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IMMUNE RESPONSES TO Streptococcus agalactiae IN RED TILAPIA, Oreochromis spp. FOLLOWING VACCINATION WITH NON-ADJUVANTED AND ADJUVANTED VACCINE INCORPORATED FEED PELLETS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of Requirement for the Degree of Master of Science

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Chairman : **Md. Sabri Mohd Yusoff, DVM, MSc, PhD**

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Streptococcosis is an important bacterial disease in tilapia in many countries, including Malaysia. The two common *Streptococcus* species causing the disease are *S. agalactiae* and *S. iniae*. In Malaysia, outbreaks of streptococcosis in red tilapia (*Oreochromis* spp.) were due to *Streptoccoccus agalactiae* while infection by *Streptococcus iniae* has never been reported. Mass mortalities among red tilapia in cage-culture system were reported to occur usually during the dry months of the year, between April and August when water temperature was high.

This study was conducted to investigate the effect of high water temperature on the susceptibility of red tilapia to infection by *Streptococcus agalactiae*, the immune response by red tilapia following exposure to live *S. agalactiae* and the protection following vaccination against streptococcosis. To achieve the first objective, eight groups of red tilapias of approximately 100 g were infected intraperitoneally with 0.5 mL of live *S. agalactiae* at 6.3×10^6 CFU/mL, 6.3×10^7 CFU/mL, 6.3×10^8 CFU/mL and 6.3×10^9 CFU/mL. Four groups were kept at normal water temperature of 27 °C ± 2°C while the rest were kept in high water temperature of 33°C± 2°C for a period of one week. The high water temperature caused an increased in the susceptibility of red tilapia to *S. agalactiae* infection as indicated by the rapid rate of mortality at lethal dose 50 (LD_{50}). The period to achieve 100% mortality in the high temperature group was faster than the normal water temperature group, in four days compared to seven days. The lethal dose 50 (LD_{50}) for groups that were kept in high water temperature was 5.68×10^6 CFU/mL, significantly ($p<0.05$) lower than those that were kept in normal water temperature (2.29×10^7 CFU/mL). The clinical signs included loss of appetite, lethargy, unilateral or bilateral exophthalmia, cloudy eyes, erratic swimming and inflammation of the skin.

Once the susceptibility was determined, formalin killed whole-cell *S. agalactiae* at the concentration of 6.7×10^6 CFU/mL was incorporated homogenously into fish pellet as feed vaccine (FNV vaccine). The vaccine was then administered orally to the red tilapia in three different vaccination regimes; once (F1D), thrice (F3D) and five times a week (F5D). Body mucus and blood serum were sampled every week for eight weeks to analyze the

mucosal and systemic antibody responses by using ELISA. Immunization by FNV vaccine resulted in significant ($p<0.05$) increase in the serum and mucus antibody levels (IgM) as early as week 2 in all vaccinated groups, while the control unvaccinated group (FC) showed insignificant ($p>0.05$) increase of the serum and mucus antibody levels. Group F5D showed the highest antibody levels, followed by groups F3D and F1D. At the end of the experiment, twenty fishes from each group were challenged by immersion while another twenty were challenged intra-peritoneal. Survival rate was low indicating poor protective immune response in all groups of tilapia tested. Gut samples were obtained at the end of the experiment and subjected to histological analyses to examine the presence of gut-associated lymphoid tissue (GALT). According to the analyses, exposure at the rate of once a week to FNV vaccine was sufficient to stimulate GALT and skin mucus antibody responses. However, more frequent exposures stimulated better responses by GALT as observed in red tilapias of groups F3D and F5D. As expected, unvaccinated red tilapias failed to stimulate any GALT development. In conclusion, vaccination using FNV vaccine stimulated mucosal and systemic immunities but the protection provided was unsatisfactory.

Following the episode of poor protection provided by oral administration of FNV vaccine, the vaccine was further modified by adding Freund's Incomplete Adjuvant (FIA) into the vaccine, a known potent immune response enhancer at both humoral and cellular levels. The feed adjuvanted vaccine (FAV) was found to improve the mucosal immune response and elicited excellent systemic immune response. Apart from higher body mucus,

both gut lavage fluid and blood serum antibody titers were also higher than the FNV vaccine. The FAV vaccine also provided 100% protection following challenged with 3.4 x 10^9 CFU/mL of live *S. agalactiae,* significantly ($p \geq 0.05$) higher than protection provided by FNV vaccine. Similarly, the size of GALT and the number of lymphocytes in the FAV-vaccinated group were significantly ($p<0.05$) greater compared to the FNV-vaccinated group.

