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DEVELOPMENT OF MONOVALENT RECOMBINANT VACCINE AGAINST *Streptococcus* INFECTION IN HYBRID RED TILAPIA (*Oreochromis* spp.)

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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 MONOVALENT RECOMBINANT VACCINE AGAINST *Streptococcus* INFECTION IN HYBRID RED TILAPIA (*Oreochromis* spp.)

By

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Tilapia (*Oreochromis niloticus*) is one of the most cultured species in the world. However, bacterial disease caused by *Streptococcus* spp. is one of the major problems affecting farmed tilapia worldwide. Hybrid red tilapia is highly susceptible to this disease and mortality may reach up to 70% within a week. Thus, the study was conducted to develop a recombinant vaccine of *S. agalactiae,* Millud II and *S. iniae*, TSK_2 previously isolated from infected tilapia. The isolates were successfully characterised as *S. agalactiae* and *S. iniae* respectively by species-specific PCR assay. The result confirms the amplification of 300 bp amplicons of 16S rRNA gene and sequence analysis demonstrated 98% similarity with complete genome of *S. iniae,* TSK_2 in BLASTn study. In addition, PCR amplification with primers specific for *S. agalactiae* 16S rRNA confirmed the target amplicons of 220 bp from positive control of bacterial DNA for *S. agalactiae,* Millud II.

In order to analyse the antigenicity of *S. iniae* and *S. agalactiae* proteins, outer surface proteins (OSPs) of these two bacteria were extracted by sodium lauryl-sarcosinate method and separated by SDS-PAGE. Protein profiling revealed 40 and 52 kDa for *S. iniae,* while only 52 kDa was observed for *S. agalactiae* as major proteins. Western immunoblot results demonstrated that rabbit anti-serum of *S. iniae* could able to detect the antigenic proteins for *S. iniae* and *S. agalactiae* in both the homologous and heterologous reactions whereas; the antiserum of *S. agalactiae* was unable to detect cross-antigenicity. Thus, the immunogenic gene encoding 52 kDa of *S. iniae* was successfully cloned in pET-32 Ek/LIC plasmid and transformed into *E. coli* BL21 (*DE3*). The cloned sequence was identified as 1305 bp nucleotide sequence of sip gene coding for polypeptides of 434 amino acid residues with 99% similarity to other surface immunogenic gene (sip) of *S. agalactiae* in GenBank.

To evaluate efficacy of developed vaccine, 450 fish was vaccinated via intraperitoneal injection with an inoculum containing 250 µl of 1.0 x 10^7 CFU/ml per fish. Group 1 (90 fish) was vaccinated with formalin killed (FK) recombinant vaccine, group 2 (90 fish) with FK whole cell of *S. iniae*, group 3 (90 fish) with FK whole cell of *S. agalactiae,* group 4 (90 fish) with FK *E. coli* and group 5 (90 fish) was unvaccinated control. Finally all fish were i.p. challenged on week 4 with an inoculum containing 1.32 × 10^9 CFU/ml live *S. agalactiae* and *S. iniae* in different aquariums. Results showed that the recombinant vaccine provides 60% protection against *S. agalactiae* whereas 100% defence against *S. iniae.* ELISA assay of serum, mucus and gut lavage

fluid of vaccinated and post challenged fish demonstrated significant ($p < 0.05$) level of systemic and mucosal immunity in recombinant vaccine (group 1). Overall, the developed recombinant vaccine is highly effective to control *S. iniae* virulence in hybrid red tilapia but have moderate efficacy when challenged with *S. agalactiae*, suggesting that the vaccine is partially cross protective. Therefore, further improvement may be carried out in the future to induce outstanding protection against streptococcosis.

Keywords: streptococcosis, antigenicity, recombinant vaccine, tilapia

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PEMBANGUNAN VAKSIN REKOMBINAN MONOVALEN TERHADAP JANGKITAN *Streptokokus* DALAM TILAPIA MERAH HIBRID (*Oreochromis* spp.)

