FIELD EVALUATION OF FEED-BASED RECOMBINANT PROTEIN-ADJUVANTED VACCINE AGAINST STREPTOCOCCOSIS IN RED HYBRID TILAPIA (Oreochromis sp.)

NADIRAH ABU NOR

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By

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Chairman : Assoc. Prof. Md Sabri Mohd Yusoff, PhD

Faculty : Veterinary Medicine

Streptococcosis is a fish diseases that are reported in aquaculture systems and can occur in both marine and freshwater fish. This disease is an important bacterial disease in tilapia in many countries including Malaysia. High mass mortalities of Red hybrid tilapia (*Oreochromis* sp.) have been associated with *S. agalactiae*, frequently recorded between April and July which depicts the dry season, where the bacterium was isolated from infected Red hybrid tilapia in cage-culturing system in Malaysia. Oral vaccination is a good technique since it required no handling fish, not stressful method, does not require extensive labour, less time consuming and it is may be very effective in fish industry. There is paucity of information on the efficacy of the feed-based recombinant protein- adjuvant vaccine (FRAV) against streptococcosis in tilapia with special regard to humoral and mucosal antibody responses. Therefore, this study was to evaluate the efficacy of the said vaccine, utilizing serum IgM antibody, mucus IgM antibody and gut lavage IgM antibody responses as well as development of gut-associated lymphoid tissue (GALT) in Red hybrid tilapia (*Oreochromis* sp.) in Kenyir Lake, Hulu Terengganu, Terengganu, Malaysia. This study also conducted to determine the oxidative stress level and mortality in tilapia under heat stress.

A commercial Red hybrid tilapia was experimented with *S. agalactiae* infection under influences of heat stress using plasma malondialdehyde (MDA) and erythrocyte superoxide dismutase (SOD) as biomarkers of stress. To achieve these objectives, 110 fish in good health were divided into five groups of 22 fish in each group. Group A was challenged with 2.3×10^9 CFU of *S. agalactiae* and heat stress at $33 \pm 0.5°C$ on day 1. Group B was challenged on day 1 as in Group A but heat stress was introduced on day 7

post challenge (pc). Group C was exposed to heat on day 1 and challenged on day 7 pc while group D was challenged on day 1 with no heat stress apply and served as a positive control group. Group E was neither introduced to heat nor challenged (negative control group). Blood samples were collected at day 0, 3, 7, 10 and 14 for MDA and SOD analysis. Overall, Group A, Group B, Group C and Group D showed pattern of increment in MDA analysis as early as on day 3 and decrement pattern for SOD analysis. Group E did not show any significant difference in MDA activities throughout the study period.

Besides, study of the immune response of tilapia vaccinated against *Streptococcus. agalactiae* using a FRAV in a field trial both in rainy and dry seasons was evaluated and mucosal immunity specifically gut-associated lymphoid tissue (GALT) of tilapia was assessed too. To achieve these objectives, 4000 of Red hybrid tilapia were divided into two groups with duplicate. Before the start of experiment, 100 fish was screening by sacrificed them for bacterial isolation. Group 1 and Group 2 were vaccinated group and Group 3 and Group 4 were served as control groups. At month 1, all fish from vaccinated groups were fed with FRAV thrice on day 1. Fish in the control unvaccinated groups were fed with a standard commercial pellet. Booster dose was performed in month 2 of post-vaccination (pv). Fifty fish from each group were sacrificed monthly for sampling by taking their serum, body mucus and gut-lavage fluids to evaluate for antibody responses using indirect ELISA, gut for identification the development of GALT and bacterial isolation for bacteria identification. The result of ELISA showed significant increment ($P < 0.05$) of all types of antibodies in vaccinated groups when compared to the control groups throughout the study. According to the analyses, exposure to the FRAV was sufficiently to stimulate the aggregation of lymphocytes and development of GALT in vaccinated groups while for control groups; Group 3 and Group 4 only normal scattered of lymphocytes were observed. Bacterial isolation was also performed from the captured fish, which brain, eyes and kidney are used for isolation of *S. agalactiae*. None of the groups studied, shows the presence of *S. agalactiae*.

