



UNIVERSITI PUTRA MALAYSIA

**DEVELOPMENT OF ORAL AVIAN INFLUENZA VIRUS SUBTYPES H5
DNA VACCINES USING ATTENUATED *SALMONELLA TYPHIMURIUM*
AND SILVER NANOPARTICLES AS CARRIERS**

SEYED DAVOUD JAZAYERI

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By

SEYED DAVOUD JAZAYERI

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**DEVELOPMENT OF ORAL AVIAN INFLUENZA VIRUS SUBTYPES H5 DNA
VACCINES USING ATTENUATED SALMONELLA TYPHIMURIUM AND SILVER
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Chairman: Professor Abdul Rahman bin Omar, PhD

Institute: Institute of Bioscience

Recent epizootics of highly contagious OIE List A diseases, such as foot and mouth disease, classical swine fever and avian influenza (AI), have led to the implementation of stamping out policies resulting in the depopulation of millions of animals. In order to avoid the destruction of large numbers of animals, the possibility of pursuing different control strategies should be considered. Hence, with the advances in modern vaccine technologies, several different vaccines are currently available against AI virus (AIV) subtype H5N1. DNA vaccines are potentially safer than other traditional vaccines, such as, the whole-killed viral vaccines, are relatively inexpensive to manufacture and store, and have the potential for simultaneous immunization against multiple antigens or pathogens. Administration of DNA vaccine has been shown to stimulate immune responses, including both humoral and cellular responses. Although DNA

vaccines offer several advantages, among the major setbacks are: the vaccine is unable to induce strong immunity and is not applicable for mass vaccination of poultry. Hence, there is a need to develop better delivery systems and/or adjuvants. In this study, *S. typhimurium* strain SV4089 and green synthesis silver nanoparticle (AgNP) were used as carriers for DNA vaccine expressing the H5 gene (pcDNA3.1/H5) of AIV A/Ck/Malaysia/5858/04 (H5N1). The safety and immunogenicity of the formulated DNA vaccines were evaluated based on cytotoxicity, humoral and cellular immune responses as well as cytokine production.

Attenuated *Salmonella enterica* sv. *typhimurium* (*S. typhimurium*) strain SV4089 is a double mutant (Dam⁻ and phoP⁻) derived from wild type *S. typhimurium* strain SL1344, was used as carrier for oral H5 DNA vaccine, pcDNA3.1/H5. Primary chick cells from 18 days specific-pathogen-free (SPF) embryo were used to determine the level of mRNA cytokine expression following *in vitro* infection with live and killed from both the attenuated and wild type *S. typhimurium* strains SV4089 and SL1344, respectively by using multiplex quantitative gene expression assay. Live *S. typhimurium* SL1344 showed strong expression of pro-inflammatory (GM-CSF, IL-1 β , TGF- β , TNFSF13B, IL-12 β , IL-6 and IL-8), Th1 (IFN- γ , IL-2, IL-15, IL-18) and Th2 (IL-4, IL-10) cytokines from infected primary chick cells. Although attenuated *S. typhimurium* SV4089 is also able to induce all the examined cytokines, the levels of expression were significantly low ranging from 1.3 to 28 fold compared to the wild type. Unlike the live wild and attenuated strains, infection of primary cells with killed strains of *Salmonella* associated with little or no induction of pro-inflammatory (IL-8), Th2 (IL-10, IL-4) and Th1 (IL-18) expression. pcDNA3.1/H5 was formulated using green synthesis of silver nanoparticles (AgNP) with polyethylene glycol (PEG). The AgNP were successfully synthesized, uniformly dispersed

with size in the range of 4 to 18 nm with an average size of 11 nm by using moderated temperature. Cytotoxicity of the prepared AgNP was evaluated *in vitro* and *in vivo* using MCF-7 cells and chicken splenic cytokine expression, respectively. At concentration of $-5 \log_{10}$ of AgNP, no cytotoxic effects were detected in MCF-7 cells with 9.5 % cell death compared to the control. One-day old SPF chicks immunized once by oral gavage with 10 μ l of pcDNA3.1/H5 (2000 ng/mL) coated with 40 μ l AgNP (3.7×10^{-2} μ g of Ag) did not show any clinical manifestations.

Attenuated *S. typhimurium* SV4089 as a potential DNA vaccine carrier in young chickens was carried out in newly hatched SPF chicks immunized once by oral gavage with 10^9 *Salmonella* colony-forming units. No deaths nor side effects were found at post-immunization with the attenuated strain. Viable bacteria were detected as soon as 3 days after inoculation by plating, fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR) from spleen, liver and cecum. Flow cytometry analysis of oral inoculated chickens against attenuated *Salmonella* on peripheral mononuclear blood cells (PBMC) did not show any significant responses on $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells. Regardless of the duration, immunized chickens with attenuated *Salmonella* strain, showed expression on IL-1 β , IL-6, TNFSF13B (pro inflammatory cytokines) and IL-15, IL-18 and IL-12 β (Th1 cytokine) although no significantly fold changes were recorded during 35 days experiment.

