UNIVERSITI PUTRA MALAYSIA

EVALUATION OF NATURAL IMMUNOSTIMULANTS FOR GROWTH PROMOTION AND PROTECTION AGAINST Aeromonas hydrophila IN JUVENILE RED HYBRID TILAPIA (Oreochromis sp.)

NUR HIDAYAHANUM HAMID

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By

NUR HIDAYAHANUM HAMID

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

May 2014

DEDICATION

With appreciation and respect, this thesis is dedicated to:

My beloved parent, brothers and sisters.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in partial fulfillment of the requirement for the degree of Master of Science

EVALUATION OF NATURAL IMMUNOSTIMULANTS FOR GROWTH PROMOTION AND PROTECTION AGAINST *Aeromonas hydrophila* IN JUVENILE RED HYBRID TILAPIA (*Oreochromis* sp.)

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Chairman : Associate Professor Hassan Hj. Mohd Daud, PhD

Faculty : Veterinary Medicine

Aeromonas hydrophila is widely known as one of the common bacteria species in freshwater habitats and occasionally been recognized as a fish pathogen. In Malaysia, the use of immunostimulants especially in aquaculture industry has not been studied widely due to the lack of promotion and education especially to fish farmers. Hence, this study was conducted to identify *A. hydrophila* from disease freshwater fish. The bacteria were also used to perform antimicrobial sensitivity tests. Two types of immunostimulants (Lipopolysaccharide and ɓ-glucan) were used to evaluate the changes in the haematology, non-specific immune response, disease resistance and growth performance of juvenile Red hybrid tilapia (*Oreochromis* sp.). In the present study, the isolates of *A. hydrophila* were subjected for Gramôs staining and the identity of the isolates were confirmed by using API 20E® kit in combination with oxidase and catalase tests. The isolates were also inoculates on horse blood and Rimler-Shotts agar. Antimicrobial sensitivity test was also done by using Kirby-Bauer method. Then, pathogenicity of *A. hydrophila* was expressed as median lethal dose (LD_{50}) and moribund fish were proceed to histopathological examination. After that, the efficacy of lipopolysaccharide (LPS) and ɓ-glucan was studied in juvenile Red hybrid tilapia (*Oreochromis* sp.) against *A. hydrophila*. The fish were divided into seven different groups of test including control which consisting of 15 fish per group. Each group had two replicates. Different concentration of LPS and ɓ-glucan were used for intraperitoneal injection (i.p.), long-term bath exposure and oral feeding, they were 50 εg/fish, 50 εg/L and 25 mg/kg, respectively. For intraperitoneal injection and long-term bath exposures, LPS and ɓ-glucan were administered to the test fish on day 1, 7 and 14. While for oral feeding, the test fish were fed with commercial pellet added with LPS and ɓ-glucan daily until day 40. On day 7, blood samples were collected from each group to examine the haematological parameters of the test fish. Control and test fish were challenged with LD_{50} concentration of *A. hydrophila* by i.p. on day 16 (i.p injection and bath exposures) and on day 41 (oral). Mortality rate and RPS (Relative Percentage Survival) were