In conclusion, this study proved that this FRAV can confer possible protection against streptococcosis to the Red hybrid tilapia either in dry or rainy seasons in Malaysia. Vaccination using FRAV also can stimulate both mucosal and systemic immunities. Therefore, FRAV is the best alternative choice of candidate for the control of streptococcosis in Red hybrid tilapia particularly in Malaysia.

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

PENILAIAN LAPANGAN UNTUK VAKSIN PROTEIN- ADJUVAN REKOMBINAN TERHADAP STREPTOCOCCOSIS PADA IKAN TILAPIA HIBRID MERAH (*Oreochromis* sp.)

Oleh

NADIRAH BINTI ABU NOR

April 2015

Pengerusi : Profesor Madya Md Sabri Mohd Yusoff, PhD

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Streptococcosis adalah salah satu penyakit ikan yang telah dilaporkan dalam sistem akuakultur dan streptococcosis boleh berlaku dalam marin dan air tawar. Di Malaysia, jisim kematian Tilapia Hibrid Merah (*Oreochromis* sp.) adalah disebabkan oleh *S. agalactiae* biasa berlaku diantara bulan April dan Julai dimana kemuncak musim panas di Malaysia. Vaksinasi melalui oral adalah teknik yang bagus kerana teknik ini tidak memerlukan pengendalian terhadap ikan, kaedah yang tidak memberi tekanan kepada ikan dan sangat efektif dan berkesan dalam industri ikan. Terdapat kekurangan maklumat tentang keberkesanan vaksin protein-adjuvan rekombinan terhadap tindak balas antibodi dalam mukus dan usus ikan. Oleh itu, kajian ini adalah untuk menilai keberkesanan vaksin tersebut, menggunakan antibodi IgM pada serum, antibodi IgM pada mukus dan antibodi IgM pada usus selain tindak balas "gut-associated lymphoid tissue" (GALT) terhadap Tilapia Hibrid Merah (*Oreochromis* sp.) di Tasik Kenyir, Hulu Terengganu, Terengganu, Malaysia. Kajian ini juga dijalankan untuk mengenalpasti tahap tekanan oksidatif terhadap tilapia selain mengenalpasti kematian ikan dibawah pengaruh tekanan haba.

Tilapia Hibrid Merah komersial telah dikaji dengan menjangkitan *S. agalactiae* dibawah pengaruh tekanan haba menggunakan "plasma malondialdehyde" (MDA) dan "erythrocyte superoxide dismutase" (SOD) sebagai penanda bio-tekanan. Untuk mencapai objektif ini, seratus sepuluh ekor ikan telah dibahagikan kepada lima kumpulan yang berbeza, dimana terdapat 22 ekor ikan bagi setiap kumpulan. Kumpulan A telah dicabar dengan 2.3 x 10^9 CFU *S. agalactiae* dan diberi tekanan haba pada 33± 0.5ºC pada hari pertama. Kumpulan B telah dicabar pada hari pertama dan tekanan haba telah

diberikan pada hari ke 7 "post-challenge" (pc). Kumpulan C telah didedahkan dengan haba pada hari pertama dan cabaran pada hari ke-7 pc dimana Kumpulan D pula telah dicabar pada hari pertama tanpa tekanan haba dan bertindak sebagai kawalan positif. Kumpulan E bertindak sebagai kawalan negatif. Sampel darah telah diambil untuk analisa MDA dan SOD. Secara keseluruhan, Kumpulan A, Kumpulan B, Kumpulan C dan Kumpulan D menunjukkan corak kenaikan dalam analisa MDA seawal hari ke 3 dan menunjukkan corak penurunan dalam analisa SOD. Kumpulan E tidak menunjukkan apa-apa perubahan terhadap aktiviti MDA sepanjang tempoh kajian.

