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DEVELOPMENT OF DNA AND ORAL VACCINES AGAINST CHICKEN ANEMIA VIRUS

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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To my wife, Niloufar

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF DNA AND ORAL VACCINES AGAINST CHICKEN ANEMIA VIRUS

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October 2010

Chairman : Prof. Datin Paduka Khatijah Mohd Yusoff, PhD

Faculty : **Biotechnology and Biomolecular Sciences**

Chicken anemia virus (CAV) belongs to the genus *Gyrovirus* of *Circoviridae* family, which causes an economically important disease in the poultry industry worldwide. Commercially available vaccines against CAV infection which are based on non-attenuated virulent CAV propagated in chicken embryos or attenuated live vaccine cannot be used in chickens before 8 weeks of age and within 21 days of slaughter and sometimes spreading of the modified viruses to young chickens may cause disease. Therefore, the development of DNA and oral vaccines against this virus was considered in the current study.

For development of DNA vaccine, the VP1 and VP2 genes of CAV were amplified and cloned into pBudCE4.1 to construct two DNA vaccines, namely, pBud-VP1, and pBudVP2-VP1. *In vitro* and *in vivo* studies showed that co-expression of VP1 with VP2 is required to produce the essential neutralizing form of VP1 to induce significant levels of antibody against CAV. Subsequently, the vaccines were tested in 2-week-old specific pathogen free (SPF) chickens which were inoculated with the

DNA plasmid constructs by the intramuscular route. Serum antibody titers against CAV were determined 10 days after vaccination by ELISA. Chickens immunized with the DNA-plasmid pBudVP2-VP1 showed positive antibody titer (1853) against CAV. Furthermore, proliferation induction of splenocytes and also high serum levels of Th1 cytokines, IL-2 (78.2 pg/ml) and IFN-γ (534.5 pg/ml) were detected in the pBudVP2-VP1-vaccinated group indicating the induction of cell-mediated immune response in the vaccinated chickens. These results suggest that the recombinant plasmid pBudVP2-VP1 can be used as a potential DNA vaccine against CAV infection.

To enhance CAV-specific immune responses, the use of the VP22 gene of Marek's disease virus type-1 (MDV-1) linked to the CAV VP1 gene was also investigated. This was achieved by constructing a recombinant DNA plasmid, namely pBudVP2-VP1/VP22 encoding the fusion protein VP1/VP22 synchronously with the CAV VP2 and testing its effectiveness in specific pathogen free chickens. Chickens vaccinated with pBudVP2-VP1/VP22 exhibited a significant increase in antibody titers to CAV, VP1-stimulated proliferative induction of splenocytes and also higher levels of IL-2 and IFN-γ when compared to the chicken vaccinated with pBudVP2-VP1 ($P<0.05$). These observations showed that the MDV-1 VP22 is capable of enhancing the potency of DNA vaccine against CAV when fused with CAV VP1 gene expressing simultaneously with CAV VP2.

In the second part of this study, *Lactobacillus acidophilus* (ATCC 53672) carrying the VP1 protein of CAV was used as live delivery vehicles for oral immunization against CAV. The binding domain of AcmA anchor protein of *Lactococcus lactis*

MG1363 were used to display the VP1 protein of CAV on the surface of *Lb. acidophilus*. One and two repeats of the cell wall binding domain of *acmA* gene were amplified from *L. lactis* MG1363 genome and then inserted into co-expression vector, pBudCE4.1 to construct plasmids pETacma1 and pETacma2, respectively. Thereafter, the VP1 gene of CAV was fused to the *acmA* sequences and the CAV VP2 gene was cloned into the second multiple cloning site on the same vector before transformation into *Escherichia coli* BL 21 (DE3). The expressed recombinant proteins were purified using a His-tag affinity column and mixed with a culture of *Lactobacillus acidophilus*. Whole cell ELISA and immunofluorescence assay showed binding of the fusion proteins containing the CAV VP1 protein on the surface of the cells. The *lactobacilli* cells carrying the VP1 protein were used to immunize SPF chickens through oral route. The presence of anti-CAV antibodies was detected by ELISA. The immunized chickens showed significantly ($P<0.05$) higher level of neutralizing antibody against CAV compared to those from the controls. VP1-specific proliferative response was also observed in splenocytes of the chickens after oral immunization. Furthermore, high levels of Th1 cytokines, IL-2 and IFN-γ were detected in the immunized chickens. These results showed that the *lactobacilli* cells carrying the VP1 protein of CAV could induce immune response against CAV suggesting *lactobacilli* as live delivery vehicle for oral immunization purposes.

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

PENGHASILAN VAKSIN DNA DAN VAKSIN ORAL TERHADAP VIRUS ANEMIA AYAM

Oleh

Hassan Moeini

Oktober 2010

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Virus anemia ayam (CAV), tergolong dalam genus *Gyrovirus*, yang merupakan ahli family *Circoviridae*, adalah penyebab penyakit serius yang menggugat ekonomi pengunggasan di seluruh dunia. Vaksin komersil yang terdapat didalam pasaran terhadap jangkitan CAV yang berdasarkan CAV virulen yang dikultur dalam embrio ayam atau vaksin di mana CAV dilemahkan tidak dapat digunakan untuk ayam yang berumur kurang daripada 8 minggu dan dalam 21 hari sebelum penyembelihan. Kadang kala, penggunaan vaksin yang telah diubahsuaikan terhadap anak ayam yang belum cukup matang boleh mendatangkan penyakit. Justeru, dalam kajian ini, perhatian diberi untuk pengembangan vaksin DNA dan vaksin oral terhadap virus tersebut.