then calculated. Daily mortality was recorded for a week. From the study, 11 isolates of bacteria were successfully identified by using morphological, biochemical (conventional and commercial kit) and physiological tests. The bacteria isolates were characterized as Gram-negative, motile, catalase positive, oxidase positive, possessed straight rod cell; approximately up to 3 µm in length which appeared in singles and pairs. The isolates showed ϐ-haemolysis on horse blood agar and were able to grow on selective agar (Rimler-Shott agar). All bacteria isolates were found to be sensitive towards streptomycin, kanamycin, chloramphenicol and gentamicin but showed resistant to amoxicillin. In a pathogenicity test run by intraperitoneal injection, the infection caused marked clinical sign of abnormalities such as exophthalmia, lethargy, enlargement of kidney, spleen and liver. Histopathological examinations showed marked congestion and haemorrhages in the spleen, liver and kidney tissues. The median lethal dose (LD_{50}) at 96 hours of the isolate for juvenile Red hybrid tilapia (*Oreochromis* sp.) was 6.3×10^6 cfu/ml**.** Administration of LPS and ϐ-glucan by intraperitoneal injection, long-term bath exposure and oral feeding significantly enhanced the RPS. The survivability was higher in fish treated with both compounds, which was significantly high (more than 50%) when compared to control group (less than 50%). The results indicated that oral feeding had the highest survival among the treatment groups followed by i.p injection and bath due to the long duration of feeding exposure. In haematological assay, there were no significant difference between control and treated groups. However, the fish administered with LPS and ϐ-glucan showed significant increased in total leucocytes count ($P < 0.05$) and also significantly ($P < 0.05$) improve the growth performance of tested fish compared with the control group. In histopathological examinations, different types of MMC (melano-macrophage centre) were observed in spleen tissues of control and treated groups. The present study revealed that the presence of numerous hemosiderin granules (brownish yellow) was higher in the spleen of control group compared to treated groups. The MMC in the treated group was more enriched in melanin pigment which is dark pigment-containing cells (macrophage).The findings in this study indicates that *A. hydrophila* infection has become an important health issue in tilapia farms. This study also demonstrates that LPS and ϐ-glucan administration through injection, long-term bath exposures and oral feeding effectively stimulates the non-specific cellular as well as growth performances and offers protection against *A. hydrophila* infection in juvenile Red hybrid tilapia (*Oreochromis* sp.). Data from biochemical tests, haematological and histological studies provide valuable and previously unknown information associates with *A. hydrophila* in cultured juvenile Red hybrid tilapia (*Oreochromis* sp.). In addition, this study also reveals that LPS and ϐ-glucan could be used as an alternative for prophylaxis against *A. hydrophila* infection and the important of long term exposure of immunostimulants to obtain maximum protection against bacterial infection.

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

PENILAIAN KE ATAS IMMUNOSTIMULASI SEMULAJADI DALAM MENGGALAKKAN TUMBESARAN DAN PERLINDUNGAN MENENTANG *Aeromonas hydrophila* PADA REGA IKAN TILAPIA MERAH HIBRID (*Oreochromis* sp.)

Oleh

NUR HIDAYAHANUM HAMID

Mei 2014

Pengerusi : Prof. Madya Hassan Hj. Mohd Daud, PhD

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Aeromonas hydrophila terkenal dengan meluas sebagai spesies bakteria yang biasa dalam habitat air tawar dan kadangkala dikenali sebagai patogen ikan. Di Malaysia, penggunaan immunostimulasi terutamanya dalam industry akuakultur kurang dikaji kerana kurangnya promosi dan pengetahuan terutamanya para penternak ikan. Oleh itu, kajian ini dilakukan untuk mengenalpasti *A. hydrophila* daripada ikan air tawar yang dijangkiti penyakit. Bakteria tersebut juga digunakan untuk ujian sensitivity antimikrobial. Dua jenis immunostimulasi (Lipopolisakarida dan ɓ-glukan) digunakan untuk menilai keberkesanan bagi memeriksa perubahan dalam hematologi, sel tak-spesifik, daya rintang penyakit dan penilaian tumbesaran rega ikan tilapia merah hibrid (*Oreochromis* sp.). Dalam kajian ini, isolate *A. hydrophila* melalui perwarnaan Gram, Identiti isolate *A. hydrophila* dipastikan dengan API 20E® dengan kombinasi ujian oksidasi dan katalase. Kemudian bacteria tersebut diinokulasi atas agar darah kuda dan agar Rimler-Shotts. Ujian sensitiviti antimikrobial dijalankan menggunakan kaedah Kirby-Bauer. Selepas itu, kepatogenan *A. hydrophila* diungkap sebagai median dosis maut (LD_{50}) dan ikan nazak diteruskan dengan ujian histopatologi. Daripada kajian ini, keberkesanan LPS dan ɓ-glukan turut dikaji pada rega tilapia merah hibrid (*Oreochromis* sp.) menentang patogen bakteria, *A. hydrophila*. Sekumpulan ikan dibahagi kepada tujuh kumpulan yang berbeza termasuk kawalan dimana setiap kumpulan mengandungi 15 ekor ikan. Setiap kumpulan memiliki dua replika. Kepekatan LPS dan ɓ-glukan yang berbeza digunakan untuk suntikan (i.p.), mandian dalam jangka masa panjang dan pemakanan secara oral iaitu 50 µg/ikan, 50µg/L dan 25 mg/kg, setiap satu. Untuk suntikan (i.p.) dan mandian, LPS dan ɓ-glukan diberi pada hari pertama, ke-tujuh dan ke-empat belas. Sementara, kumpulan ikan yang diberi makanan secara oral diberi makan pellet komersial yang ditambah LPS atau ɓ-glukan setiap hari sehingga 40 hari. Pada hari ke-tujuh, sampel darah ikan diambil daripada setiap kumpulan untuk ujian hematologi danimunologi. Ikan kawalan dan kajian dijangkitkan dengan *A. hydrophila* berkepekatan LD_{50} pada hari ke-16 (suntikan intraperitoneal dan

