UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND EVALUATION OF RECOMBINANT VECTOR CELLS CARRYING CELL WALL SURFACE ANCHOR FAMILY PROTEINS AS A VACCINE AGAINST STREPTOCOCCOSIS IN RED HYBRID TILAPIA (OREOCHROMIS SPP)

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By

NUR NAZIFAH MANSOR

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairman : Md. Sabri Mohd Yusoff, DVM, MVSc, PhD

Faculty : Veterinary Medicine

Tilapia is one of the most common cultured fish in many countries. However, productions of tilapia might decrease due to various diseases, including streptococcosis that can kill 100% of the fish. In Malaysia, outbreaks of streptococcosis are frequently observed during the dry months, particularly between April and August when the water temperature is high. Streptococcosis in fish is caused by either *Streptococcus agalactiae* or *S. iniae.* Although vaccination is practiced to control streptococcosis, the protection remains unclear. Thus, this project attempted to develop and evaluate recombinant cell for vaccine preparation to improve vaccine efficacy against streptococcosis. The hypothesis of this study are major outer surface protein of *Streptococcus agalactiae* is suitable as vaccine candidate and can be cloned and expressed in

the *Escherichia coli* prokaryotic system to produce the recombinant vaccine. The newly develop feed based recombinant vaccine can elicit certain level of systemic and mucosal antibody and gave protection against Streptococcosis in red hybrid tilapia.

The outer surface proteins (OSPs) of *S. agalactiae* isolated earlier from fish that died of streptococcosis were extracted, purified and characterized. The 48kDa protein band was found to be the most antigenic following SDS-PAGE and Western immunoblotting methods. Therefore, the 48kDa protein band was selected for further studies as a vaccine candidate.

The 48kDa band was processed for N-terminal sequencing, and the results revealed that the protein was the cell wall surface anchor family protein of the outer surface protein (OSP) of *S. agalactiae,* which was encoded by a gene of approximately 1263bp in size. The DNA gene was amplified by the polymerase chain reaction (PCR) method, purified and cloned in pET-32 Ek/Lic vector. The successful clones were then transformed into Novablue *Escherichia coli* strain before the positive clones were screened for the end product of 1335bp; the vector contributed 72bp. Plasmid extraction was performed prior to the expression of the cell wall surface anchor family protein in the BL21 (DE3) *Escherichia coli*. Overnight Express™ Autoinduction system 1 (Novagen, USA) was used in expression. Successful protein expression was analysed by SDS-PAGE and western immunoblotting and was found to be approximately 65.8kDa, consisted of the 48kDa cell wall surface anchor family protein and the 17.8kDa of tagged protein.

The successful expression of the protein of interest paved the way for preparation of crude recombinant vaccine against streptococcosis. Next was the study on humoral and mucosal immune responses by red hybrid tilapia following exposure to the inactivated recombinant cell vaccine. The newly developed recombinant vaccine was incorporated homogenously into fish pellet at a concentration of $1x10^6$ CFU/g as feed-based vaccine against streptococcosis. To vaccinate, red hybrid tilapia were given the feed-based recombinant vaccine orally before a booster dose was given 2 weeks later. The results showed that the newly develop recombinant vaccine elicited high levels of systemic and mucosal immunity from week-5 until the end of the 12-week study period.

Lastly, the efficacy of the newly developed feed-based recombinant vaccine against Streptococcosis in red hybrid tilapia (*Oreochromis* spp.) was tested. Following initial vaccination and booster dose at week 2, the fish were challenged intraperitoneal on week 6 with an inoculum containing $2.27x10^9$ CFU/mL of live *S. agalactiae*. This was followed by heat stress at 31°C throughout the entire 3-week challenge period, which ended on week 9. The newly develop feed-based recombinant vaccine stimulated both mucosal and systemic immunity when the immunoglobulin levels were higher, the size of GALT was bigger and the number of lymphocytes was greater. The stimulations, however, were able to provide only 70% protection. Nevertheless, the efficacy of the newly develop recombinant vaccine was significantly better than the whole cell vaccine.

