CHARACTERIZATION OF T-LYMPHOCYTE POPULATIONS AND SELECTED CYTOKINE EXPRESSIONS OF CHICKENS VACCINATED WITH H5-RECOMBINANT FOWL POX VIRUSES CO-EXPRESSING IL-15 GENE

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By

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Fowl pox virus (FPV) has been modified to express avian influenza virus (AIV) antigens since the late 1980s. A more advanced approach would be to co-express a novel host cytokine from such recombinants and characterize its immune response. In this study, previously constructed H5-recombinant Fowl pox viruses co-expressing host IL-15 (rFPV) was inoculated into specific-pathogen-free (SPF) chickens. T-lymphocyte populations and selected cytokine expression namely IL-15 and IL-18 of the vaccinated chickens were evaluated to add the relatively limited knowledge of chicken IL-15 cytokine gene compared to that of mammalian. It is hypothesized that vaccination with H5-recombinant Fowl pox viruses co-expressing IL-15 gene is able to show a higher population percentage of CD4+ and CD8+ T lymphocytes, and high IL-15 and IL-18 expressions, compared to H5-recombinant vaccines alone, in chickens.

Prior to in vivo characterization, recombinant viruses were propagated in Chicken Embryonic Fibroblast (CEF) primary cell line. Stability of H5 gene from influenza strain A/Chicken/Malaysia/5858/2004 (1695 kb), and IL-15 (695 kb) gene integrations was confirmed by using Polymerase Chain Reaction (PCR) with specific primers after three passages. Propagations and plaque assays were done until desired titres of recombinant viruses were obtained.

Parental (FP9 wild-type) and recombinant virus vaccines (10^5 PFU) were inoculated subcutaneously into one-day-old SPF chickens. The immunogenicity of the recombinant viruses was analyzed based on evaluation of T-lymphocytes cell population via flow cytometry, from Peripheral Blood Mononuclear Cell (PBMC) of 14 and 28-days-old vaccinated chickens. Chickens inoculated with rFPV/H5/IL-15 had a higher increased in CD4+ T cells population relative to rFPV/H5 in both time points. However, the result showed that rFPV/H5/IL-15 was not significant ($P>0.05$) in inducing CD8+ T cells. In

general, the percentage of CD4+ and CD8+ lymphocytes cell population in chickens immunized with rFPV/H5/IL-15 were statistically higher compared to chickens immunized with rFPV/H5 and FP9 wild-type virus ($P<0.05$). Specific gene expressions of SPF chickens inoculated with rFPV were analyzed by quantitative real-time PCR (qRT-PCR), following extraction of spleen from 14-day-old SPF chickens at days 2, 4 and 6 post-infection. Two target genes chosen were IL-15 and IL-18 genes. The rFPV/H5/IL-15 group showed an increased level of IL-15 and IL-18 genes expression up to 2 and 3.5 folds, respectively, within 6 days post-vaccination, compared with other inoculated groups. rFPV/H5 group showed an increased level of IL-15 gene expression at day 2 and maintained at day 4 until day 6, while the IL-18 expression was decreasing within 6 days. Overall, the FP9 wild type group showed a low cytokine expression level as compared to the recombinant virus groups. While histopathology results showed successful vaccination of rFPV into chicken cells, weekly weighing suggested that inoculation with rFPV might not influence any weight changes.

In summary, this study showed modulation immunogenicity of FP9 Wild Type, rFPV/H5, and rFPV/H5/IL-15, with rFPV/H5/IL-15 being the best vaccine candidate compared to others.

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

PENCIRIAN POPULASI T-LIMFOSIT DAN UNGKAPAN SITOKIN TERPILIH PADA AYAM YANG DISUNTIK DENGAN VIRUS FOWL POX REKOMBINAN-H5 BERSAMA UNGKAPAN GEN IL-15