In conclusion, this study demonstrated that adjuvanted vaccine (FAV) was more effective compared to the non-adjuvanted vaccine (FNV). The FAV vaccine effectively stimulated both mucosal and systemic immunity and enhanced protection against *S. agalactiae* infection in red tilapia. Therefore, FAV vaccine is the best candidate for control of streptococcosis in red tilapia.

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

TINDAKBALAS IMUN TERHADAP *Streptococcus agalactiae* DIDALAM IKAN TILAPIA MERAH, *Oreochromis* spp. SETELAH DIVAKSINASI DENGAN PELLET BERCAMPUR VAKSIN TANPA ADJUVANT (FNV) DAN PELLET BERCAMPUR VAKSIN DENGAN ADJUVANT

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Streptococcosis merupakan penyakit bakteria yang utama pada ikan tilapia di seluruh dunia, termasuklah Malaysia. Dua jenis spesis Streptococcus yang biasa menyebabkan penyakit streptococcosis adalah *Streptococcus agalactiae* and *Streptococcus iniae*. Di Malaysia, wabak streptococcosis di kalangan ikan tilapia merah (*Oreochromis* spp) telah di kenal pasti berpunca dari jangkitan *S. agalactiae* manakala jangkitan oleh *S. iniae* tidak pernah dilaporkan. Kematian secara besar-besaran ikan tilapia merah yang diternak dalam sistem sangkar biasanya dilaporkan berlaku semasa musim kemarau di antara bulan April hingga Ogos dimana suhu air adalah sangat tinggi pada waktu itu.

Kajian telah dilakukan untuk mengenalpasti kesan suhu air yang tinggi terhadap kerentanan ikan tilapia merah kepada jangkitan *S. agalactiae,* tindakblas imun tilapia merah setelah didedahkan kepada *S. agalactiae* hidup dan perlindungan yang diberi apabila dicabar setelah diberi vaksinasi untuk melawan streptococcosis. Untuk mencapai objektif pertama, lapan kumpulan ikan tilapia seberat kira-kira 100 g telah dijangkitkan melalui suntikan intraperitonium dengan 0.5 mL *S. agalactiae* hidup yang mengandungi 6.3 x 10^6 CFU/mL, 6.3 x 10^7 CFU/mL, 6.3 x 10^8 CFU/mL dan 6.3 x 10^9 CFU/mL. Empat kumpulan ikan tilapia merah dibela pada suhu air biasa (27 °C ± 2°C) dan yang selebihnya pada suhu air yang tinggi (33°C± 2°C) selama seminggu. Suhu air yang tinggi telah menyebabkan peningkatan kerentanan ikan tilapia merah terhadap jangkitan *S. agalactiae* seperti mana ditunjukkan melalui keputusan kadar cepatnya kematian dan dos yang mematikan 50 (LD_{50}). Jangkamasa kadar kematian 100% untuk kumpulan ikan yang dibela pada suhu air yang tinggi adalah lebih cepat berbanding kumpulan ikan yang dibela pada suhu air biasa. Kadar dos yang mematikan 50 (LD_{50}) bagi kumpulan ikan yang dibela pada suhu air yang tinggi adalah 5.68 x 10^6 CFU/mL, secara signifikannya lebih rendah ($p<0.05$) berbanding kumpulan ikan yang dibela pada suhu air biasa, 2.29 x 10^7 CFU/mL. Tanda-tanda klinikal yang diamati sepanjang kajian perkembangan penyakit ini termasuk hilangnya selera makan, kelihatan lesu, unilateral dan bilateral exophthalmia, mata beselaput, berenang tak menentu dan inflamasi pada kulit. Tanda-tanda yang diamati sangat berguna untuk mengenalpasti wabak apabila ia berlaku.