In conclusion, the 70% protection provided by the newly developed recombinant vaccine was considered as moderate. Therefore, vaccine enhancement should be carried out in the future to produce an excellent protection against streptococcosis. Adding oil adjuvant to the vaccine can be considered as one of the approaches to further enhance the efficacy of the recombinant vaccine.

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

PEMBANGUNAN DAN PENILAIAN SEL REKOMBINAN VEKTOR YANG MEMBAWA "PROTEIN FAMILI DINDING SEL PERMUKAAN SAUH" SEBAGAI VAKSIN TERHADAP PENYAKIT STREPTOCOCCOSIS DALAM IKAN TILAPIA MERAH HIBRID (*OREOCHROMIS* SPP)

Oleh

NUR NAZIFAH BINTI MANSOR

Julai 2013

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Tilapia adalah sejenis ikan yang biasa diternak di kebanyakan negara. Walau bagaimanapun, pengeluaran ikan tilapia mungkin berkurangan disebabkan oleh pelbagai penyakit termasuk penyakit Streptococcosis yang boleh menyebabkan 100% kematian. Di Malaysia, wabak penyakit ini berlaku semasa musim kemarau antara bulan April dan Ogos setiap tahun iaitu apabila suhu air adalah tinggi. Penyakit Streptococcosis pada ikan adalah disebabkan oleh *S. agalactiae* atau *S. iniae*. Walaupun kaedah pevaksinan telah digunakan untuk mengawal penyakit ini, hasilnya masih tidak jelas. Oleh itu, projek ini telah dijalankan bagi membangunkan dan menilai sel rekombinan yang akan digunapakai dalam penyediaan vaksin untuk meningkatkan keberkesanan vaksin terhadap

penyakit Streptococcosis. Hipotesis bagi kajian ini adalah protin permukaan luar dari *Streptococcus agalactiae* yang major sesuai dijadikan sebagai calon vaksin dan boleh diklon dan diekspresikan dalam system prokaryotik *Escherichia coli* bagi penghasilan vaksin rekombinan. Vaksin rekombinan berasaskan makanan yang baru dibangunkan ini dipercayai boleh menghasilkan tahap antibody sistemik dan mukosal tertentu dan memberi perlindungan kepada ikan tilapia merah hibrid terhadap penyakit Streptococcosis.

Protin permukaan luar (OSPs) dari *S. agalactiae* yang dipencil daripada ikan Tilapia yang telah mati disebabkan oleh penyakit Streptococcosis telah diekstrak, disucikan dan dicirikan. Protin 48 kDa yang dijalurkan adalah protin yang paling antigenik yang ditentusahkan menggunakan kaedah analisis "SDS PAGE" dan Pemblotan Western. Oleh itu, jalur protin 48 kDa telah dipilih untuk dikaji dengan lebih terperinci dan dijadikan sebagai calon vaksin.

Jaluran protin 48 kDa telah diproses untuk penjujukan Terminal-N dan keputusannya menunjukkan ianya adalah " Protin keluarga dinding sel permukaan sauh" dari protin permukaan luar (OSPs) yang terdapat pada *S. agalactiae.* Protin ini mengkodkan jujukan gen DNA bersaiz 1263 bp. Gen DNA tersebut telah diamplifikasi menggunakan kaedah tindakbalas rantaian polimerase (PCR), disucikan dan diklonkan di dalam vektor pET-32 Ek/LIC. Klon yang berjaya dihasilkan telah dimasukkan kedalam bakteria Novablue *E. coli*. Klon positif telah diskrin menggunakan kaedah PCR dan menghasilkan jujukan bersaiz 1335 bp; 72 bp disumbangkan oleh vektor. Kemudian, pengekstrakan plasmid telah dijalankan supaya dapat digunakan dalam proses

ekspresi protein bagi “protin keluarga dinding sel permukaan sauh” di dalam bakteria BL21 (DE3) *E. coli*. Pengaruhan ekspresi protin dijalankan dengan menggunakan kit “ Overnight Express™ Autoinduction System 1” (Novagen, USA). Protin yang telah berjaya diekspreskan, dianalisa dengan menggunakan kaedah analisis “SDS PAGE” dan Pemblotan Western. Protin tersebut adalah bersaiz 65.8 kDa iaitu berpadanan dengan saiz “Protin keluarga dinding sel permukaan sauh”, 48 kDa dan protin.Tag bersaiz 17.8 kDa.

