UNIVERSITI PUTRA MALAYSIA

NATURAL AND EXPERIMENTAL INFECTION OF STREPTOCOCCUS SP. IN RED HYBRID TILAPIA (OREOCHROMIS SP.) AND SELECTED EFFECTIVE HERBAL PROPHYLACTICS

MILUD ALSAID ALSHAAFAI

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By

MILUD ALSAID ALSHAAFAI

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

May 2013

DEDICATION

With appreciation and respect, this thesis is dedicated to:

My beloved parents, wife, sons, daughter, brothers and sisters

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

NATURAL AND EXPERIMENTAL INFECTION OF *STREPTOCOCCUS* SP. ON THE RED HYBRID TILAPIA (*OREOCHROMIS* SP.) AND SOME EFFECTIVE HERBAL PROPHYLACTICS.

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May 2013

Chairman : Associate Professor. Madya Hassan Hj Mohd Daud, PhD

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Streptoccocosis, a septicaemic disease which is caused by *Streptococcus* bacteria is a major bacterial disease in tilapia due to the intensification of aquaculture and had caused significant economic losses in many countries, including Malaysia. Hence, the purpose of this study were to isolate and confirm *Streptococcus agalactiae* as a natural pathogen in Malaysian aquaculture and investigate the effect of different environmental parameters on the susceptibility of the Red hybrid tilapia to infection and the haematological, biochemical and histopathological responses of the fish following experimental infection. The *in vitro* and *in vivo* antimicrobial properties of herbal extracts against *S. agalactiae* were also studied to see whether selected herbal extracts could protect the fish from infection. To achieve the first objective, four intensive red hybrid tilapia (*Oreochromis* sp) grow-out farms were studied in the state of Selangor, Malaysia.

From 600 fish samples collected, 410 isolates were successfully cultured and identified as β-haemolytic group B *Streptococcus agalactiae* using standard conventional methods, biochemical characteristic tests, Lancefield serogrouping as well as a PCR method to detect and confirm the pathogens involved in the infection.

The median lethal dose (LD_{50}) value in red hybrid tilapia injected intraperitoneally (IP) with selected *S. agalactiae* (strain ML333) within 120 hours was 1.5×10^5 cfu/ml. A series of experiments were conducted in order to determine the effects of a variety of environmental factors on the susceptibility of the red hybrid tilapia following *S. agalactiae* exposure. The mortality rate was significantly higher ($P<0.05$) in tilapia experimentally infected with *S. agalactiae* which were maintained at 33°C than those held at 20, 25 and 30°C; and in water of pH 6 compared to those held in more alkaline water. An experimental infection were also carried out in adult Red hybrid tilapia to determine the haematological, biochemical and pathological changes that occur during the disease. The erythrocytes counts (RBC), haemoglobin concentration (Hb) and haematocrit (PCV) of the experimentally infected tilapia decreased significantly ($P<0.05$) at 3, 5 and 7 dpi compared with the healthy fish. In contrast, leucocyte counts significantly increased from 4.20±1.8 ($x10^3/\mu L$) in the control fish to 13.2±5.5 at 7 day post infection. Experimental infection caused marked clinical sign abnormalities such as exophthalmia, lethargy, gallbladder enlargement, mucoid fecal casts and pale liver. Histopathological examinations showed marked mononuclear cell infiltration, congestion and haemorrhages in the spleen, liver, kidney, brain

and heart tissues. In the present study, water extracts of ten herbs were screened to identify whether they have antimicrobial activities against *S. agalactiae* using the disk diffusion assay. The water extracts of *Cinnamomum verum*, *Allium sativum*, *Syzygium aromaticum* and *Thymus vulgaris* displayed antimicrobial properties. The minimum inhibitory concentration (MIC) values for herbal extracts were also determined by utilizing agar diffusion method. The lowest MIC value with high efficacy against *S. agalactiae* was 150 µg/ml, which was obtained from *C. verum* extract. The *in vivo* antimicrobial effect of *C. verum* was then tested by feeding tilapia fingerlings with feed supplemented with different ratios of *C. verum* bark extract and bark powder for 7 days before challenging with 0.1 ml of LD_{50} concentration (1.5×10^5 cfu/ml) of *S. agalactiae* via intraperitoneal injection. The cumulative mortality was significantly lower ($P<0.05$) in the fish fed on feed supplemented with *C. verum* extract with a ratio of 3:26 (w/w) as compared to other groups.