Disamping itu, kajian tentang tindakbalas imun ikan tilapia selepas divaksinkan dengan *S. agalactiae* menggunakan vaksin protein-adjuvan rekombinan berdasarkan makanan dalam percubaan lapangan di kedua-dua musim hujan dan panas telah di nilai dan pertahanan mukosa terutamanya "gut-associated lymphoid tissue" (GALT) ikan tilapia yang telah divaksinkan dengan vaksin protein-adjuvan rekombinan telah ditentukan juga. Untuk mencapai objektif-objektif ini, 4000 ikan Tilapia Hibrid Merah telah dibahagikan kepada dua kumpulan dengan duplikasi. Sebelum menjalankan kajian, 100 ekor ikan telah disaring dengan dibunuh untuk pemencilan bakteria. Kumpulan 1 dan Kumpulan 2 adalah kumpulan yang telah divaksinkan dengan vaksin protein-adjuvan rekombinan dan Kumpulan 3 dan Kumpulan 4 adalah sebagai kumpulan kawalan dan diberi makan pelet komersial yang biasa. Penvaksinan Kumpulan 1 dan Kumpulan 2 telah diberikan vaksin sebanyak tiga kali sehari pada hari pertama dalam bulan pertama kajian. Dos penggalak telah diberikan pada bulan ke 2 pos-pemvaksinan. 50 ekor ikan di setiap kumpulan telah dikorbankan setiap bulan. Serum, mukus dan air usus telah dikumpulkan untuk dinilaikan tindakbalas antibodi mengunakan kaedah ELISA tidak langsung, usus ikan untuk mengenalpasti kewujudan GALT dan pemencilan bakteria untuk mengenalpasti kehadiran bakteria *S. agalactiae*. Kumpulan yang telah divaksinkan menunjukkan peningkatan antibodi IgM dalam semua sampel (serum, mukus dan cecair usus) berbanding kumpulan kawalan. Berdasarkan keputusan analisa, pembentukan kumpulan limfosit dan pengembangan GALT dapat dilihat pada Tilapia Hibrid Merah dalam kumpulan yang telah divaksinkan manakala hanya taburan "lymphocyte" biasa dan normal dapat dilihat dalam kumpulan kawalan; Kumpulan 3 dan Kumpulan 4. Pemencilan bakteria turut dilakukan daripada ikan yang telah dikumpulkan, iaitu daripada otak, mata dan buah pinggang untuk memastikan kehadiran *S. agalactiae*.

Kesimpulannya, kajian ini menunjukkan bahawa vaksin protein-adjuvan rekombinan ini mampu memberikan perlindungan terhadap penyakit streptococcosis terhadap Tilapia Hibrid Merah dalam kedua-dua musim di Malaysia iaitu musim panas dan musim hujan. Penvaksinan menggunakan FRAV juga boleh merangsang kedua-dua sistem imun iaitu mukosa dan sistemik. Oleh itu, FRAV adalah alternatif yang terbaik untuk mengawal streptococcosis terhadap Tilapia Hibrid Merah.

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I certify that a Thesis Examination Committee has met on 23 April 2015 to conduct the final examination of Nadirah Binti Abu Nor on her thesis entitled "Field Evaluation of Feed-Based Recombinant Protein-Adjuvanted Vaccine Against Streptococcosis in Red Hybrid Tilapia (*Oreochromis* sp.)" in accordance with the Universities and University College 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

BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CAT	Catalase
CP	Capsular Polysacharides
CFU	Colony Forming Units
DI	Direct Immersion
ELISA	Enzyme Link Immunosorbent Assay
FRAV	Feed-based Recombinant Protein-Adjuvanted Vaccine
GALT	Gut-Associated Lymphoid Tissue
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
H&E	Hematoxylin and Eosin
HI	Hyperosmotic Infiltration
MDA	Malondialdehyde
OD	Optical Density
PBS	Phosphate Buffer Saline
PC	Post- challenge
PV	Post- vaccination
ROM	Reactive Oxygen Metabolite
ROS	Reactive Oxygen Species
rpm	Revolution per Minute
SOD	Superoxide Dismutase
S. agalactiae	*Streptococcus agalactiae*
S. inae	*Streptococcus inae*

CHAPTER 1

INTRODUCTION

Streptococcus agalactiae is one of the causative agents associated with warm-water streptococcosis that produces massive mortality in aquaculture (Nazrin *et al*., 2012) and these outbreaks can occur in both marine and freshwater fish. This disease can cause high mortality rates of more than 50% (Yanong and Francis Floyd, 2006). From source of Aquatic Community Tropical Fish (2008), fish weighing at least 100 grams are more susceptible to streptococcosis compared to smaller fish.