Untuk penghasilan vaksin DNA, gen VP1 dan VP2 CAV diamplifikasi dan diklonkan ke dalam pBudCE4.1 untuk pembentukan dua vaksin DNA, iaitu, pBud-VP1, dan pBudVP2-VP1. Kajian *in vitro* dan *in vivo* yang telah dilaksanakan membuktikan bahawa penzahiran kedua-dua VP1 dan VP2 diperlukan untuk

menghasilkan bentuk VP1 yang neutral supaya mengaruhkan tahap antibodi yang signifikan terhadap CAV. Vaksin DNA tersebut yang dihasilkan dikaji dalam anak ayam SPF berusia 2 minggu melalui cara intraotot. Serum antibodi terhadap CAV dikenalpasti 10 hari selepas pemvaksinan melalui ELISA. Ayam yang menerima vaksin pBudVP2-VP1 menunjukkan titer antibodi positif (1853) terhadap CAV. Tambahan pula, pembahagian sel-sel limpa dan peningkatan tahap serum Th1 yang tinggi seperti IL-2 (78.2 pg/ml) dan IFN-γ (534.5 pg/ml) dikesan dalam kumpulan ayam ini menunjukkan pengaruhan keimunan sel. Keputusan ini mencadangkan bahawa plasmid rekombinan pBudVP2-VP1 mempunyai potensi sebagai vaksin DNA terhadap jangkitan CAV.

Untuk menguatkan gerak balas imun yang spesifik terhadap CAV, gen VP22 daripada penyakit Marek jenis-1 (MDV-1) yang disambungkan dengan gen VP1 daripada CAV turut dikaji. Ini dilakukan dengain pembentukan plasmid rekombinan DNA, iaitu pBudVP2-VP1/VP22 yang mengkodkan kedua-dua VP1/VP22 bersamaan dengan VP2 daripada CAV. Tahap keberkesanan vaksin ini dikaji dalam ayam SPF. Ayam yang diberi vaksin pBudVP2-VP1/VP22 menunjukkan peningkatan tahap antibodi yang signifikan terhadap CAV. VP1 juga mengaruhkan pembahagian sel-sel limpa dan kenaikan tahap IL-2 dan IFN-γ apabila dibandingkan dengan ayam yang diberi vaksin pBudVP2-VP1 ($P < 0.05$). Keputusan ini menunjukkan MDV-1 VP22 berupaya meningktakan kekuatan vaksin DNA terhadap CAV apabila dicantumkan dengan VP1 yang dihasilkan bersama VP2 daripada CAV.

Dalam bahagian kedua kajian ini, *Lactobacillus acidophilus* (ATCC 53672) yang mengandungi protein VP1 daripada CAV digunakan sebagai jentera penghantaran vaksin untuk imunisasi oral terhadap CAV. Domain pengikatan protein AcmA daripada *Lactococcus lactis* MG1363 digunakan untuk pempameran protein VP1 daripada CAV di permukaan *Lb. acidophilus*. Satu dan dua ulangan domain pengikatan gen AcmA diamplifikasikan daripada genom *L. lactis* MG1363 dan dimasukkan dalam vektor pengekspresan bersama, pBudCE4.1untuk pembentukan pETacma1 dan pETacma2 masing-masing. Selepas itu, gen VP1 daripada CAV dicantumkan kepada domain AcmA dan gen VP2 CAV diklonkan dalam MCS kedua dalam vektor yang sama sebelum transformasi ke dalam *Escherichia coli* BL 21 (DE3). Protein rekombinan yang telah diekspreskan dilalui proses penulenan menggunakan kolum His-tag dan dicampurkan dengan kultur *Lactobacillus acidophilus*. ELISA seluruh sel dan esei imunofloresen menunjukkan protein-protein mengandungi VP1 protein daripada CAV dipamerkan di permukaan sel. Sel lactobacilli mengandungi protein VP1 digunakan untuk imunisasi ayam SPF melalui cara oral. Kehadiran antibodi anti-CAV dikesan melalui ELISA. Ayam yang diberi vaksin menunjukkan tahap antibodi peneutrelan yang signifikan ($P<0.05$) terhadap CAV apabila dibandingkan dengan sampel kawalan. Respons terhadap VP1 yang spesifik turut didapati dalam sel-sel limpa ayam yang telah diberi imunisasi oral. Tahap sitokin yang tinggi seperti IL-2 dan IFN-γ turut dikesan dalam ayam-ayam ini. Keputusan ini menunjukkan sel laktobasili yang mengandungi protein VP1 daripada CAV berupaya mengaruh imun respons terhadap CAV. Ini mencadangkan laktobasili berpotensi sebagai jentera penhantaran vaksin untuk tujuan imunisasi.

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I certify that a Thesis Examination Committee has met on 8 October 2010 to conduct the final examination of Hassan Moeini on his thesis entitled "Development of DNA and oral vaccines against chicken anemia virus" 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 degree of Doctor of Philosophy.

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The thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as the fulfilment requirement for the degree of Doctor of Philosophy. The members of the Supervisor Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for the quotation and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institution.

HASSAN MOEINI

Date: 8 Oct. 2010

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