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STUDIES ON MYCOBACTERIOSIS IN SIAMESE FIGHTING FISH *Betta splendens* REGAN

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STUDIES ON MYCOBACTERIOSIS IN SIAMESE FIGHTING FISH *Betta splendens* REGAN

by

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LIST OF ABBREVIATIONS

Bd	=	buoyant density
CFU	=	colony forming unit
cm	=	centimetre
DNA	=	deoxyribonucleic acid
G+C	=	Guanine + Cytosine
G.I.	=	gastrointestinal
H&E	=	Haematoxylin-Eosin
MBC	=	Minimal Bactericidal Concentration
MIC	=	Minimal Inhibitory Concentration
ml	=	millilitre
mM	=	millimolar
OD	=	optical density
nm	=	nanometre
PAS	=	Periodic Acid Shiff
pH	=	potential of hydrogen
ppm	=	parts per million
RNA	=	ribonucleic acid
SDS	=	sodium dodecyl sulfate
SSC	=	Standard saline citrate
Tm	=	Melting temperature
W	=	Watts
µg	=	microgram
%	=	percentage
µg/ml	=	microgram per millilitre

Abstract of dissertation to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

STUDIES ON MYCOBACTERIOSIS IN SIAMESE FIGHTING FISH *Betta splendens* REGAN

By

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February 1994

Chairman : Prof. Dr. Mohamed Shariff Mohamed Din

Faculty : Fisheries and Marine Science

Siamese fighting fish, *Betta splendens* Regan mortalities in Thailand are usually associated with mycobacteriosis. The present study was undertaken to isolate and characterise mycobacteria from Siamese fighting fish, examine their sensitivity to selected antibiotics and to test the efficacy of kanamycin sulphate as a chemotherapeutic agent against mycobacteria. Histopathological studies on the Siamese fighting fish, experimentally and naturally infected with mycobacteria, were also conducted.

Ten acid-fast, rapidly growing, photochromogenic strains of mycobacteria were isolated from apparently healthy fish and fish showing gross lesions of mycobacteriosis. Prevalence of mycobacteria in farm reared

Siamese fighting fish ranged from 0-8%. Primary isolation of mycobacteria were successful on Ogawa egg medium and Löwenstein Jensen medium. The isolates grew within 5-7 days at 28°C on Ogawa egg medium and showed a temperature preference of 15-17°C. No growth was observed at 42°C. Optimum incubation temperature was 30°C. They were considered as mesophyllic forms. The organism produced mycolic acid which melted between 50.5-70°C. On the basis of their biochemical and physiological properties and the percentage guanine plus cytosine, the pathogen was confirmed as *Mycobacterium* sp. and was analogous to "*M. piscicida*" with similarity of 94%, but differed from the reference strains *M. chelonae* sub sp. *chelonae*, *M. fortuitum*, *M. marinum*, *M. parafortuitum* and *M. vaccae* by many characteristics.

An antibiotic sensitivity test was conducted using seventeen antimicrobial agents against ten mycobacteria strains isolated from Siamese fighting fish. Kanamycin at 30 μg was the most effective antibiotic against mycobacteria. MIC and MBC values of kanamycin sulphate were 6.25-12.50 and 12.5-25.0 ppm, respectively. However, treatment of mycobacteriosis with kanamycin, *in vivo*, was unsuccessful.

Siamese fighting fish experimentally infected with *Mycobacterium* sp.(SFF90/1) by intramuscular injection of bacteria at a concentration of 10^8 cell per ml did not exhibit any external lesions when observed up to 28 days. However, various sizes of hard and soft granulomas containing clumps of acid-fast bacteria were observed. Granuloma formation began at the site of injection, then spread to the haemopoietic tissues in the kidney and the spleen. No calcification in the granulomas was observed but lipofuscin and melanin pigment were found in the cytoplasm of the macrophages in some granulomas. Foreign body type giant cells were seen on the fifth day of infection. In naturally affected fish, numerous granulomas were found in various organs and parts of body. The kidney and spleen were the most seriously and consistently affected organs.