Ekspesi protin telah berjaya dihasilkan dan membuka jalan dalam penyediaan vaksin rekombinan mentah yang sesuai digunakan bagi mengawal penyakit Streptcoccosis. Seterusnya adalah kajian yang dijalankan bagi menentukan tindakbalas imun humoral dan mukosa terhadap sel rekombinan vaksin yang telah dilemahkan yang digunapakai pada ikan Tilapia merah. Rekombinan vaksin yang baru dibangunkan ini digaul secara rata ke dalam pellet makanan ikan berkepekatan 1 x 10^6 CFU/g menjadikannya vaksin rekombinan berasaskan makan bagi mengawal penyakit Streptococcosis. Pada pemvaksinan, ikan Tilapia merah telah diberikan vaksin rekombinan berasaskan makanan secara oral dan kemudiannya dos peningkatan diberikan dua minggu kemudian. Keputusan menunjukkan bahawa rekombinan vaksin yang baru dibangunkan berjaya menghasilkan kadar tindakbalas imun humoral dan mukosa yang tinggi dari minggu ke lima sehingga ke akhir eksperimen iaitu minggu ke dua belas.

Keberkesanan rekombinan vaksin berasaskan makanan yang baru dibangunkan terhadap Tilapia merah hibrid (*Oreochromis sp.*) diuji. Setelah diberikan

pemvaksinan awal dan dos peningkatan dua minggu kemudiannya, ikan Tilapia merah telah diuji secara suntikan intraperitonium pada minggu keenam menggunakan inokulum mengandungi 2.27 x 10^9 CFU/mL *S. agalactiae* hidup. Ikan juga diberikan tekanan dengan kenaikan suhu air kepada 31^{o}C bermula minggu keenam hingga minggu kesembilan; iaitu minggu terakhir eksperimen. Rekombinan vaksin berasaskan makanan yang baru dibangunkan merangsang kedua-dua imun mukosa dan sistemik. Ketika tahap immunoglobin adalah tinggi, saiz "sel limfoid berkaitan usus" (GALT) adalah besar, dan bilangan limfosid juga adalah tinggi. Walau bagaimanapun, dengan stimulasi tinggi yang dihasilkan, vaksin rekombinan berasaskan makanan yang baru dibangunkan ini mampu memberi 70% perlindungan. Namun, keberkesanan vaksin rekombinan berasaskan makan yang baru dibangunkan ini secara ketaranya memberikan perlindungan yang lebih baik berbanding vaksin keseluruhan sel.

Kesimpulannya, 70% perlindungan yang diberikan oleh vaksin rekombinan berasaskan makanan yang baru dibangunkan diklasifikasikan sebagai sederhana. Oleh itu, vaksin ini perlulah ditambahbaik di masa hadapan bagi menghasilkan perlindungan yang menyeluruh terhadap penyakit Streptococcosis. Penambah minyak Adjuvant terhadap vaksin tersebut boleh dijadikan sebagai salah satu langkah penambahbaikkan bagi meningkatkan lagi keberkesanan vaksin rekombinan bersasakan makanan ini.

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I certify that a Thesis Examination Committee has met on 17 July 2013 to conduct the final examination of Nur Nazifah binti Mansor on her thesis entitled "Development And Evaluation of Recombinant Vector Cells Carrying Cell Wall Surface Anchor Family Proteins as a Vaccine Against Streptococcosis in Red Hybrid Tilapia (Oreochrimis Spp)" 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 Doctor of Philosophy.

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

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DECLARATION

I hereby declare that the thesis is based on my original work accept for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrent submitted for any other degree at Universiti Putra Malaysia or other institutions.

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NUR NAZIFAH MANSOR

Date: 17 July 2013

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