Streptococcus sp. which can cause diseases in fish includes *S. agalactiae* (Suanyuk *et al*., 2005), *Streptococcus iniae* (Shoemaker *et al*., 2000) and *Streptococcus difficile* (Berridge *et al*., 2001). Fish usually become stressed due to heat stress, high nitrate level and low dissolved oxygen and these predisposing factors do increase the occurrence of streptococcosis outbreak. The physical signs of streptococcosis include erratic swimming, anorexia, exophthalmia and ascites (Evans *et al*., 2002; Salvador *et al*., 2005) and meningoencephalitis (Eldar *et al*., 1994). Pathogenesis of the disease in infected fish includes organs colonization of *S. agalactiae* such as brain, kidney, nares and intestines (Pasnik *et al*., 2005) occurred by infected dead fish or fishes with wound or cut besides septicaemia. Therapeutic measures which involved chemicals medicine are usually ineffective and therefore, development of vaccines is essential for the control of the disease (Carmen *et al*., 2004).

During last 10 to 20 years vaccination has become an important method in preventing the infectious diseases in farmed fish (Gudding *et al*., 1999). Craig *et al*. (2009) reported that development of vaccines in aquaculture have reduced the used of antibiotic in fish production. Besides, vaccination is a cheaper method and the most effective in preventing infectious diseases (Lombard *et al.,* 2007). Inactivating the microorganisms either by heat or chemical made the whole-cells vaccine and this type of vaccine may not always induce an immune response nor long lived. Since recombinant vaccine is just a single protein, it is more preferable because no chance of the host becoming ill from the agent. Oral vaccines was proposed as a good technique since it required no handling fish, not stressful method, not require extensive labour and less time consuming (Newman, 1993; Alabi *et al*., 1999). Oral immunization systems may be very effective in fish industry, especially when vaccination through injection is not practicable (Jaime *et al*., 2011). From oral vaccination, when the antigens reach the fish intestine, it may stimulate the development of GALT. Gut-associated lymphoid tissues were present in all part of the gut either in small aggregations of lymphocytes or scattered individual lymphocytes cells in the lamina propria or epithelium (Dogget and Harris, 1991).

For oral vaccination, the protection provided is slightly longer than immersion vaccine which is within eight months, and is adequate for tilapia production cycle (Le Breton, 2009). Stress to the fish must be prevented because it can depress aspects of the body's

immune system and also reduce the vaccine effectiveness of the vaccine (Alan *et al*., 1994; Ellis, 1988). Adjuvant is also believed to promote better protection in immune responses as it is also an immuno-enhancer that enhances the protective immunity on targeted diseases. Xu-Dong *et al*. (2010) stated that this is because of the main role of Freund's adjuvant, which has strong promoter effect on both cellular and humoral immune response. The role of adjuvant is to improve the presentation of antigen to immune-competent cells and slow down the release of antigens (Audibert and Lise, 1993). IgM has pentameric shape in cartilaginous fish and higher vertebrates (Kobayashi *et al.*, 1984), tetrameric shape in teleosts (Acton *et al.*, 1971). IgM is the first antibody to appear in fish evolution and commonly the only antibody class described in fish (Bergljót and Buvisindi, 1998). That is the reason why only IgM was study in this project.

Currently there is limited information on the field trial of the feed-based recombinant protein-adjuvant vaccine in both rainy and dry seasons which temperature will be the most important factor in contributing heat stress in fish. The need to evaluate the efficacy of the feed-based recombinant protein-adjuvant vaccine against *S. agalactiae* infection in a field trial and/or the pathogenicity of the disease is expedient, thus, the objectives of this study were:

1. to determine the influence of heat stress on oxidative status of Red hybrid tilapia (*Oreochromis* sp.) infected with *S. agalactiae.*
2. to determine the immune response of tilapia vaccinated against *S. agalactiae* using a feed-based recombinant protein-adjuvanted vaccine in both rainy and dry seasons in field trial.
3. to determine the mucosal immunity specifically gut-associated lymphoid tissue (GALT) of tilapia vaccinated with a feed-based recombinant protein-adjuvanted vaccine against *S. agalactiae*.