Abstrak dissertation yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat keperluan Ijazah Doktor Falsafah

KAJIAN MIKOBAKTERIOSIS KE ATAS IKAN PELAGA SIAM *Betta splendens* REGAN

Oleh

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Februari 1994

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Mortaliti ikan pelaga Siam (*Betta splendens* Regan) biasanya dikaitkan dengan mikobakteriosis. Kajian ini adalah bertujuan untuk mengasing dan mencirikan mikobakteria dari ikan pelaga Siam, memeriksa tahap sensitiviti terhadap antibiotik yang terpilih dan untuk menguji keberkesanan kanamisin sulfat sebagai agen rawatan kimia. Kajian histopatologi ke atas ikan pelaga Siam, melalui jangkitan secara eksperimen dan secara semulajadi dengan mikobakteria telah juga dilakukan.

Sepuluh strain mikobakteria yang tahan asid, mempunyai tumbesaran yang cepat serta fotokromogenik telah diasingkan dari ikan sihat dan ikan yang menunjukkan lesi mikobakteria. Kekerapan hadirnya mikobakteria di dalam ikan pelaga Siam yang dikultur berjulat antara 0-8%.

Pengasingan primer mikobakteria telah berjaya dilakukan dengan menggunakan media telur Ogawa dan media Löwenstein Jensen. Isolat timbul dalam tempoh 5-7 hari pada suhu 28°C dalam media telur Ogawa dan menunjukkan suhu pilihan pada 15-17°C. Tidak ada tumbesaran pada suhu 42°C. Suhu pengeraman optimum isolat ini adalah 30°C dan dianggap berbentuk mesofilik. Organisme ini menghasilkan asid mikolik yang lebur pada suhu 50.5-70°C. Isolat dicirikan dengan sifat biokimia dan fisiologinya serta peratus kandungan guanin campur sitosin. Patogen dikenal pasti sebagai *Mycobacterium* sp. dan adalah menyerupai 94% dengan *"M. piscicida"*. Walau bagaimanapun ia berbeza dengan strain-strain rujukan lain seperti *M. chelonae* sub sp. *chelonae, M. fortuitum, M. marinum, M. parafortuitum* and *M. vaccae* kerana perbezaan dalam banyak ciri.

Satu ujian sensitiviti antibiotik menggunakan tujuh belas agen antimikrob telah dijalankan terhadap sepuluh strain mikobakteria yang diasingkan dari ikan pelaga Siam. Keputusan menunjukkan bahawa kanamisin pada sukatan 30 μg adalah antibiotik yang paling berkesan bagi merawat mikobakteria. Nilai-nilai MIC dan MBC kanamisin sulfat adalah masing-masing 6.25-12.50 dan 12.50-25.00 ppm. Walau bagaimanapun rawatan mikobakteriosis dengan kanamisin secara *in vivo* tidak berjaya.

Ikan pelaga Siam yang dijangkiti *Mycobacterium* sp. (SFF90/1) melalui suntikan intraotot dengan bakteria pada kepekatan 10^8 sel per ml tidak menunjukkan apa-apa lesi luaran walaupun selepas 28 hari. Walau bagaimanapun, granuloma berbagai saiz, berbentuk keras dan lembut dan mengandungi gumpalan bakteria tahan asid telah diperhatikan. Pembentukan granuloma berlaku di tempat suntikan, kemudian merebak ke tisu-tisu hemopoietik dalam ginjal dan limpa. Klasifikasi dalam granuloma tidak berlaku tetapi pigmen lipofusin dan melanin telah dijumpai dalam sitoplasma sel raksaksa pada hari kelima suntikan. Dalam ikan yang dijangkiti secara semulajadi, mikobakteria menghasilkan banyak granuloma di berbagai organ. Organ ginjal dan limpa merupakan organ-organ yang paling serius yang kerap dijangkiti.