mandian) dan hari ke-41 (oral). Peratus tahan hidup relatif (RPS) dan kesan modulasi imun lipopolisakarida dan ɓ-glukan dikaji pada rega hybrid ikan tilapia merah (*Oreochromis* sp.) menentang bacteria patogen, *A. hydrophila*. Mortaliti direkodkan setiap hari selama seminggu dan RPS dikira. Daripada kajian ini, sebelas *A. hydrophila* isolasi bacteria telah Berjaya dipencilkan melalui ujian morfologi, ujian biokimia (konvensional dan kit komersial) dan ujian pisiologi. Bakteria yang diisolasi dikenalpasti sebagai Gram-negatif, motil, positif katalase, positif oksidase, terdiri daripada sel morfologi rod lurus lebih kurang 3 µm panjang, terdapat dalam bentuk tunggal dan pasangan. Bakteria tersebut menunjukkan ɓ-hemolisis di atas agar darah kuda dan boleh tumbuh di atas agar selektif (agar Rimler-Shott). Kesemua isolate bacteria adalah peka terhadap streptomisin, kanamisin, kloramfenikol dan gentamisin, tetapi rintang terhadap amoxicillin. Dalam ujian patogenisiti secara suntikan intraperitoneal, jangkitan menyebabkan tanda-tanda klinikal abnormal seperti exoftalmia, letargi, pembesaran ginjal, limpa dan hati. Dalam ujian histopatologi, terdapatnya congesti dan hemoraj di dalam tisu ginjal, limpa dan hati. Median dosis maut, LD_{50} pada 96 jam oleh pencilan bakteria (*A .hydrophila*) bagi rega tilapia merah hibrid (*Oreochromis* sp.) secara suntikan intraperitoneal adalah $6.3x10^6$ cfu/ml**.** RPS bertambah secara signifikan dengan pemberian LPS dan ɓ-glukan secara suntikan intraperitoneal, mandian dan pemberian secara oral. Keupayaan tahan hidup adalah tinggi padaikan yang diberi rawatan kedua-dua sebatian LPS dan ɓ-glukan (lebih daripada 50%) berbanding dengan kawalan (kurang daripada 50%). Hasil kajian menunjukkan pemberian secara oral mempunyai tahan hidup yang tertinggi berbanding kumpulan rawatan yang lain kerana pemberian makanan dalam jangkamasa yang panjang. Bagi ujian hematologi, nilainya adalah tidak signifikan untuk ikan yang diberi LPS dan ɓ-glukan dengan kumpulan kawalan. Jumlah leukosit keseluruhan serta pelaksanaan pertumbuhan juga bertambah secara signifikan untuk ikan yang diberi LPS dan ɓ-glukan. Dalam kajian histopatologi, MMC (pusat melano-makrofaj) yang berlainan jenis dapat dilihat di dalam tisu limpa. Kajian ini mendedahkan banyaknya kehadiran hemosiderin (coklat keperangan) di dalam tisu limpa bagi kumpulan kawalan berbanding kumpulan yang dirawat. MMC dalam kumpulan yang dirawat lebih kaya dengan pigmen melanin dimana ia adalah pigmen gelap mengandungi sel (makrofaj). Kajian ini mendapati bahawa LPS dan ɓ-glukan yang diberi secara suntikan, mandian da npemakanan secara oral merangsang dengan efektif sel tak-spesifik serta pelaksanaan pertumbuhan dan member perlindungan terhadap jangkitan *A. hydrophila* pada rega tilapia merah hybrid (*Oreochromis* sp.). Hasil daripada ujian biokimia, hematologi dan histology member maklumat yang bernilai dan tidak diketahui sebelum ini mengenai *A. hydrophila* pada rega tilapia merah hibrid (*Oreochromis* sp.).Tambahan lagi, kajian ini turut mendedahkan bahawa LPS dan ɓ-glukan boleh digunakan sebagai profilaksis alternative menentang infeksi *A. hydrophila* dan pentingnya pendedahan jangka masa panjang immunostimulasi untuk mendapat pelindungan maksimum terhadap jangkitan bakteria.