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Virus Fowl pox (FPV) telah diubahsuai untuk mengungkap antigen virus selesema burung (AIV) sejak penghujung 1980 an. Pendekatan yang lebih maju adalah ungkapan bersama dengan hos sitokin asal dari rekombinan tersebut dan mengkaji reaksi imunnya. Dalam kajian ini, virus Fowl pox rekombinan-H5 ungkapan bersama dengan IL-15 (rFPV) perumah yang dihasilkan sebelum ini telah diinokulasikan ke dalam ayam bebas-patogen-spesifik (SPF). Data populasi T-limfosit dan ungkapan sitokin yang dipilih iaitu IL-15 dan IL-18 daripada ayam-ayam yang divaksinasikan dianggarkan sebagai tambahan kepada maklumat yang terhad terhadap gen IL-15 sitokin ayam berbanding dengan maklumat pada mammalia. Hipotesis menyatakan bahawa vaksinasi dengan virus Fowl pox rekombinan-H5 dengan ungkapan bersama gen IL-15 boleh menunjukkan penambahan peratusan populasi CD3+ dan CD4+ T-limfosit, IL-15 dan IL-18, berbanding dengan vaksin rekombinan-H5 sahaja, di dalam ayam.

Sebelum pencirian in vivo, virus rekombinan telah dibiakkan dalam titisan sel utama embrionik fibroblast ayam (CEF). Kestabilan integrasi gen H5 daripada strain influenza A/Chicken/Malaysia/5858/2004 (1695 kb), dan gen IL-15 (695 kb) telah dipastikan dengan menggunakan Tindakan Polimerase Berantai (PCR) dengan primer khas selepas tiga kali laluan. Pembiakan dan ujian plak telah dilakukan sehingga titer yang dikehendaki untuk virus rekombinan dicapai.

Induk (FP9 wild-type) dan vaksin rekombinan (10^5 PFU) telah diinokulasi secara subkutaneus kepada ayam-ayam SPF yang berumur 1 hari. Keimunogenan virus rekombinan telah dianalisis berdasarkan penilaian populasi sel T-limfosit melalui sitometri aliran, daripada lapisan sel darah mononuklear periferal (PBMC) ayam bervaksinasi berumur 14 dan 28 hari. Ayam-ayam yang diinokulasi dengan rFPV/H5/IL-15 menunjukkan peningkatan lebih tinggi dalam populasi sel-sel T CD4+ berbanding dengan rFPV/H5 pada kedua-dua titik masa. Walau bagaimanapun, keputusan

menunjukkan bahawa rFPV/H5/IL-15 tidak signifikan ($P>0.05$) dalam mendorong sel-sel T CD8+. Secara umum, peratusan populasi sel-sel limfosit CD4+ dan CD8+ dalam ayam-ayam diimunisasikan dengan rFPV/H5/IL-15 adalah tinggi secara statistik berbanding dengan ayam-ayam yang diimunisasikan dengan rFPV/H5 dan virus FP9 wild-type ($P<0.05$). Ungkapan gen khas daripada ayam-ayam SPF yang disuntik dengan rFPV telah dianalisis dengan kuantitatif PCR masa-nyata (qRT-PCR), diikuti dengan pengekstrakan limpa dari ayam SPF berumur 14 hari pada hari kedua, keempat, dan keenam selepas pemvaksinan. Dua gen sasaran pilihan adalah gen IL-15 dan gen IL-18. Kumpulan rFPV/H5/IL-15 menunjukkan peningkatan tahap ungkapan gen IL-15 dan gen IL-18 sehingga 2 dan 3.5 kenaikan setiap satu-satunya, dalam masa 6 hari selepas pemvaksinan, berbanding dengan kumpulan suntikan yang lain. Kumpulan rFPV/H5 menunjukkan penigkatan tahap ungkapan gen IL-15 pada hari kedua dan kekal sehingga pada hari keempat sehingga hari keenam, manakala ungkapan gen IL-18 menurun sehingga hari keenam. Kumpulan FP9 Wild Type menunjukkan kadar ungkapan yang rendah berbanding dengan kumpulan virus rekombinan. Sementara itu keputusan histopatologi menunjukkan kebolehan vaksinasi rFPV kepada sel ayam, dan keputusan berat ayam mingguan menunjukkan inokulasi dengan rFPV mungkin tidak mempengaruhi apa-apa perubahan kepada berat badan ayam.

Kesimpulannya, kajian ini menunjukkan perbezaan immunogenan daripada FP9 Wild Type, rFPV/H5, dan rFPV/H5/IL-15, dengan rFPV/H5/IL-15 menunjukkan sebagai calon vaksin terbaik berbanding dengan yang lain.