Hypothesis:

1. H_O: Feed-based recombinant protein- adjuvanted vaccine is unable to give protection against streptococcosis in Red tilapia hybrid (*Oreochromis* sp.) in rainy and dry seasons in field trial
   H_A: Feed-based recombinant protein- adjuvanted vaccine is able to give protection against streptococcosis in Red hybrid tilapia (*Oreochromis* sp.) in rainy and dry seasons in field trial

2. H_O: Challenge with *S. agalactiae* and/or administration of the feed-based recombinant protein- adjuvanted vaccine against *S. agalactiae* does not induce development of gut-associated lymphoid tissue (GALT).
   H_A: Challenge with *S. agalactiae* and/or administration of the feed-based recombinant protein- adjuvanted vaccine against *S. agalactiae* could induce development of gut-associated lymphoid tissue (GALT).

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Appendix A

Antigen and Buffers Used for ELISA

Appendix A1: Preparation of Antigen for ELISA

1. Stock culture of *Streptococcus agalactiae* isolated and characterized from outbreak of streptococcosis in Kenyir Lake was obtained from the microbiology laboratory of the faculty of veterinary medicine, University Putra Malaysia. The bacteria were culture onto blood agar (10% caprine blood) and incubated at 30°C for 24 hours.
2. Ten same sized colonies were selected and inoculated into 50 ml brain-heart infusion broth and then incubated at 30°C for 24 hours. The bacteria colony-forming units (CFU) of the grow *Streptococcus agalactiae* in brain-heart infusion broth was estimated using standard total plate count method.
3. The inoculum was then washed for three times with phosphate buffered saline (PBS) to get rid of the brain-heart infusion broth. In between each washing, the inoculums was centrifuged at 6000 rpm for 30 minutes.
4. The pellet was the re-suspend in carbonate bicarbonate coating buffer (pH 9.6) and boiled in water bath at 98°C for 20 minutes.
5. The suspension was then cooled and dispersed into 1ml vials and kept frozen at -20°C until used in ELISA procedure.

Apppendix A2: Phosphate Buffer Saline (PBS)

Sodium Chloride	8.00g
Potassium di-Hydrogen Orthophosphate	0.20g
Sodium Hydrogen Orthophosphate Dodecahydrate	2.90g
Potassium Chloride	0.20g
H_2O	1 Liter

Dissolve all the salts into distilled water and adjust pH to pH 7.4, then autoclaved at 121°C for 15 minutes

Appendix A3: Buffer for ELISA

1. Coating Buffer (Carbonate/bicarbonate, 0.05M, pH 9.6)

Sodium Carbonate	1.59g
Sodium bi-Carbonate	2.93g
H_2O to 1 Liter	

2. Washing Buffer (pH 7.4)

Sodium Chloride	8.00g
Potassium di-Hydrogen Orthophosphate	0.20g
Sodium Hydrogen Orthophosphate Dodecahydrate	2.90g
Potassium Chloride	0.20g
Tween 20	0.5 ml
H_2O to 1 Liter	

3. Phosphate Buffer Saline/ Bovine Serum Albumin, Tween 20 (pH 7.4)

Sodium Chloride	8.00g
Potassium di-Hydrogen Orthophosphate	0.20g
Sodium Hydrogen Orthophosphate Dodecahydrate	2.90g
Potassium Chloride	0.20g
Tween 20	0.5 ml
Bovine Serum Albumin (BSA)	10.0g
H_2O to 1 Liter	

4. Substrate Buffer

Add 0.5M citric acid to 500 ml of 0.6M sodium acetate until pH 6.0. Adjust volume to 600 ml. Before use, dilute 1:5 distilled water to give 0.1M sodium acetate/ citric acid buffer of pH 6.0.