CHAPTER 1

GENERAL INTRODUCTION

Siamese fighting fish, *Betta splendens* Regan, is perhaps the most beautiful species within the genus *Betta*. Its shape and colour patterns attract fish hobbyist and it has become one of the most popular aquarium fishes (Plate 1). Thailand produces more than five million of these fish per year for export and it is now considered to be one of the most economically important ornamental fish species in Thailand.

Plate 1 The normal Siamese fighting fish *Betta splendens* Regan

Siamese fighting fish are widely distributed in natural reservoirs in Thailand. The intensive culture of this species is restricted to the central region of the country, including Bangkok, Nakornpathom and Rajaburi provinces. Under intensive culture, these fish are reared in cement ponds containing adequately clean water and are generally fed with water flea, *Moina macrocopa*. One of the main constraints in the intensive culture of Siamese fighting fish is the common occurrence of bacterial and parasitic diseases. Among the important bacterial diseases of *Betta splendens*, tuberculosis or mycobacteriosis ranks as number one. This disease has caused considerable mortalities among cultured *B. splendens* over the past years and has resulted in substantial economic losses.

Fish tuberculosis was first described in Thailand in 1983, in snakehead, *Channa striatus* (Fowler), by Limsuwan, Chinabut, Pawaputanon and Lawhavinit. Later, this disease was observed in a number of aquarium fish species, including *B. splendens*. The etiological agent of fish tuberculosis has been identified as *Mycobacterium* spp., belonging to order Actinomycetales, family Mycobacteriaceae. This bacterium is an acid fast, weakly Gram-positive, extremely pleomorphic, non-motile, non-spore forming, non-branching slender rod and usually requires enriched media to facilitate growth (Wayne and Kubica, 1986). In general, fish mycobacteria have an optimal

temperature range of 25-30°C. *Mycobacterium* spp. can be differentiated using their colony morphology, pigmentation, growth rate, carbohydrate fermentation, utilization of organic acids and nitrogen compounds, sensitivity to certain antimicrobial agents, and the presence of certain enzymes (Tsukamura, 1984).

Mycobacterium chelonei, *M. fortuitum* and *M. marinum* are commonly isolated from both infected fish and humans. As infected fish may not necessarily show clinical signs of mycobacteriosis, isolation and identification of *Mycobacterium* sp. is essential for definitive diagnosis. Histopathological evidence of focal granulomas within the infected tissue confirms the diagnosis.

Mycobacteriosis is a cosmopolitan disease of fish. The spread of the causative agent, *Mycobacterium* sp., and resulting outbreaks of mycobacteriosis are generally associated with the uncontrolled movement of infected fish with no clinical signs, and feeding fish with uncooked infected fish or fish offal. Transovarian spread of the disease has also been suggested (Nigrelli and Vogel, 1963).

The control of fish mycobacteriosis is a difficult task. There has been little or no work done on the treatment and control of this bacterial disease. Further, there is little information available, especially in

Thailand, on the isolation, characterization and antibiotic sensitivity of fish mycobacteria. The knowledge on histopathology of controlled infections of *Mycobacterium* spp. and the understanding of the defence mechanism of *B. splendens* against *Mycobacterium* spp. are far from adequate. Therefore, the present study was designed to elucidate the following:

- To characterize acid-fast bacteria (presumably *Mycobacterium* spp.) isolated from *B. splendens* in Thailand and to compare them with six reference strains of *Mycobacterium* sp.

- To study the temperature dependent growth of the above mentioned acid-fast bacteria.

- To determine the antimicrobial sensitivity and to study the minimal inhibitory concentration of the above mentioned acid-fast bacteria isolated from *B. splendens* in Thailand.

- To evaluate the efficacy of kanamycin sulphate as a chemotherapeutic agent against mycobacteriosis in *B. splendens*.

- To investigate the histopathological changes in *B. splendens* following natural and experimental infection of *Mycobacterium* spp.