Oleh

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Tilapia (*Oreochromis niloticus*) adalah salah satu spesies paling berbudaya di dunia. Walau bagaimanapun, penyakit bakteria yang disebabkan oleh *Streptococcus spp*. adalah salah satu masalah utama yang mempengaruhi tilapia ternakan di seluruh dunia. Ikan tilapia merah hybrid sangat mudah terdedah kepada penyakit ini dan kematian mungkin mencapai sehingga 70% dalam masa seminggu. Oleh itu, kajian ini dijalankan untuk membangunkan vaksin rekombinan *S. agalactiae*, Millud II dan *S. iniae*, TSK_2 yang dipencil daripada tilapia yang dijangkiti dengan bakteria *Streptococcus*. Pengasingan telah berjaya dicirikan sebagai *S. agalactiae* dan *S. iniae* masing-masing oleh spesifik PCR esei. Hasil keputusan mengesahkan penguatan 300 bp daripada 16S rRNA gen dan analisis jujukan menunjukkan persamaan 98% dengan genom lengkap *S. iniae*, TSK_2 dalam analisis BLASTn. Tambahan pula, amplifikasi PCR dengan primer spesifik untuk *S. agalactiae* 16S rRNA mengesahkan 220 bp dari kawalan positif DNA bakteria untuk *S. agalactiae*, Millud II.

Untuk menganalisis antigenisiti protein *S. iniae* dan *S. agalactiae*, protein permukaan luar (OSP) kedua-dua bakteria telah diekstrak dengan kaedah natrium lauryl-sarcosinate dan dipisahkan oleh SDS-PAGE. Analisi profil protein menunjukkan 40 dan 52 kDa untuk *S. iniae*, sementara hanya 52 kDa diperhatikan untuk *S. agalactiae* sebagai protein utama. Hasil immunoblot barat menunjukkan bahawa anti-serum *S. iniae* dapat mengesan protein antigen untuk *S. iniae* dan *S. agalactiae* dalam kedua-dua tindakbalas homolog dan heterolog manakala antiserum *S. agalactiae* tidak dapat mengesan tindakbalas *cross-antigenicity* untuk protein *S. inae* . Oleh itu, pengkodan gen immunogenik 52 kDa *S. iniae* telah berjaya diklonkan dalam plasmid pET-32 Ek / LIC dan diubah menjadi *E. coli* BL21 (*DE3*). Jujukan klon dikenal pasti sebagai jujukan nukleotida 1305 bp pengekstrakan gen sip untuk polipeptida daripada 434 residu asid amino dengan kesamaan 99% kepada gen immunogenik permukaan lain (sip) *S. agalactiae* dalam GenBank.

Untuk menilai keberkesanan vaksin yang telah dibangunkan, 450 ikan telah divaksin melalui suntikan intraperitoneal dengan inokulum yang mengandungi 250 µl 1.0 x 10^7 CFU/ml per ikan. Kumpulan 1 (90 ikan) telah diberi vaksin dengan vaksin rekombinan formalin terbunuh (FK), kumpulan 2 (90 ikan) dengan FK seluruh sel *S. iniae*, kumpulan 3 (90 ikan) dengan FK seluruh sel *S. agalactiae*, kumpulan 4 (90 ikan) dengan FK *E. coli* dan kumpulan 5 (90 ikan) adalah sebagai kawalan . Di akhir eksperimen, semua ikan adalah diinfeksi secara i.p pada minggu ke 4

dengan inokulum yang mengandungi dos hidup *S. agalactiae* dan *S. iniae* sebanyak 1.32×10^9 CFU/ml di akuarium yang berbeza. Keputusan menunjukkan bahawa vaksin rekombinan menyediakan perlindungan 60% terhadap *S. agalactiae* manakala pertahanan 100% terhadap *S. iniae*. Ujian ELISA serum, lendir dan cecair lavage ikan yang divaksin dan pasca-infeksi menunjukkan tahap imuniti sistemik dan mukosa tinggi dalam vaksin rekombinan (kumpulan 1).

Hasil kajian secara keseluruhannya menunjukkan vaksin rekombinan yang dihasilkan adalah berkesan untuk mengawal virulensi *S. iniae* dalam tilapia merah hibrid tetapi mempunyai keberkesanan yang sederhana apabila diinfeksi dengan *S. agalactiae.* Ini mencadangkan bahawa vaksin rekombinan itu sebahagiannya melindungi pelindung. Oleh itu, penambahbaikan selanjutnya boleh dilakukan pada masa akan datang untuk mendorong perlindungan yang luar biasa terhadap streptococcosis.

Kata kunci: streptococcosis, antigenisiti, vaksin rekombinan, tilapia

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I certify that an Examination Committee has met on 14 December 2018 to conduct the final examination of Muhammad Rahmatullah on his PhD thesis entitled "Development of monovalent recombinant vaccine against streptococcus infection in hybrid red tilapia (*Oreochromis* spp.)" 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 (Fish Health Management).

