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MOLECULAR CHARACTERIZATION OF Aeromonas hydrophila AND DEVELOPMENT OF RECOMBINANT CELLS VACCINE EXPRESSING OUTER MEMBRANE PROTEINS AGAINST ITS IN AFRICAN CATFISH (Clarias gariepinus Burchell)

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By

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

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MOLECULAR CHARACTERIZATION OF *Aeromonas hydrophila* AND DEVELOPMENT OF RECOMBINANT CELLS VACCINE EXPRESSING OUTER MEMBRANE PROTEINS AGAINST ITS IN AFRICAN CATFISH (*Clarias gariepinus* Burchell)

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September 2015

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Aeromonas hydrophila act as primary pathogen causing high mortality rate especially in freshwater fish, resulting big losses to farmers. Vaccination is one of the approaches to prevent bacterial infection in fish. At present, there is no commercial vaccine for protecting farmed fish against *A. hydrophila*, although studies have proved that vaccination may provide protection. The studies were conducted to develop a recombinant cells vaccine expressing the immunogenic genes of OmpTs and OmpW of *A. hydrophila* strain Ah1sa5 and to determine the protective efficacy of the developed vaccine. Five strains of *Aeromonas* sp. isolated from diseased freshwater fish were identified as *A. hydrophila* by phenotypic identification, characterized by 16S rRNA and Internal Transcribed Spacer Region (ITS) genes sequencing. The nBLAST analysis showed high percentage of similarity with other *A. hydrophila* strains published in Genbank obtained between 95 % - 98 %. A single strain, Ah1sa5 was selected, and used for the construction of recombinant cells vaccine by identifying the immunogenic genes of interest, the outer membrane protein (OMP) OmpTs and OmpW. The genes were amplified by PCR using specific primers and cloned into pET102/D-TOPO® vector. The OmpTs and OmpW genes showed approximately 1000 bp and 600 bp of open reading frame (ORF) and encoded a protein of 265 and 204 amino acid residues respectively. Expression of the target proteins done by SDS-PAGE and Western immunoblotting analysis revealed the expressed fusion proteins of pET102/D-OmpTs and pET102/D-OmpW were approximately 60 kDa and 45 kDa. *In-vivo* study was conducted to investigate the efficacy of the developed vaccine in African catfish (*Clarias gariepinus*). The fishes were divided into five groups: control, placebo, and three vaccinated groups with inactivated recombinant cell vaccines which were recombinant cells OmpTs, OmpW and bivalent OmpTs+OmpW. The experiment was conducted for five weeks where fishes were vaccinated intraperitoneally at week 0 and booster at week 2. All fishes were challenged with *A. hydrophila* at week 4 and observation was done until week 5. The RPS

was significantly higher (P<0.05) for all vaccinated groups (100 %) compared to placebo vaccine group (29.42 %). In conclusion, the inactivated recombinant cell vaccines expressing the OmpTs and OmpW protect African catfish (*C. gariepinus*) against *A. hydrophila*.

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

GAMBARAN SIFAT *Aeromonas hydrophila* SECARA MOLEKULAR DAN PEMBINAAN VAKSIN SEL REKOMBINAN MENGEKSPRESIKAN PROTIN LUAR MEMBRAN MENENTANGNYA DI DALAM KELI AFRIKA (*Clarias gariepinus* Burchell)

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Aeromonas hydrophila bertindak sebagai patogen utama menyebabkan kadar kematian yang tinggi terutamanya terhadap ikan air tawar, mengakibatkan kerugian yang besar kepada para penternak. Vaksinasi adalah salah satu pendekatan untuk mencegah jangkitan bakteria terhadap ikan. Sehingga kini, tiada vaksin yang dikomersilkan untuk melindungi ikan ternakan menentang *A. hydrophila,* walaupun kajian telah membuktikan pemvaksinan boleh memberikan perlindungan. Kajian ini telah dijalankan bagi membina vaksin sel rekombinan yang mengekspresi imunogen OmpTs dan OmpW dari *A. hydrophila* jenis Ah1sa5 dan untuk mengetahui keberkesanannya. Lima jenis *Aeromonas* sp. tempatan di saring daripada ikan air tawar yang berpenyakit dikenalpasti sebagai *A. hydrophila* melalui kaedah pengenalpastian fenotipik dan dicirikan melalui jujukan gen 16S rRNA dan Ruang Tertranskripsi Dalaman (ITS). Analisis nBLAST menunjukkan peratus kesamaan yang tinggi di antara 95 % - 98 % dengan *A. hyrophila* jenis lain yang telah diterbitkan di GenBank. *Aeromonas hydrophila* jenis Ah1sa5 telah di pilih untuk pembinaan vaksin sel rekombinan melalui pengenalpastian imunogen protin luar membran iaitu OmpTs dan OmpW. Amplifikasi reaksi rantaian polymerase (PCR) dilakukan terhadap gen tersebut menggunakan primer khusus yang telah direka dan seterusnya diklonkan ke dalam vektor pET102/D-TOPO®. Gen OmpTs dan OmpW yang diperolehi menunjukkan jujukan penuh lebih kurang 1000 bp dan 600 bp setiap satu dan setiap gen merangkumi protin asid amino 265 dan 204. Ekspresi protin menggunakan analisis natrium dodesil sulfat-poliakrilamida agar elektroforesis (SDS-PAGE) dan pemedapan Western mendedahkan protin lakuran lebih kurang 60 kDa bagi pET102/D-OmpTs dan 45 kDa bagi