Setelah kerentanan ditentukan, sel-sel bakteria *S. agalactiae* yang dimatikan dengan formalin pada kepekatan 6.7 x 10^6 CFU/mL dicampurkan sehingga rata bersama pelet makanan ikan tilapia merah (Vaksin FNV) untuk menentang streptococcosis. Vaksin FNV kemudiannya diberi makan kepada ikan tilapia merah dalam tiga rejim vaksinasi berlainan, sekali (F1D), tiga kali (F3D) dan lima kali dalam seminggu (F5D). Sampel lendir badan dan serum darah diambil setiap minggu sehingga minggu kelapan untuk menganalisis aras antibodi ikan tilapia merah selepas divaksinasi menggunakan teknik ELISA. Pemvaksinan dengan vaksin FNV pada ikan tilapia merah telah menghasilkan peningkatan yang signifikan ($p<0.05$) pada aras antibodi serum dan mukus seawal minggu 2 dalam kumpulan-kumpulan yang divaksinasi, sementara tiada peningkatan signifikan ($p>0.05$) dalam kumpulan kawalan (FC). Aras antibodi dalam kumpulan F5D merupakan yang tertinggi, diikuti oleh kumpulan F3D dan F1D. Pada penghujung eksperimen, 20 ekor ikan tilapia merah dari setiap kumpulan dicabar secara rendaman untuk menilai perlindungan yang diperolehi daripada keimunan mukosa, sementara 20 ekor ikan tilapia yang selebihnya dari setiap kumpulan dicabar secara suntikan intraperitonium untuk menilai perlindungan yang diperolehi daripada keimunan sistemik. Kadar survival adalah rendah dan ini menandakan perlindungan hasil daripada tindak balas keimunan yang lemah dalam kumpulan-kumpulan yang diuji. Sampel usus ikan tilapia merah diambil pada minggu terakhir eksperimen dan dianalisis secara histologi untuk mengesan kehadiran "Sel Limfoid Berkaitan Usus" atau "Gut-Associated Lymphoid Tissue" (GALT). Berdasarkan analisis tersebut, pendedahan pada vaksin untuk sekali sahaja sudah mencukupi

untuk merangsang penghasilan GALT dan tindakbalas antibodi mukus. Walau bagaimanapun, lebih kerap pendedahan yang diberi merangsang tindakbalas GALT yang lebih bagus seperti apa yang dapat dilihat dalam kumpulan F3D dan F5D. Seperti dijangka, ikan tilapia merah yang tidak divaksinasi gagal untuk menghasilkan tindak balas GALT. Kesimpulannya, pemvaksinan menggunakan vaksin FNV dapat merangsang tindak balas keimunan mukosa dan sistemik tetapi perlindungan yang dihasilkan adalah tidak memuaskan. Oleh yang demikian, pemvaksinan perlu dipertingkatkan lagi untuk mendapatkan perlindungan keimunan sistemik yang berkesan pada ikan tilapia merah untuk menentang jangkitan dari *S. agalactiae.*

Susulan episod perlidungan keimunan sistemik yang tidak memuaskan diperolehi dari vaksinasi oral FNV, vaksin tersebut telah diberi penambahbaikan dengan memasukkan Freund Incomplete Adjuvant (FIA), penggalak tindak balas imun yang kuat pada peringkat humoral dan selular ke dalam formulasi vaksin. Keputusan kajian menunjukkan vaksin yang ditambah dengan adjuvant (FAV vaksin) tersebut telah meningkatkan respon keimunan mukosa dan juga berjaya menghasilkan respon keimunan sistemik yang baik. Disamping memberi aras antibodi yang lebih tinggi didalam lendir badan, cairan usus dan serum darah, vaksin FAV juga memberikan 100% perlindungan setelah dicabar dengan 3.4×10^9 *S. agalactiae* hidup, dimana secara signifikan ($p<0.05$) lebih tinggi berbanding dengan perlindungan yang diberikan oleh vaksin FNV. Saiz GALT dan jumlah limfosit yang dikira dalam kumpulan ikan yang diberi vaksin FAV juga secara signifikannya ($p<0.05$) adalah lebih tinggi berbanding dengan kumpulan ikan yang diberikan vaksin FNV. Kesimpulannya, kajian ini menunjukkan vaksin FAV adalah lebih efektif

berbanding vaksin FNV. Vaksin FAV secara efektifnya merangsang kedua-dua sistem keimunan, mukosa dan sistemik, dan meningkatkan perlindungan dari jangkitan *S. agalactiae* pada ikan tilapia merah. Oleh yang demikian, vaksin FAV merupakan calon vaksin terbaik dalam mengawal streptococcosis pada ikan tilapia merah.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

BHI	Brain Heart infusion
BSA	Bovine Serum albumin
CFU	Colony Forming Units (bacteria)
ELISA	Enzyme-Linked Immunosorbent Assay
FNV	Vaccine Incorporated Pellet
FAV	Adjuvant Vaccine Incorporated Pellet
GALT	Gut-associated Lymphoid Tissue
H&E	Hematoxylin and Eosin
M	Molar
PBS	Phosphate-Buffered Saline
rpm	Revolutions per minute
RM	Malaysian Ringgit
OD	Optical Density
S. agalactiae	*Streptococcus agalactiae*
S. iniae	*Streptococcus iniae*
USD	United States Dollar

