kilo Dalton
L  Liter
LB  Luria-bertani
LPS  Lipopolysaccharides
M  Molar
Mg  Milligram
MgCl₂  Magnesium chloride
MHC  Major histocompatibility 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
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<td>SDS-PAGE</td>
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<td>SPF</td>
<td>Specific-pathogen-free</td>
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<td>Ss</td>
<td>single stranded</td>
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<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
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<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
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<td>v/v</td>
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<td>VRI</td>
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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) caused 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 pandemic 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).


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, Jahrsdorfer & 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