pET102/D-OmpW. Kajian '*in-vivo'* telah dijalankan untuk menyiasat keberkesanan vaksin yang telah dibina terhadap ikan keli Afrika (*Clarias gariepinus*). Ikan dibahagikan kepada lima kumpulan iaitu kumpulan kawalan, plasebo dan tiga lagi kumpulan yang divaksinasikan dengan vaksin sel rekombinan yang tidak aktif iaitu sel rekombinan OmpTs, OmpW dan dwi valen OmpTS+OmpW. Kajian telah dijalankan dalam tempoh lima minggu di mana ikan telah divaksinasikan secara suntikan intraperitoneum pada minggu 0 dan dos penggalak pada minggu kedua. Kesemua ikan di cabar dengan *A. hydrophila* pada minggu keempat dan pemerhatian dijalankan sehingga minggu kelima. Kemandirian peratus relative (RPS) disignifikasikan dengan perbezaan bererti yang tinggi (P<0.05) untuk kesemua kumpulan ikan yang divaksinasi (100 %) berbanding kumpulan plasebo (29.42 %). Kesimpulannya, vaksin sel rekombinan yang tidak aktif mengekspresikan protin OmpTs dan OmpW memberikan perlindungan terhadap ikan keli Afrika (*C. gariepinus*) menentang *A. hydrophila*.

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I certify that a Thesis Examination Committee has met on 22 September 2015 to conduct the final examination of Saleema Binti Matusin on her thesis entitled "Molecular characterization of *Aeromonas hydrophila* and development of recombinant cells vaccine expressing outer membrane proteins against it in African catfish (*Clarias gariepinus* Burchell)" 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 Master of Science.

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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

dNTPs	Nucleoside triphosphates containing deoxyribose
BSA	Bovine Serum Albumin
LB	Luria Bertani Broth/Agar
PBS	Phosphate-Buffered Saline
TBS	Mixture of Tris-Buffered Saline
TBST	Mixture of Tris-Buffered Saline and Tween 20
TCBS	Thiosulfate-Citrate-Bile salts-Sucrose agar
TMB	3,3′,5,5′-Tetramethylbenzidine
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
S.O.C	Super Optimal broth with Catabolite repression
H.E.	Haematoxylin and Eosin
O/F	Oxidase or Fermantase
OD600	Optical Density of 600
IP	Intraperitoneal
ORF	Open Reading Frame
DNA	DeoxyriboNucleic Acid
rDNA	ribosomal DeoxyriboNucleic Acid
rRNA	ribosomal RiboNucleic Acid
OMP	Outer Membrane Protein
LPS	Lipopolysaccharide
ITS	Internal Transcribe Spacer region
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
EMS	Early Mortality Syndrom
MAS	Motile Aeromonad Septicemia
Pfu	*Pyrococcus furiosus*
RBC	Red Blood Cell
RPS	Relative Percentage Survival
FAO	Food and Agriculture Organization
DOFM	Department of Fisheries Malaysia
MALT	Mucosa-Associated Lymphoid Tissue
GALT	Gut-Associate Lymphoid Tissue
GIALT	Gill-Associated Lymphoid Tissue
SALT	Skin-Associated Lymphoid Tissue
AFLP	Amplified Fragment Length Polymorphisms
ERIC	Enterobacterial Repetitive Intergenic Consensus
gyrB	Housekeeping gene use for taxonomical identification
rpoD	Housekeeping gene use for taxonomical identification
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
PFGE	Pulsed-Field Gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information

CHAPTER 1

INTRODUCTION

Aquaculture has recently been recognized as a potential fisheries sector that [illegible] achieved through commercial scale with intensification of production and sustainable management (De et al., 2014). *Aeromonas* species is an important pathogen in aquaculture systems and millions of dollars are estimated to be lost per annum due to diseases caused by this bacterium (Maiti et al., 2012). It has been reported to cause mass mortalities in several species of cultured and wild fish living in fresh, brackish and marine water environments including African catfish (*Clarias gariepinus*) (Anyanwu et al., 2014), Red tilapia (*Oreochromis* sp.) (Marcel et al., 2013), Nile tilapia (*O. niloticus*) (Lukkana et al., 2012), gilthead seabream (*Sparus aurata*) (Reyes-Becerril et al., 2011) and ornamental fish such as Dwarf Gourami (*Trichogaster lalius*), Discus Cichlids (*Symphysodon* sp.), and Tiger Barb (*Puntigrus tetrazona*) (Musa et al., 2008).