Serum samples collected from the intramuscular (IM) immunized group elicited low hemagglutination-inhibition (HI) titres, while chickens immunized with attenuated *S. typhimurium*/pcDNA3.1/H5 and AgNP/pcDNA3.1/H5 showed rapidly increasing antibody

against H5 on day 14 after immunization. The highest average antibody titres were detected on day 35 after immunization via IM, attenuated *S. typhimurium*/pcDNA3.1/H5 and AgNP/pcDNA3.1/H5 immunized groups, at 4.0 ± 2.8 , 51.2 ± 7.5 , and 51.2 ± 7.5 % respectively.

Attenuated *S. typhimurium*/pcDNA3.1/H5 and AgNP/pcDNA3.1/H5 also elicited both CD4⁺ and CD8⁺ T cells in immunized chickens as early as day 14 after immunization, at 20.5 ± 2.0 (CD4⁺), 22.9 ± 1.9 % (CD8⁺) and 7.5 ± 2.0 % (CD4⁺), and 20 ± 1.9 % (CD8⁺), respectively. Meanwhile, the CD4⁺ and CD8⁺ T cells in chickens vaccinated intramuscularly were low, at 5.9 ± 0.9 and 8.5 ± 1.3 %, respectively. Regardless of the duration, immunization of chickens with attenuated *S. typhimurium*/pcDNA3.1/H5 enhanced IL-1 β , IL-6, TNFSF13B, TGF- β , IL-12 β , IL-15 and IL-18 expressions although no significant differences were recorded in chickens vaccinated via IM. Immunization of chickens with AgNP/pcDNA3.1/H5 also induced similar cytokine expression profiles except for IL-6 and TNFSF13B. Although no significant differences were recorded in chickens inoculated with AgNP and AgNP/pcDNA3.1.

Hence, single oral administrations of the attenuated *S. typhimurium* containing pcDNA3.1/H5 and AgNP/pcDNA3.1/H5 could induce strong antibody, T cell and Th1-like cytokine responses against avian influenza virus in chickens. This study provide valuable information for further study to determine the efficacy of the vaccine to induce protection against challenge with virulent H5N1 virus.

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

**PEMBANGUNAN VAKSIN ORAL VIRUS SELESEMA BURUNG SUBTIP H5
MENGUNAKAN SALMONELLA TYPHIMURIUM ATTENUAT DAN PARTIKEL
NANO PERAK SEBAGAI PEMBAWA**

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Februsri 2012

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Baru-baru ini, penyakit epizotik yang mudah berjangkit dalam senarai A OIE seperti penyakit kuku dan mulut, *classical swine fever* dan selesema burung (AI), telah membawa kepada pelaksanaan polisi *stamping out* yang menyebabkan pemusnahan berjuta-juta haiwan. Untuk mengelakkan pemusnahan haiwan secara besar-besaran, pelaksanaan strategi kawalan yang berlainan perlu ditimbang. Oleh itu, dengan kemajuan teknologi vaksin moden, beberapa jenis vaksin yang berbeza kini boleh digunakan untuk AI H5N1. Vaksin DNA adalah lebih selamat jika dibandingkan dengan vaksin tradisional seperti vaksin berasaskan virus yang dibunuh, lebih jimat kos pengeluaran dan penyimpanan secara relatif, dan mempunyai potensi untuk imunisasi serentak terhadap antigen atau pelbagai patogen. Inokulasi vaksin DNA telah menunjukkan rangsangan tindakbalas imun, termasuk kedua-dua gerak balas humor dan sel. Walaupun vaksin DNA menawarkan pelbagai kelebihan, antara kekurangan utama adalah

kegagalan untuk merangsang keimunan yang kuat dan vaksinasi tidak dapat dijalankan secara besar-besaran dalam ayam. Oleh itu, terdapat keperluan untuk membangunkan sistem penyampaian dan/atau adjuvan yang lebih berkesan. Dalam kajian ini, *S. typhimurium* strain SV4088 dan sintesis menggunakan teknologi mesra alam partikel nano perak (AgNP) telah digunakan sebagai pembawa untuk vaksin DNA ekspresi gen H5 (pcDNA3.1/H5) daripada AIV A/Ck/Malaysia/5858/04 (H5N1). Keselamatan dan keimunan formulasi vaksin DNA tersebut telah dinilai berdasarkan kajian sitotoksisiti, gerak balas imun humor dan sel serta penghasilan sitokin.