The findings obtained in this study indicated that *S. agalactiae* infection has become an important health issue in tilapia farms. This study demonstrated that environmental changes increase the susceptibility of the Red hybrid tilapia to *S. agalactiae* infection. Results from haematological, biochemical and histological studies provided valuable and previously unknown information on the extensity of pathology associated with streptococcosis in cultured Red hybrid tilapia. This study also showed that antimicrobial properties of *C. verum* extract could be used as an alternative biomedicine for prophylaxis against streptococcosis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagi memenuhi keperluan untuk ijazah Doktor Falsafah

JAGKITAN SEMULAJADI DAN UJIKAJI *STREPTOCOCCUS* SP. TERHADAP KACUKAN TILAPIA MERAH (*OREOCHROMIS* SP.) DAN PROFILAKSI HERBAL TERPILIH YANG EFEKTIF

Oleh

MILUD ALSAID ALSHAAFAI

Mei 2013

Pengerusi: Prof. Madya Hassan Hj Mohd Daud, PhD

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Streptoccocosis adalah sejenis penyakit septisemik yang berpunca daripada jangkitan bakteria *Streptococcus* dan merupakan penyakit bakteria utama bagi tilapia yang dikultur secara intensif. Ianya telah mengakibatkan kerugian ekonomi yang tinggi di banyak negara termasuk Malaysia. Oleh itu, tujuan kajian ini adalah untuk mengasingkan dan mengesahkan bahawa *S. agalactiae* adalah patogen yang semulajadi dalam akuakultur di Malaysia dan untuk menyiasat kesan faktor persekitaran yang berbeza terhadap kepekaan tilapia kacukan merah terhadap jangkitan dan juga tindakbalas hematologi, biokimia dan histopatologikal dalam ikan yang dijangkiti secara ujikaji. Sifat-sifat *in vitro* dan *in vivo* antimikrobial ekstrak herba terhadap *S. agalactiae* juga telah dikaji untuk melihat sama

ada ekstrak herba yang dipilih boleh melindungi ikan daripada jangkitan. Untuk mencapai objektif pertama, Empat ladang ternakan intensif ikan tilapia kacukan merah (*Oreochromis* sp.) telah dikaji di negeri Selangor, Malaysia. Daripada 600 ekor sampel ikan yang dikumpul, 410 isolat berjaya dipencil dan dikenalpasti sebagai *S. agalactiae* *β*-hemolytic kumpulan B dengan menggunakan ujian biokimia kit komersil iaitu BBL Crystal™ Identification Systems, kaedah serologi seperti aglutinasi slaid, kaedah PCR untuk mengesan dan mengesahkan patogen-patogen yang terlibat dalam jangkitan.

Nilai dos kematian purata (LD_{50}) dalam tilapia kacukan merah disuntik secara intraperitoneal (IP) dengan *S*. *agalactiae* (strain ML333) yang dipilih dalam tempoh 120 jam adalah (1.5×10^5 cfu /ml). Beberapa ujikaji telah dijalankan untuk menentukan kesan pelbagai faktor persekitaran terhadap kerentanan tilapia kacukan merah berikutan pendedahan pada *S. agalactiae*. Kadar kematian adalah lebih tinggi dan signifikan (*P≤0.05*) bagi tilapia yang dijangkiti *S. agalactiae* secara ujikaj dan dikultur pada suhu 33°C berbanding dengan tilapia yang diletak pada suhu 20, 25 dan 30°C; dan juga dalam air pada pH 6 berbanding dengan tilapia yang diletakkan dalam air yang lebih beralkali. Jangkitan secara ujikaji juga dijalankan pada tilapia kacukan merah yang dewasa untuk menentukan perubahan-perubahan hematologi, biokimia dan patologi yang berlaku semasa menghidap penyakit ini. Penilaian hematologi, biokimia dan histopatologikal telah dijalankan pada hari ke-0, 1, 3, 5 dan 7 selepas suntikan intraperitoneal dengan kepekaan 1 mL *S. agalactiae* ($1x10^4$cfu/ikan, strain ML333). Bilangan sel darah merah (RBC), kepekatan hemoglobin (Hb), hematokrit (PCV) pada ikan tilapia kacukan