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LIST OF ABBREVIATIONS

µL	Microliter
µg	Microgram
%	Percentage
^{0}C	Degree Celsius
1x	One time
β	Beta
λ	Lamda
µm	Micrometer
µM	Micromolar
BLAST	Basic local alignment search tool
BHI	Brain heart infusion
BSA	Bovine serum albumin
Bp	Base pair
Cfu	Colony forming unit
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide triphosphate
ds	Double stranded
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorband acid
EtBr	Ethidium Bromide
Fk	Formalin killed
g	Gram
GALT	Gut-associated lymphoid tissue
h	hour
hpi	hour post inoculum
H_2O	Water
H&E	Hematoxilin and Eosin
i.e.	For example
IgM	Immunoglobulin M
in vitro	In an experimental situation outside the organism
in vivo	In a living cell or organism
IPTG	Isopropyl-β-D-thiogalacosidase
Kb	Kilobase pair
kDa	Kilodalton
LB	Luria broth
L	Liter
M	Molar
mA	Miliampere
MAb	Monoclonal antibody
MCS	Multiple cloning site
Mg	Milligram
$MgCl_2$	Magnesium choloride
mRNA	Messenger ribonucleic acid
ml	Milliliter
mM	Milimolar
MW	Molecular weight
ng	Nanogram
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
OD	Optical density

ORF	Open reading frame
OSP	Outer surface protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain recation
pH	Puissance hydrogen
PVDF	Polyvinylidene difluoride
RCF	Relative centrifuge force
RE	Restriction enzyme
RM	Malaysian Ringgit
RPM	Rotation per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
R vac	Recombinant vaccie
rRNA	Ribosomal ribonucleic acid
S. agalactiae	*Streptococcus agalactiae*
S. iniae	*Streptococcus iniae*
Sa vac	*Streptococcus agalactiae* vaccine
Si vac	*Streptococcus iniae* vaccine
SDS-PAGE	Sodyum dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus YT-1*
TBE	Tris-Borate-EDTA
TBS	Tris-buffer saline
TE	Tris-EDTA buffer
Tm	Melting temperature
U	Unit
UV	Ultra-violet
V	Volt
V vac	Vector vaccine
v/v	Volume per volume
WC	Whole cell
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background

Tilapia is one of the most important cultured species worldwide and emerging to be very popular because of its fast growth, tolerant to high stocking density, and excellent adaptability to a wide range of environment and affordable to customers (El-Sayed 2006 and Fitzsimmons 2016). Aquaculture production of tilapia increased worldwide 12 percent annually, from less than 0.5 million tonnes in early 1990s to 5.7 million tonnes in 2015 (FAO 2016). Tilapia *Oreochromis* spp. is one of the maor aquaculture products in Malaysia with total annual production of 35, 400 mt (DFM, 2015). However, intensive farming and rapid expansion of aquaculture industry in Malaysia has lead to severe outbreaks of bacterial diseases which reducing the tilapia production and causing great financial loss (Zamri-Saad 2014).

Streptococcosis is considered as the most devastating disease in fish which is responsible for massive death of large size fishes and high economic losses for the farmers. *Streptococcus agalactiae* is a group β streptococcus and was reported to cause streptococcal infection in fish including farmed tilapia (Ferguson et al. 1994; Toranzo et al. 2005; Roberts 2012). *Streptococcus iniae* also causes mortality but to a lesser extent (CFSPH 2005).

Streptococcus agalactiae and *S. iniae* in tilapia were proven to be pathogenic to cultured tilapia in Malaysia, causing septicaemia and meningitis and causes trespassing infections in saltwater and freshwater fish and occasional zoonosis. (Abuseliana et al. 2010; 2011). Streptococcosis in fish can generate high mortality rates (> 50%) over a period of three to seven days (Yanong and Francis-Floyd 2006). Some outbreaks of streptococcal disease are more persistent in nature and mortalities may expand into several weeks, with only few fish dying each day (Roy et al. 2002). Streptococcal-based infections resultant a significant mortalities in aquaculture industries of Asia Pacific region. In Thailand estimated mortalities due to streptococcosis was 16,270 tonnes which worth about USD 26.57 million. Furthermore, 289,440 tonnes total loss was estimated throughout the region in 2014 that valued at USD 480 million (applying an average Asian price of USD 1,657/tonne) (Shinn et al. 2016).

Antibiotics are coomonly used to treat streptococcosis in Malaysia (Najiah et al. 2009). However, antibiotic treatment is only effective in healing streptococcal diseases if the medication is applied in early fish stages. In most incidents, the oral antibiotic is not effective as diseased tilapia experience less appetite (Zamri-Saad et al. 2014). Based on Agriculture Research Service (ARS) data, the United States Departments of Agriculture (USDA), no antibiotics are available to deal with streptococcosis effectively (Agriculture Research Service, 2010).