5. Substrate (pH 6.0)

Tetramethylbenzidine (TMB) 10.0µg	
Dimethyl Sulphoxise (DMSO)	100µl
0.1M sodium acetate/ citric acid buffer	9.9 ml
Hydrogen Peroxide (33% w/v)	1.5µl

- Dissolve TMB in DMSO before adding into buffer.
  Note: Make fresh every time used

6. Sulphuric Acid (2M)

Sulphuric acid ml	109.9
H_2O to 1 Liter	

Appendix B

Medium for Bacterial Culture and Determination of Colony Forming Unit (CFU)

Appendix B1: Blood Agar (DIFCO Lab ® Colombia Blood Agar (EH # 0790-17-5)

1. Suspend 37g of Blood Agar Base in 1 Litre distilled water and mixed well.
2. Sterile by autoclave at 121°C for 15 minutes.
3. Cool to 45°C-50°C in water bath.
4. Aseptically add 5% defibrinated caprine blood and mix homogenously.
5. Pour into petri dish and store at 4°C.

Blood agar was used for isolating, culturing and determination of colony – forming unit (CFU).

Appendix B2: Brain – Heart Infusion (BHI) Broth (Oxoid Code CM375)

1. Suspend 37g of Brain – Heart Infusion in 1 Litre distilled water and mixed well.
2. Transfer into bottle.
3. Sterile by autoclave at 121°C for 15 minutes.

Brain – Heart Infusion (BHI) Broth was used to prepare inoculums during determination of colony forming unit (CFU)

Appendix B3: Peptone water (Oxoid 500g)

1. Suspend 15g Peptone Water powder in 1 litre distilled water and boil to dissolve homogenously.
2. Distribute into universal bottles.
3. Sterile by autoclave at 121°C for 15 minutes.

Peptone water was used to dilute *Streptococcos agalactiae* inoculums during determination of CFU

Appendix C
Gram Stain Protocol

Appendix C1: Gram Staining

1. Add one drop of normal saline onto glass slide
2. Sterilize loop by burning in flame and take one colony from pure culture and mix with normal saline on glass slide. Spread equally.
3. Let the slide dry at room temperature.
4. Flood slide with Crystal Violet solution for one minute.
5. Wash slide with running tap water for 5 seconds.
6. Rinse off excess water and flood the slide with Lugol's Iodine solution for one minute.
7. Wash slide with running tap water for 5 seconds.
8. Decolorize the slide by using Acetone (95%) for two second and wash immediately with running tap water.
9. Rinse off excess water and flood slide with Safranin Solution for 30 seconds.
10. Wash slide with running tap water for 5 seconds.
11. Dry the slide before examine under microscope.

BIODATA OF THE STUDENT

Nadirah Binti Abu Nor was born and raised in Kampung Belimbing Dalam, Durian Tunggal, Melaka, Malaysia, on February 3, 1990. She is a daughter of Abu Nor Bin Abdul Raoh and Rahmah Binti Ibrahim. She completed her primary school in Sekolah Kebangsaan Belimbing Dalam, Melaka, Malaysia (1997-2002). She then went to Sekolah Menengah Kebangsaan Durian Tunggal, Melaka, Malaysia for her secondary school from Form 1 to Form 3 (2003-2005) and continued her Form 4 to Form 5 in Sekolah Menengah Sains Muar, Muar Johor, Malaysia (2006-2007) where she obtained her Sijil Pelajaran Malaysia (SPM). She continued her studied in Matriculation level for 1 year in Life Sciences at Kolej Matrikulasi Johor (2008). In 2009, she pursued her study at Universiti Putra Malaysia, Selangor, Malaysia where she graduated with Bachelor (Honours) in Microbiology.

She commenced the Master of Science Degree in the field of Immunology under the supervision of Associate Professor Dr. Md. Sabri Bin Mohd Yusoff at Faculty of Veterinary Medicine, Universiti Putra Malaysia in September 2012. She was involved in a research project to study the field evaluation of efficacy of feed-based recombinant protein-adjuvanted vaccine against streptococcosis in Red hybrid tilapia.

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