According to Bastardo et al. (2012), fish can be infected with *Aeromonas* due to several factors and stressors such as environmental changes, high stocking density, sudden temperature fluctuations, inappropriate handling, hypoxic condition, malnutrition, inflammation, and ulceration which give high possibilities of infections. Similarly with Osman et al. (2009) which added transportation, drugs treatment and poor water quality as high potential factors. The general signs of infection observed in fish include swelling of tissues, dropsy, red sores, necrosis, ulceration, and haemorrhagic septicaemia (Xu et al., 2012; Mu et al., 2011). Based on a review previously, Joseph and Carnahan (1994) concluded that *A. hydrophila* is the most virulent among the aeromonads followed with *A. sobria* and *A. caviae* appear to be non virulent. In addition, it cause Motile Aeromonas Septicemia (MAS) syndrome that lead to acute fatal septicemia occur rapidly and resulting fish mortality before any clinical signs can be seen (Xu et al., 2012).

Application of antibiotics to control disease outbreaks is no longer effective where only several type of antibiotics are allowed to be used. Furthermore, the pathogens fast development by emergence of drug resistance strains making the application of antibiotics ineffective towards diseases management plus, its negative effect of immunosuppressive in fish (Mu et al., 2011). Thus, many researches had been done recently to substitute antibiotics with more effective and efficient way to prevent disease outbreaks. One of the most concerned ways is through vaccination (Vivas et al., 2004). Recently, vaccination is the successful strategy to be applied in intensive culture of salmonid fish (*Salmonidae*), catfish (*Silurus asotus*), sea bream (*S. auratus*), halibut

(*Hippoglossus hippoglossus*), turbot (*Scophthalmus maximus*), red sea bream (*Chrysophrys major*), and yellow tail (*Acanthopagrus latus*) because it can reduce infection, prevents clinical disease and slowly decreases dependency on antibiotics (Thorarinsson and Powell, 2006). Moreover, the immunization can last for longer periods, give higher levels of protection and at the same time reduce cost expenses to immunize fish (Austin, 2012).

There are many approaches to develop vaccines. Some of them are traditional vaccines: live attenuated vaccines, whole inactivated vaccines, and genetically engineered vaccines: toxoid vaccines, conjugate vaccines, DNA vaccines and subunit vaccines or recombinant vaccines (National Institute of Allergy and Infectious Disease (NIAID), 2012; Poland et al., 2001). Despite of the effectiveness of traditional vaccines, safety issues and inconvenient application lead to the emergence of genetically engineered vaccines especially the current trend of using subunit vaccines which is recombinant protein vaccine (Poland et al., 2001). Recombinant protein vaccine expressing OMPs gene have been proven to provide protective efficacy in preventing fish diseases (Maiti et al, 2011; Guan et al., 2011; Khushiramani et al 2007a), where the OMPs was very immunogenic and stimulate the immune response (Khushiramani et al., 2012). As examples, recombinant vaccine Omp48 of *Aeromonas hydrophila* induced immunity to striped catfish (*Pangasius hypophthalmus*) when challenged with *A. hydrophila* (Pham and Nguyen, 2014) and recombinant vaccine OmpK of *Vibrio harveyi* effectively protect Orange-spotted grouper (*Epinephelus coioides*) against virulent *V. harveyi* (Ningqiu et al., 2008). In addition, some other studies showed diseases prevention using the application of polyvalent or bivalent vaccine produced better protection towards fish compared to monovalent vaccine (Guo et al., 2013; Shoemaker et al., 2012) where according to Guo et al. (2013), bivalent OMPs from *A. hydrophila* and *Edwardsiella tarda* give protection to American eels (*Anguilla rostrota*) against the two pathogens.

However, there is still no commercial vaccine available against *Aeromonas* spp. (Pham and Nguyen, 2014). This may be due to an inability of these vaccines to cross-protect against different isolates of *Aeromonas* spp.

Therefore, the objectives of this study were:

1. To genotypically characterize the *A. hydrophila* isolated from diseased freshwater fish
2. To construct vaccine candidate using recombinant cells expressing immunogenic OMPs of *A. hydrophila* strain Ah1sa5.
3. To determine protective efficacy of developed monovalent and bivalent recombinant cell vaccines tested in cultured African catfish (*Clarias gariepinus*) against *A. hydrophila*.

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