Vaccination is a simple, potential and preventive method of controlling diseases in fish. It has no toxic side effect and pathogen will not develop resistance. There are many vaccines that have been developed, commercialized and used successfully worldwide, but the development of vaccine that can be used against more than one pathogen at a time should be given more attention, especially for aquaculture sector in Malaysia. For instance, a variety of pathogens survive in water bodies and the administration of number of vaccines may be tiresome and uneconomical. Moreover, antigenic competition can affect the viral or bacterial vaccine

efficiency when the whole bacterium is used as a vaccine (Pross et al. 1974; Hunt et al. 1995). The antigens commonly found in different pathogens can be precious vaccine candidates that provide cross protection against bacterial pathogens.

Streptococcus agalactiae capsular polysaccharides have attracted most attention as an encapsulated bacteria with regards to vaccine development. But, it was reported that the defence provided by capsular polysaccharides is specific to the type of the pathogen (Brodeur et al. 2000). There are several vaccines have been developed using outer membrane protein (OMP) or outer surface protein (OSP). OSP of group B streptococcus (GBS) has also been regarded as potential virulence components and vaccine ingredients. Some of the outer surface proteins like alpha, beta c protein, Rib and R protein were recommended as vaccine candidates in several experiments but these surface proteins were not obtained in all serotypes of *S. agalactiae* (Rioux et al. 2001). Group B streptococcus (GBS) surface immunogenic protein (Sip) was reported to be available in all serotype of GBS isolates (Brodeur et al. 2000) and highly conserved (Rioux et al. 2001; Huang et al. 2014). Therefore, Sip is a potential vaccine component that may have cross-protective ability against different strains of *Streptococcus* spp.

In order to treat streptococcosis, there are several commercial vaccines available in the market such as formalin killed *S. agalactiae* (Department of agriculture, USA), AQUAVAC® Strep Sa vaccine from attenuated *S. agalactiae* (Merck Animal Health Company, USA), Aquavac™ Garvetil™ vaccine against *Lactococcus* and *S. iniae* (Intervet/Schering-Plough Animal Health) and NORVAX® STREP Si vaccine, the monovalent vaccine carrying an inactivated *S. iniae* strain (Merck Animal Health Company, USA). However, most of the commercial vaccines are species specific which means limited to one specific strain only.

To our knowledge, there is no report regarding the availability of vaccine that can confer cross protection both *S. agalactiae* and *S. iniae* infection in hybrid red tilapia *Oreochromis* spp. Thus, the development of a monovalent vaccine prepared from the most antigenic outer membrane protein of two prominent pathogenic bacteria, *S. agalactiae* and *S. iniae* can be a novel vaccine strategy for the prevention of streptococcosis in the aquaculture sector of Malaysia.

1.2 Objectives

The main objective of this study was to develop a vaccine through recombinant DNA technology which can protect against two major pathogens *Streptococcus agalactiae* and *Streptococcus iniae* in hybrid red tilapia (*Oreochromis* spp.).

The four specific objectives of this research were:

1) To characterize *Streptococcus iniae,* TSK_2 strain isolated from hybrid red tilapia and to re-confirm *Streptococcus agalactiae*, Millud II strain, previously isolated from fish farm based on biochemical and molecular analysis.
2) To determine the electrophoretic profiles and analyze antigenicity of outer cell layer proteins of *S. iniae,* TSK_2 strain and *S. agalactiae*, Millud II strain.
3) To construct and develop a recombinant cells vaccine expressing surface immunogenic gene of *S. iniae,* TSK_2 strain.
4) To evaluate the protective efficacy of developed recombinant cells vaccine following the bacterial challenge with *S. iniae,* TSK_2 and *S. agalactiae*, Millud II.

1.3 Hypothesis of the study

Hypotheses of this study as stated below:

H_0:

Outer surface proteins of *Streptococcus* spp. do not have cross protective ability against streptococcus infection and cannot be a potential vaccine candidate for hybrid red tilapia.

H_1:

Outer surface proteins of *Streptococcus* spp. do have cross protective ability against streptococcus infection and have potentiality to be used as vaccine candidate for hybrid red tilapia.

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LIST OF PUBLICATIONS

Rahmatullah, M., Ariff, M., Kahieshesfandiari, M., Daud, H.M., Sabri, M.Y., Amal, M.N.A., Zamri-Saad, M. & Ina-Salwany, M.Y. (2017). Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid tilapia in Malaysia. *Journal of Aquatic Animal Health*, 29(4), 208-213.

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