merah yang dijangkit secara ujikaji telah menurun dengan signifikan (*P≤0.05*) pada 3, 5 dan 7 hari selepas dijangkiti (hlj) berbanding dengan ikan yang sihat. Sebaliknya, bilangan sel darah putih dalam ikan kawalan meningkat secara signifikan dari 4.2±1.8 ($x10^3$/µl) hingga 13.2±5.5 pada hari ke-7 hlj. Jangkitan secara ujikaji mengakibatkan petanda klinikal abnormal seperti berenang dengan tidak menentu, eksoptalmia, letargi, pembesaran pundi hempedu, tinja bermukoid dan hepar pucat. Pemeriksaan histopatologikal menunjukkan berlakunya penyusupan sel mononuklear, kongesi dan hemoraj dalam limfa, hepar, ginjal, otak dan tisu jantung. Dalam kajian ini, ekstrak air daripada sepuluh herba telah disaring untuk mengetahui samada ianya mempunyai aktiviti antimikrobial terhadap *S. agalactiae* dengan menggunakan asei resapan piring. Ia menunjukkan bahawa ekstrak air daripada *Cinnamomum verum, Allium sativum, Syzygium aromaticum* dan *Thymus vulgaris* memaparkan sifat-sifat antimikrobial. Ekstrak air daripada kulit *C. verum* menunjukkan sifat-sifat antimikrobial yang paling tinggi terhadap *S. agalactiae* dengan zon perencatan 18 mm. Nilai kepekatan minima perencatan (MIC) bagi ekstrak herba juga dikenalpasti dengan menggunakan kaedah peresapan agar. Nilai MIC yang terendah dengan keberkesanan yang tinggi terhadap *S. agalactiae* adalah 150 µg/ml yang diperolehi daripada ekstrak *C. verum*. Kesan *in vivo* antimikrobial daripada *C. verum* seterusnya diuji dengan memberi anak ikan tilapia dengan makanan yang ditambah dengan beberaba nisbah ekstrak kulit *C. verum* selama 7 hari sebelum diinteksi dengan 0.1 ml *S. agalactiae* dengan kepekatan LD_{50} (1.56×10^5cfu/ml) melalui suntikan intraperitoneal.

Mortaliti kumulatif adalah lebih rendah tetapi signifikan (*P≤0.05*) pada ikan yang diberi makanan yang ditambah dengan ekstrak *C*. *verum* pada nisbah 3:26 (w/w) berbanding dengan kumpulan-kumpulan yang lain.

Hasil dari kajian ini menunjukkan bahawa jangkitan *S. agalactiae* telah menjadi satu isu kesihatan yang penting diladang ternakan tilapia. Kajian ini menunjukkan bahawa perubahan persekitaran meningkatkan kerentanan ikan tilapia kacukan merah, *Oreochromis sp.* terhadap *S. agalactiae*. Keputusan daripada kajian hematologi, biokimia dan histopatologi memberikan maklumat yang bermanfaat dan juga informasi yang belum diketahui untuk kesinambungan kajian dalam patologi yang berkaitkan dengan streptococcosis dalam kultur ikan tilapia kacukan merah. Kajian ini juga menunjukkan sifat-sifat antimikrobial ekstrak *C. verum* boleh digunakan sebagai profilaksis alternatif untuk bioperubatan terhadap streptococcosis.

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I certify that a Thesis Examination Committee has met on 20 May 2013 to conduct the final examination of Milud Alsaid Alshaafai on his thesis entitled "Natural and experimental infection of *Streptococcus* sp. In Red hybrid tilapia (*Oreochromis* sp.) and selected effective herbal prophylactics" 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 degree of Doctor of Philosophy (PhD).

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DECLARATION

I declare that the thesis is my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MILUD ALSAID ALSHAAFAI

Date**:** 20 may 2013

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BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
bp	Base pair
CAMP	Christie, Atkins and Munch-Petersen
cfu	Colony-forming unit
cm	Centimeter
DDW	Double distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DO	Dissolved oxygen
dpi	Days post inoculation
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
GBS	Group B *Streptococcus agalactiae*
g	Gram
h	Hour
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
hpi	Hours post inoculation
IP	Intraperitoneal
L	Liter

LD_{50}	Median lethal dose
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Millimeter
NaOH	Sodium hydroxide
NaCl	Sodium chloride
nm	Nanometer
PCR	Polymerase chain reaction
ppt	Parts per thousand
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBA	Sheep blood agar
TBE	Tris-borate-EDTA
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UV	Ultra violet
w/v	Weight per volume
w/w	Weight per Weight
µg	Microgram
µl	Microlitre
µm	Micrometer
°C	Degree centigrade (Celsius)

CHAPTER 1

INTRODUCTION

Aquaculture of finfish, crustaceans, molluscs and seaweed are most rapidly growing food production sectors. Several species of marine, brackish water and freshwater fishes have been successfully cultured worldwide and farmed-fish production has tremendously increased every year. Aquaculture has grown considerably around the world in all its forms during the last fifty years and currently comprises approximately 50% of the world's fish production for food (De Silva and Davy, 2010). In the 1950's annual production was less than one million tones, but the production had increased to approximately 79 million tonnes in 2010 with an annual growth rate of almost 7% and a value of approximately US $125 billion (FAO, 2008).