CHAPTER 1

INTRODUCTION

Streptococcus agalactiae is an important pathogenic bacterium that infects various species of animals. *Streptococcus agalactiae* has a broad host range and is pathogenic to mammals, reptiles, amphibians and fish (Elliott *et al.*, 1990). *Streptococcus agalactiae* causes bovine mastitis in cow, characterized by inflammation of the parenchyma of the mammary gland in the presence of significantly increased leukocyte content in milk (Meiri-Bendek *et al.*, 2002). In human, *S. agalactiae* causes sepsis in neonates and their mothers, may lead to overwhelming infection without much localization (septicemia) or predominantly localized in the lung causing pneumonia or the brain causing meningitis (Fanrong *et al.*, 2005). *S. agalactiae* infection or streptococcosis was reported to cause significant mortalities among numerous wild and cultured fish species, including menhaden (*Brevoortia patronus*) (Plumb *et al.*, 1974), bullminnows (*Fundulus grandis*) (Rasheed and Plumb, 1984), striped bass (*Morone saxatilis*) (Baya *et al.*, 1990), mullet (*Liza klunzingeri*), and tilapia (*Oreochromis niloticus*) (Evans *et al.*, 2002).

Streptococcosis is a septicaemic disease affecting both captive and wild fish in all water bodies (Evans *et al.*, 2002). It affects large-sized tilapias, particularly those weighing at least 100 grams (Amal *et al.*, 2009). Currently, the disease has become a major problem in many tilapia farms and there is still no effective control measure. The two common *Streptococcus* sp. found

to infect tilapia were *S. agalactiae* (Pasnik *et al.*, 2005) and *S. iniae* (Intervet, 2006).

Streptococcosis has proven to be very difficult to control with antibiotics. According to Agriculture Research Service (ARS), United States Department of Agriculture (USDA), no antibiotic is available to treat the pathogen at this time (Agriculture Research Service, 2010). Moreover, the infection is usually transmitted from fish to fish where the bacteria were released from dead and dying fish that was considered as the most important source of infection (Kitao, 1993). Hence, vaccination is considered as the effective way to control the disease. In Malaysia, high incidences of *S. agalactiae* infection in red tilapia were reported during dry months between April and July, where water temperature was high (Siti-Zahrah *et al.*, 2005; Amal *et al.*, 2008).

Vaccination of fish by intraperitonial injection and bath immersion against bacterial infections has been proven to be a commercial success (Lamers, 1985). However, oral vaccination, with antigen being incorporated into a feed, is potentially the most appropriate method for mass vaccination of fishes of all sizes. This route of vaccination avoids the stress and reduces time and labour cost whilst allowing a more flexible approach to the formulation of immunization regime, particularly important during handling large numbers of fish (Hart *et al.*, 1987). Oral vaccination is also easy to apply on all production facilities and is effective on all sizes of fish, making it the most preferred method by fish farmers (Le Breton, 2009). Immunization through oral or anal routes resulted in antigen-specific antibodies in skin mucus, bile and intestine of several fish species (Fletcher and White, 1973; Roumbout *et al.*, 1986).

The significant response by the mucosal immune system makes oral vaccination more promising since the first contact with pathogens occurs usually through mucosal surfaces (Manganaro *et al.*, 1994).

Thus, the objectives of this study was:

1. to determine the pathogenecity of *S. agalactiae* in normal and temperature-stressed red tilapia (*Oreochromis* spp).
2. to evaluate the protective capacity provided by an orally delivered incorporated feed vaccine (FNV) against streptococcosis in red tilapia (*Oreochromis* spp).
3. to enhance the protection provided by the newly developed oral vaccine (FAV) against streptococcosis in red tilapia (*Oreochromis* spp).

Hypotheses of this study was:

1. *S. agalactiae* is more virulence in temperature-stressed red tilapia rather than in non-stressed red tilapia.
2. orally delivered incorporated feed vaccine (FNV) are able to give protection against streptococcosis in red tilapia (*Oreochromis* spp.).
3. Newly developed oral vaccine (FAV) are able to enhance the protection against streptococcosis in red tilapia (*Oreochromis* spp.).

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