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I certify that a Thesis Examination Committee has met on 22 May 2014 to conduct the final examination of Nur Hidayahanum bt Hamid on her thesis entitled "Evaluation of Natural Immunostimulants for Growth Promotion and Protection Against *Aeromonas hydrophila* in Juvenile Red Hybrid Tilapia (*Oreochromis* sp.)" 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.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia as fulfillment of the requirements for the degree of Master of Science. The members of the supervisory committee were as follows:

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cfu	Colony-forming unit
DO	Dissolved oxygen
dpi	Days post inoculation
FAO	Food and Agriculture Organization of the United Nations
H&E	Hematoxylin and eosin
i.p	Intraperitoneal
LD_{50}	Median lethal dose
MMC	Melano-macrophage centres
Ppt	Parts per thousand
SCP	single cell proteins
TSA	Trypticase soy agar
TSB	Trypticase soy broth
w/v	Weight per volume
w/w	Weight per Weight

CHAPTER 1

INTRODUCTION

1.1 Fish Diseases and Health Management in Malaysia

Aquaculture is one of important industries all over the world. From its beginning in the 1920s, aquaculture in Malaysia has developed quickly and become an important activity nowadays (FAO, 2011). Various kinds of marine and freshwater fish have been cultured and the production of cultured fish increases every year. In Malaysia, fishes are usuallycultured in enclosed system such as ponds, net cages and the latest one is in recirculating aquaculture system (RAS).Nowadays, the main aim of commercial aquaculture is to increase production by intensification; which areby increasing stocking density, increased seed production, and feeding with good quality of feed.However, disease in intensive aquaculture is said to be the great importance in Malaysia due to economic loss observed in recent years. Consequently, the use of chemicals and antibiotics had become common practices in fish farm.

Due to the intensive aquaculture practices, fish in cultured systems are susceptible to numeroustypes of bacterial, viral and parasitic diseases.The presence and development of fish disease is the result of the interaction between pathogen, host and environment. In the cultured system, poor handling and overcrowding always tend to give unfavourably affect to the fish health. These conditions may lead to the production of poor physiological environment and increase susceptibility of the cultured fish to infection disease (Sakai, 1998).Among the three major causes of disease, most frequent related to freshwater fish is bacterial infections. Hasty and uncontrolled growth of pathogens and indiscriminate use of antibiotics to prevent the emerging of pathogen have resulted in the emergence of several resistant pathogens in aquaculture. Currently, these two factors are the most fundamental concerns for both public health workers and farmers.

Even though the infectious diseases which caused by pathogenic species of bacteria in freshwater fish have been described in the majority of the existing taxonomic groups, however only a relatively small number are responsible of important economic losses in cultured fish worldwide. *Aeromonas* spp., *Pseudomonas* spp., *Streptococcus* spp. and *Flexibacter columnari* are some of the regularly bacteria isolated from fish and become primary pathogenic agents which frequently reducing the production of cultured freshwater fish. Among this pathogen of bacterial origin, motile aeromonads play an important role in freshwater systems.

1.2 *Aeromoniasis* Study in Malaysia

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world. These bacteria frequently cause diseases among cultured and wild fishes. Because ofthis, the bacteria species is said to be commonly isolated from diseased fish. *Aeromonas hydrophila* is a primary

(Esteve *et al.*, 1993), secondary (Joice *et al*., 2002) and opportunistic pathogen (Dooley and Trust, 1988) of a wide variety of aquatic and domestic animals, including humans.