The Asian and pacific regions are by far the world's leaders in aquacultural production. They produced more than 89% of the world's total aquacultural output in 2006. China was reported to be the world's top producing country by producing 67% of the total quantity and 49% of the total value of the world's aquacultural production (FAO, 2008). It is expected that the increase in population over the next two decades and production of fisheries currently in or near its peak. Thus aquaculture is the best solution to meet the growing demand for fish and other aquatic products. The ratio of per capita consumption and demand of aquatic products needs to be maintained as global aquacultural production must increase by at least 40 million tonnes by the year 2030 (FAO, 2008; De Silva and Davy, 2010).

Malaysian aquacultural production rose from 28.6 million metric tonnes in 1992 to more than 37 million metric tonnes in 2009 (Annual Fisheries Statistics, 2010). Freshwater aquaculture contributed 70,064 tonnes while marine and brackish water contributed 198,449 tonnes of the total Malaysian aquacultural production in 2007. Tilapia, catfish, carp, blood cockles, shrimp and seaweeds were considered the most important aquatic species cultured in Malaysia (Annual Fisheries Statistics, 2007).

In the middle of the 1980s, red hybrid tilapia (*Oreochromis* sp.) was first introduced to Malaysia and since, the production has increased dramatically (Siti-Zahrah *et al*., 2008). In 2007, tilapia production gave 46% of Malaysian total freshwater aquacultural production (Wing-Keong, 2009). Tilapia farming emerged as a major global industry and commodity in the 21st century. The species of tilapia that are of interest to aquaculturalists include the Nile tilapia (*Oreochromis niloticus*), Java tilapia (*O*. *mossambius*), blue tilapia (*O*. *aureus*), Zanzibar tilapia (*O*. *hornorum*) and the red tilapia (*O*. *hybrids*) which was produced by crossing those as well as other species.

Various tilapia species have been cultured at high-densities in fresh and saline water. Red tilapia strains are considered important in aquaculture (Romana-Eguia and Eguia, 1999) primarily due to market preferences over wild types. The technical advancements of red tilapia farming in Southeast Asia over the span of the past decade have been adopted by various local commercial production systems. As a result, the culture of red tilapia has a profound economical impact on a large number of fishery communities.

Bacterial disease outbreaks were increasingly recognized as a major constraint in aquacultural production and trade, reducing the economic growth of this sector in many countries of the world. Changing environmental conditions tend to affect the health status of cultured fish adversely. Currently, the livelihood and development of aquaculture is under attack due to the changing aquatic environment and the spread of fish diseases. Fish are usually cultured in enclosed spaces such as ponds, tanks and net cages. Efforts have been made to increase productivity per unit space. Changes in the aquatic environment tend to adversely affect the health condition of cultured fish.

Warm water aquaculture in Asia has problems with several bacterial diseases such as streptococcosis, columnaris, furunculosis and edwardsiellosis.Various types of Streptococci have been demonstrated to cause infections in cultured and wide fish species. *Streptococcus agalactiae* appear able to naturally infect a wide range of hosts including humans, terrestrial and aquatic animals. Several strains of the bacterium have been isolated from different fish species in natural disease outbreaks and shown to be pathogenic to various fish species (Elliott *et al*., 1990; Evans *et al*., 2002; Toranzo *et al*., 2005; Musa *et al*., 2009).

Streptococcus sp. are catalase-negative, Gram-positive cocci bacteria producing β-, α-, or non-hemolysis on sheep blood agar. They are pathogens causing septicemic streptococcosis in fish. The *S. agalactiae* is the only streptococcal species defined as Lancefield group B *S. agalactiae* has been

isolated from many fish species as a pathogen of meningoencephalitis streptococcosis or “Popeyes’ disease” (Evan *et al*., 2002).

Environmental factors such as temperature, overstocking, malnutrition, low oxygen content, unfavorable pH could weaken or exhauste the fish natural defensive capacity and possibly resulting in a predisposition to pathogens (Piper, 1986). Streptococcosis is a disease that affects both cultured and wild fish in freshwater, brackish water and marine environments. In particular, *S. agalactiae* has been reported in intensive aquaculture systems, resulting in significant economic losses in cultured tilapia (*Oreochromis* spp.) world-wide. It could cause more than 50% mortality within a one-week period (Inglis *et al*., 1993; Lio-Po and Lim, 2002; Yanong and Francis-Floyd, 2002).