Salmonella enterica sv. typhimurium (*S. typhimurium*) strain SV4088 merupakan mutan berganda (Dam- and phoP-) diperolehi daripada jenis liar *S. typhimurium* strain SL1344, telah digunakan sebagai pembawa untuk vaksin DNA H5, pcDNA3.1/H5 secara oral. Sel anak ayam primer daripada telur ayam berembrio 18 hari bebas-patogen-khusus (SPF) telah digunakan untuk menentukan tahap ekspresi sitokin selepas jangkitan secara *in-vito* dengan *S. typhimurium* yang hidup dan mati bagi strain SV4088 yang dilemahkan dan strain jenis liar SL1344, masing-masing. *S. typhimurium* SL1344 yang hidup menunjukkan ekspresi yang kuat bagi sitokin pro-inflamasi (GM-CSF, IL-1 β , TGF- β , TNFSF13B, IL-12 β , IL-6 and IL-8), Th1 (IFN- γ , IL-2, IL-15, IL-18) and Th2 (IL-4, IL-10) daripada sel anak ayam primer. Walaupun *S. typhimurium* SV4089 yang dilemahkan, juga dapat merangsangkan semua sitokin yang dikaji tetapi tahap ekspresi secara ketaranya adalah 1.3 hingga 28 kali lebih rendah dibandingkan dengan jenis liar. Tidak seperti strain yang dilemahkan dan jenis liar, jangkitan dengan *Salmonella* yang dibunuh menunjukkan sedikit atau tiada rangsangan ekspresi sitokin pro-inflamasi (IL-8), Th2 (IL-10, IL-4) and Th1 (IL-18). Plasmid DNA pcDNA3.1/H5 juga telah diformulasi menggunakan partikel

nano perak (AgNP) menggunakan kaedah sintesis mesra alam dengan polietilin glikol (PEG). AgNP telah berjaya disintesis melalui sebaran seragam dalam julat saiz di antara 4-18 nm dengan saiz purata 11 nm. Sitotoksiti AgNP yang disediakan masing-masing dikaji secara *in vitro* dan *in vivo* dengan menggunakan sel MCF-7 dan ekspresi sitokin. Pada kepekatan $-5 \log_{10}$ AgNP, tiada kesan sitotoksiti dapat dikesan dalam sel MCF-7 dengan 9.5% kematian sel berbanding kepada kawalan. Anak ayam SPF berumur 1 hari yang diimunisasi secara oral dengan partikel nano mengandungi 10 μ l pcDNA3.1/H5 (2000 ng/mL) dan 40 μ l AgNP (3.7×10^{-2} μ g Ag) tidak menunjukkan sebarang manifestasi klinikal.

Potensi *S. typhimurium* SV4089 yang dilemahkan sebagai pembawa vaksin DNA dalam ayam muda telah diuji melalui imunisasi dalam anak ayam SPF yang baru menetas secara oral dengan 10^9 CFU *Salmonella*. Tiada kematian serta kesan-kesan sampingan yang ditemui selepas imunisasi dengan strain yang dilemahkan ini. Bakteria hidup dapat dikesan 3 hari selepas inokulasi melalui plating, *fluorescent in situ hybridization* (FISH) and tindak balas rantai polimerasi (PCR) dari hati, limpa and sekum. Analisis sitometri aliran sel darah periferi mononuklear (PBMC) ayam yang diinokulasi dengan *Salmonella* yang dilemahkan secara oral, tidak menunjukkan sebarang respon sel $CD3^+/CD4^+$ dan $CD3^+/CD8^+$ yang ketara. Tanpa mengira tempoh, imunisasi ayam dengan strain *Salmonella* yang dilemahkan menunjukkan ekspresi IL- 1β , IL-6, TNFSF13B (sitokin pro inflamasi) dan IL-15, IL-18 and IL-12 β (sitokin Th1), walaupun tiada perubahan yang ketara direkod dalam eksperimen sepanjang 35 hari. Sampel serum yang dikumpul dari kumpulan imunisasi melalui intraotot (IM) mencatatkan titer HI yang rendah, manakala ayam yang diimunisasi dengan *S. typhimurium*/pcDNA3.1/H5 atenuat dan AgNP/pcDNA3.1/H5 menunjukkan peningkatan antibodi yang ketara terhadap H5 pada hari ke-

14 selepas imunisasi. Purata titer antibodi yang tertinggi dikesan pada hari ke-35 selepas imunisasi melalui IM, kumpulan yang diimmunisasi dengan *S. typhimurium*/pcDNA3.1/H5 atenuat dan AgNP/pcDNA3.1/H5 adalah 4.0 ± 2.8 , 51.2 ± 7.5 , and 51.2 ± 7.5 , masing-masing.