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I certify that a Thesis Examination Committee has met on 17 November 2016 to conduct the final examination of Nadzreeq bin Nor Majid on his thesis entitled "Characterization of T-Lymphocyte Populations and Selected Cytokine Expressions of Chickens Vaccinated with H5-Recombinant Fowl Pox Viruses Co-Expressing IL-15 Gene" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

AIV	Avian Influenza Virus
ANOVA	Analysis of Variance
CEF	Chicken Embryonic Fibroblast
CMI	Cell-mediated Immunity
CPE	Cytopathic Effect
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FPV	Fowl pox Virus
GAPDH	Glyceraldehy-3-Phosphate Dehydrogenase
HA	Hemagglutinin
HPAI	High Pathogenic Avian Influenza
IACUC	Institutional of Animal Care and Use Committee
IL	Interleukin
LGA	Low Gelling Agarose
MEM	Minimum Essential Media
MHC	Major Histocompatibility Complex
MOI	Multiple Of Infection
NA	Neuraminidase
NCS	Newborn Calf Serum
NDV	Newcastle's Disease Virus
NK	Natural Killer cell
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
rFPV	Recombinant Fowl pox Virus
RNA	Ribonucleic Acid
SEM	Standard Error of Mean
SPF	Specific Pathogen Free

CHAPTER 1

INTRODUCTION

The general aims of this project are to evaluate the effect of H5-recombinant FPV co-express IL-15 in inducing cellular responses. The aims focus on characterize the level of T-lymphocytes populations and to analyze specific genes expression upon infection with the recombinant fowl pox viruses. The IL-15 response in chickens is not well known as compared to mammalian IL-15 (Lillehoj et al., 2001). T-lymphocytes cells, specifically CD4+ and CD8+ T cells were chosen for the research because it play a central role in cell-mediated immunity response. The IL-15 plays an important role in inducing memory natural killer cell as it helps in development and maintenance of natural killer cell, innate immune responses, lymphocytes or memory phenotypic CD8 cell. A specific gene expression upon infection also was determined due to the fact that IL-15, IL-18 and IL-12 play an important role in inducing innate immune responses. Thus, addition of IL-15 gene in the recombinant Fowl pox virus might improves the immune responses in chicken. Immunosuppression is predicted to change upon vaccination of recombinant virus vaccine due to overexpression of cytokine. Therefore, vaccination with the recombinant virus vaccine might give an insight of IL-15 gene expression level. The IL-18 gene was chosen as it resides at the TH1 class similar to the IL-15 gene. Both cytokine genes produced natural killer cell thus a relation between both genes might be a clue upon vaccination of recombinant vaccines. As the recombinant virus vaccines were stored at Imperial College London for 5 years; therefore it is questionable whether the viruses still have the recombinant genes intact after a long storage. Thus, a proper propagation, titration and stability test of the viruses were proposed to ensure that the viruses' qualities are in a stable condition. It is hypothesized that vaccination with H5-recombinant Fowl pox viruses co-expressing IL-15 gene able to show a higher population percentage of CD4+ and CD8+ T lymphocytes and the expression of selected cytokines compared to H5-recombinant vaccines alone, in chickens.

1.1 Objectives

In order to address the general aim, the following specific objectives were envisaged:

I) To propagate, titrate, and confirm the stability of the recombinant viruses.

II) To enumerate the population percentage of the CD4+ and CD8+ upon vaccination of recombinant viruses into chickens.

III) To analyze the IL-15 and IL-18 gene expressions upon vaccination of recombinant viruses into chickens.

1.2 Thesis Summary

Co-expression of avian cytokines IL-15, by recombinant Fowl pox viruses already expressing AI antigens, may add to the limited laboratory studies on cytokine effects and gene expression in avian model.

Chapter 2 describes the information related to the Avian Influenza virus, Fowl pox virus, development of the vaccines and basic information of the immune system.

Chapter 3 describes the methodology of propagation, titration, gene stability study, experiment set up for enumeration of cytokine cell, and gene expression analysis of the recombinant viruses.

Chapter 4 describes result and discussion on result obtained for virus propagation and titration, cytokine cells enumeration and gene expression from host.

Chapter 5 describes the overall discussions, summary, conclusion and recommendation for future research based on the results obtained from overall studies.

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