Studies by Glibert *et al*. (2002) and Bromage and Owens (2009) showed that change in environmental conditions could introduce streptococcosis outbreaks into the gilthead sea bream (*Sparus auratus*) and Barramundi (*Lates calcarifer*). Additionally, fish streptococcosis was reported to be potentially zoonotic, which means that there might be a possibility causing disease in humans (Toranzo *et al*., 2005). Disease management strategies were based primarily on chemotherapy. Presently, numerous antibiotics like tetracycline, penicillin, streptomycin, oxytetracycline, gentamycin, etc. as well as chemicals like formalin, potassium permanganate, copper sulphate, sodium chloride were widely used for treatment but with partial success (Cruz-Lacierda, 1996). In Malaysia, antibiotics and chemotherapeutic agents as well as pesticides were commonly utilized in fish farms, as either feed

additives or immersion baths to achieve prophylaxis or therapy. They are also utilized as a common practice to avoid the overgrowth of plants and fish diseases besides promoting the rapid fish growth (Majusha *et al*., 2005; Ibrahim *et al*., 2010). The lack of regulation concerning their use in most Asian countries has contributed to their extensive use. Nevertheless, the emergence of antibiotic resistant bacteria, problems associated with antibiotic use in aquaculture and awareness towards environmental problems associated with the utilization of therapeutics have led to additional focus on alternative disease management methods. For example, there were reported on the occurrences of resistant strains of *S. agalactiae* isolated from cultured silver pomfret, *Pampus argenteus* to flumequin, gentamycin, kanamycin, neomycin, oxolinic acid and trimethoprim (Duremdez *et al*., 2004).

In recent years, the application of vaccines for disease management in aquaculture has been increasingly recognized. However, in the case of *S. agalactiae*, the main problem of vaccine development is heterogeneity of the strains which caused disease problems as well as the lack of potency of potential vaccine preparations. Vaccines are expensive and protective for only a specific pathogen and often impractical to be used in the field. Accordingly, most fish farmers have been shifting to herbal medicines as prophylactics in an attempt to try to control fish disease apart from effective water quality management in order to enhance fish production.

Recently, there has been increased interest in antibacterial activity of some herbs against bacterial pathogens in aquaculture. According to Citarasu,

(2010) more than 50 herbs could be utilized in aquaculture as immune-stimulants, anti-stress, anti-bacterials, anti-fungals and anti-virals. Additionally, the utilization of herbal medicines to prevent fish diseases gives many features and benefits. Herbal medicines come from abundant plants sources and are cheap. They have extensive functionality, including: treatment for primary and secondary aspects of disease, high safety, low toxicity, difficult to generate of drug tolerance as well as enhancing the production performance of fish and increasing the economic benefits of aquaculture (Direkbusarakom, 2004; Galina *et al*., 2009).

The present study was carried out in Red hybrid tilapia *Oreochromis* sp. farmed in the state of Selangor, Malaysia. This farmed fish were regularly affected by septicaemia disease in outbreaks due to overcrowding as well as temperature fluctuations, which resulted in heavy economic loss.

Research Hypotheses:

i. Streptococcosis bacterium (*Streptococcus agalactiae*) isolated from tilapia farms is pathogenic to cultured Red hybrid tilapia.
ii. Herbal medicines extract such as garlic (*Allium sativum*), Cinnamon (*Cinnamomum verum*), ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citrates*), thyme (*Thymus vulgaris*), curry (*Murraya koenigii*), Mustard (*Sinapis alba*), turmeric (*Curcuma longa*), Cubeb (*Cubeb pepper*) and clove (*Syzygium aromaticum*) may have antimicrobial properties and might provide prophylaxis against S. *agalactiae* infection in cultured tilapia.

iii. Water quality changes have a potent effect to increase *S. agalactiae* outbreaks in cultured tilapia.

Therefore, the objectives of this study were:

1. to isolate bacteria and characterize the agents of streptococcosis i.e., *Streptococcus* bacteria from field outbreaks.

2. to describe the clinical signs, haematological, biochemical and histopathological changes that occur in experimentally induced streptococcosis.

3. to assess the role of water quality as a predisposing factor for streptococcosis in tilapia.

4. to evaluate *in vitro* the antimicrobial potential of ten herbal extracts against *S. agalactiae*.

5. to develop fish feed supplemented with herbs as prophylactic against *Streptococcus agalactiae* infection in farmed tilapia.

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