S. typhimurium/pcDNA3.1/H5 atenuat dan AgNP/pcDNA3.1/H5 juga merangsang sel $CD4^+$ dan $CD8^+$ T pada 20.5 ± 2.0 % ($CD4^+$), 22.9 ± 1.9 % ($CD8^+$) dan 7.5 ± 2.0 % ($CD4^+$), dan 20 ± 1.9 % ($CD8^+$) masing-masing, dalam ayam seawal hari ke-14 selepas imunisasi. Sementara itu, sel $CD4^+$ dan $CD8^+$ T dalam ayam yang diimmunisasikan melalui IM adalah sangat rendah, iaitu 5.9 ± 0.9 and 8.5 ± 1.3 %, masing-masing. Tanpa mengira tempoh, imunisasi ayam dengan *S. typhimurium*/pcDNA3.1/H5 yang dilemahkan meningkatkan ekspresi IL- 1β , IL-6, TNFSF13B, TGF- β , IL-12 β , IL-15 dan IL-18 walaupun tiada perbezaan yang ketara direkodkan dalam ayam yang diimmunisasi secara IM. Ayam yang diimmunisasi dengan AgNP/pcDNA3.1/H5 juga menunjukkan peningkatan pada ekspresi sitokin yang sama kecuali IL-6 dan TNFSF13B. Tiada perbezaan yang ketara telah direkodkan dalam ayam yang disuntik dengan AgNP, dan AgNP/pcDNA3.1.

Oleh itu, pemberian tunggal *S. typhimurium* yang mengandungi pcDNA3.1/H5 dan AgNP/pcDNA3.1/H5 secara oral boleh merangsang antibodi yang kuat, sel T dan respon sitokin Th1 terhadap virus selsema burung dalam ayam. Kajian ini telah menentengahkan maklumat bermakna bagi kajian selanjutnya untuk menentukan efikasi vaksin DNA dalam mengaruh pelindungan terhadap cabaran dengan virus H5N1 virulen.

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I certify that an Examination Committee has met on to conduct the final examination of Seyed Davoud Jazayeri on his Doctor of Philosophy entitled "Development of Oral Avian Influenza Virus Subtype H5 DNA Vaccines Using Attenuated *Salmonella typhimurium* and Silver Nanoparticles as Carriers" 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 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 Universiti Putra Malaysia or other institutions.

SEYED DAVOUD JAZAYERI

Date: 10 February 2012

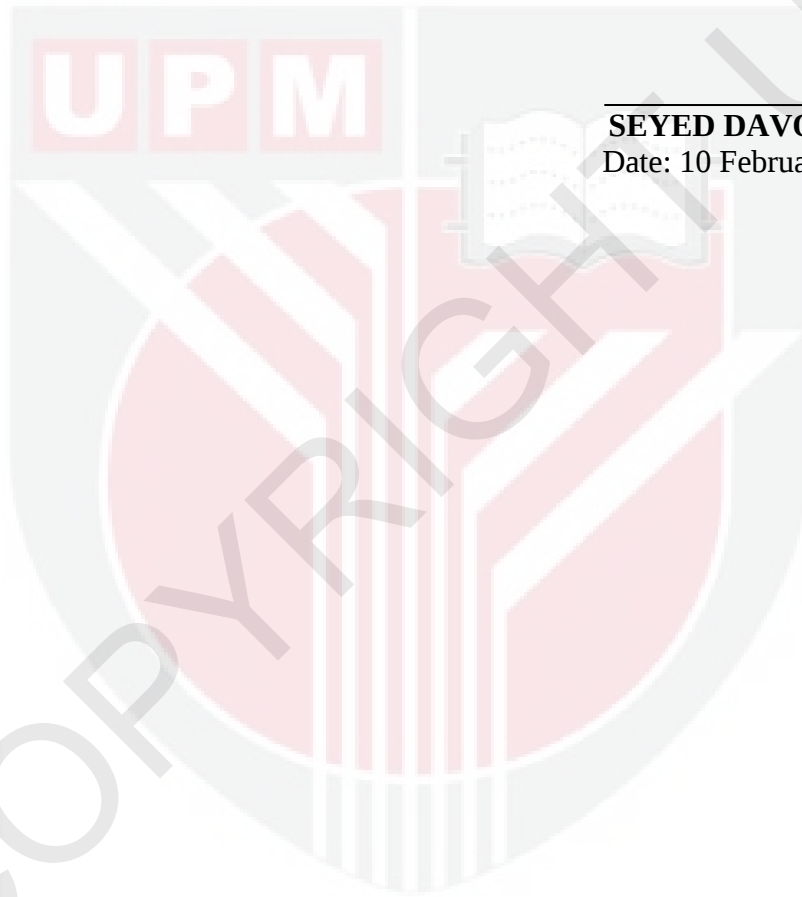


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