In Malaysia, only few report of disease outbreaks that was caused by *Aeromonas hydrophila* in aquaculture system especially in food fish have been documented. Infections due to *Aeromonas hydrophila* are common and pose a threat especially to the development of the aquaculture sector.Taufik and Wong (1990) showed that *Aeromonas hydrophila* was the major pathogenic bacterium in catfishes from paddy field Kedah and Perak, West Malaysia.In addition, Najiah *et al*.(2008) reported the presence of *Aeromonas hydrophila*not only in the food fish but also in freshwater imported ornamental fish (*Xiphophorus maculates, Barbus pentazona hexazona, Symphysodon* spp., *Colisa lalia, Gymnocorymbus ternetzi, Poecilia reticulate, Pangasuis sutchi, and Osphronemus goramy*) in retail pet shop in Kuala Terengganu.

1.3 Current Study on Fish Health Management

Various chemicals and antibiotics have been used to treat bacterial infections in cultured fish. It provides a useful means of control to the infections. However, there are many problems associated with the development of drug-resistant bacteria and the high cost of treatment. At present, preventive and management measures are fundamental concern to overcome such outbreak of diseases. Immunostimulants are considered as a helpful and effective means for enhancing immune status of cultured fish.There are several approaches to disease prevention that previously have been successfully used in other animal industries such asvaccines and immunostimulants. Both approaches also have been effectively used in some aquaculture industries and should be considered as a part of fish health management options.

Vaccination is a useful prophylaxis for infectious diseases and it is already commercially available for bacterial infections such as vibriosis, redmouth disease and furunculosis and also for viral infection such as IPN (Sakai, 1998). In the salmon industry, it have been used for about 30 years and known to be one of the major reasons that salmon production has been successful. The used of vaccine also dramatically decreased the use of antibiotics (Sommerset *et al.*, 2005). For example, in Norway (1987), before the extensive used of vaccines, almost 50,000 kg of antibiotics were used. In 1997, when vaccines had become more routine practices, antibiotic usage had decreased to less than 1000-2000 kg (Sommerset *et al.*, 2005). For the time being, even though vaccination may be the most effective method of controlling fish diseases but not all diseases can be treated with vaccine. According to Sakai (1998), the vaccine development for *Renibacterium salmoninarum*, is still not far been successful. Therefore, it is impossible for us to rely only on vaccination in order to control the fish diseases.

Nowadays the main problem in the fish rearing during larval and on-growing stages is microbial pathogens. Therefore, it is important for us to develop methods for establishing microbial control at all stages of the cultivation progress. One

possibility is immunostimulation, which includes methods of enhancing the capacities of the specific and non-specific immune systems. It is valuable for the control of fish diseases and may also be useful in fish culture. Immunostimulants increase resistance to infectious disease, not only by enhancing specific immune response but also by enhancing non-specific defense mechanisms (Sakai, 1998). There are many experiments on non-specific immunostimulation of fish that suggest the method has considerable potential for reducing losses in aquaculture, both during larval and on-growing stages.

1.4 Statement of Problem and Significance of Study

Vaccination using immunostimulant is very effective and acts as a potential approach in order to increase the immunocompetency and disease resistance of fish. Immunostimulantis said to be safer than antibiotic and chemotherapeutic as their range of efficacy are wider and broadcompared tocommon vaccine (Sakai, 1998). However, in Malaysia, the use of immunostimulant especially in aquaculture industry has not been study widely due to the lack of promotion and education especially to fish farmers. Moreover, there is a relatively few of the end product that show guarantee in a research context become available for use by the fish farmer. For aquaculture industry in Malaysia, it will lead to high return and income since this industry is expanding throughout the region. Therefore, the aim of this study is to evaluate the status and potential of immunostimulation as an element in the strategy for solving microbial problems in promoting fish health against pathogen. The immunostimulants that were used in this study were lipopolysaccharide (LPS) and β-glucan. The assay will be done on juvenile Red hybrid tilapia (*Oreochromis* sp.), but the results are also applicable to other species of fish and organisms relevant to aquaculture.

1.5 Hypothesis of Study

Natural immunostimulants which are lipopolysaccharide (LPS) and β-glucan might be used to promote health and protection against *Aeromonas hydrophila* in juvenile Red hybrid tilapia (*Oreochromis* sp.).

1.6 Objectives of the study

The objectives of this study were;

1. to isolate and identify *Aeromonas hydrophila* from freshwater fish.
2. to perform antimicrobial sensitivity test on *Aeromonas hydrophila* isolates.
3. to investigate whether the application of immunostimulants (β-glucan and LPS) can be used to improve fish health,survival against *Aeromonas hydrophila* infection as well as growth performance.

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