



UNIVERSITI PUTRA MALAYSIA

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

TEMDOUNG PUNGKACHONBOON

FPSS 1994 1

STUDIES ON MYCOBACTERIOSIS IN SIAMESE FIGHTING FISH
Betta splendens REGAN

by

Temdoug Pungkachonboon

Dissertation Submitted in Fulfillment of the
Requirements for the Degree of Doctor of Philosophy
in the Faculty of Fisheries and Marine Science
Universiti Pertanian Malaysia

February 1994



ACKNOWLEDGEMENTS

I would like to acknowledge the International Development Research Centre (IDRC) for providing me a grant to work on the research described in this thesis and the Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand for providing me leave to study at the Faculty of Fisheries and Marine Science, Universiti Pertanian Malaysia.

I gratefully acknowledge Professor Dr. Mohd. Shariff for his supervision, reading the draft manuscript and for all his assistance and the hospitality throughout the study period. I am also grateful to Dr. Hassan Mohd. Daud for being the co-supervisor.

I am greatly indebted to Dr. Kamonporn Tonguthai, Director of Aquatic Animal Health Research Institute and Dr. Supranee Chinabut for their encouragement, valuable suggestions and support.

I also wish to express my sincere thank to Prof. Dr. T. Kimura and Dr. K. Tajima, Hokkaido University, Japan for their hospitality and for providing me an opportunity



to work in their laboratory on the DNA studies and for their supervision.

My gratitude also goes to Dr. Ong-ard Lawhavinit, Faculty of Veterinary, Kasetsart University for his valuable discussions. Thanks are also due to all of the technical staff at Aquatic Animal Health Research Institute for their help and looking after the experimental fish. My gratitude also goes to Mrs. Laiat Kruachan for her patience in typing the thesis.

My deepest gratitude to my parents, my brothers and my husband Mr. Pornsawan for their love and support.



TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF PLATES	xi
LIST OF ABBREVIATIONS	xiv
ABSTRACT	xv
ABSTRAK.....	xviii
 CHAPTER	
1 GENERAL INTRODUCTION	1
2 LITERATURE REVIEW	5
Biology and characteristics of the Siamese fighting fish <i>Betta splendens</i> Regan	5
Biology and taxonomy of mycobacteria ..	7
Isolation of mycobacteria	10
Culture of mycobacteria	12
Characterization of mycobacteria	13
Group I Photochromogens	14
Group II Scotochromogens	14
Group III Nonphotochromogens	15
Group IV Rapidly growing	15
Identification of mycobacteria	16
DNA sequencing as a tool for identification of mycobacteria	16



	Page
Diagnosis of mycobacteriosis	20
Etiological agents of mycobacteriosis	22
<i>Mycobacterium marinum</i>	23
<i>Mycobacterium fortuitum</i>	25
<i>Mycobacterium chelonae</i>	27
Growth dynamics	29
Environmental factors that affect the growth of mycobacteria	31
Nutrients	32
Hydrogen ion concentration (pH) .	34
Temperature	36
Oxygen	37
Growth factor	38
Sensitivity of <i>Mycobacterium</i> spp. to antimicrobial agents	39
Kanamycin sulfate as a therapeutic agent for mycobacteriosis	41
Chemical properties of kanamycin	42
Structural formula	42
Mechanism of kanamycin action	43
Therapy of fish mycobacteriosis	43
Histopathology of fish mycobacteriosis	45
Differential diagnosis and histopathology	52



	Page
3	
CHARACTERIZATION OF <i>Mycobacterium</i> sp. FROM SIAMESE FIGHTING FISH <i>Betta splendens</i> REGAN	54
Introduction	54
Material and methods	57
Characterization of the strain ...	58
Isolation of DNA	59
Bacterial pathogenicity tests	61
Experimental fish	61
Maintenance of moina culture	62
Preparation of bacterial suspension	63
Pathogenicity testing procedure	63
Results	64
Biochemical characteristics	64
DNA isolation	65
Pathogenicity of bacteria	66
Discussion	73
4	
EFFECT OF TEMPERATURE ON THE <i>IN VITRO</i> GROWTH OF <i>Mycobacterium</i> SP.	76
Introduction	76
Material and methods	78
Results	79
Discussion	83



	Page
5 SENSITIVITY OF ANTIMICROBIAL AGENTS AND THE EFFICACY OF KANAMYCIN SULPHATE AGAINST MYCOBACTERIOSIS IN SIAMESE FIGHTING FISH	86
Introduction	86
Material and methods	89
Antibiotic sensitivity tests	89
Bacterial inoculum	89
Inoculum of plates and application of discs	90
Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)	91
Sensitivity test	93
Efficacy of kanamycin sulphate	93
Experimental fish	93
Water flea culture (<i>Moina macrocopa</i>)	94
Stock solution of kanamycin sulphate	95
Bacterial suspension	95
Treatment of test fish	95
Results	97
Antibiotic sensitivity tests	97
Efficacy of kanamycin sulphate ...	100
Discussion	101



	Page
6	
HISTOPATHOLOGY OF SIAMESE FIGHTING FISH NATURALLY AND EXPERIMENTALLY INFECTED WITH <i>Mycobacterium</i> SP.	106
Introduction	106
Material and methods	107
Experimental fish.....	107
Water flea culture	107
Bacterial suspension	108
Histopathology	108
Results	109
Experimental infections	109
Natural infections	123
Discussion	129
7	
GENERAL DISCUSSION AND CONCLUSIONS	135
REFERENCES	141
BIOGRAPHICAL SKETCH	156



LIST OF TABLES

Table		Page
1	Prevalence of mycobacterial infection of Siamese fighting fish determined by bacterial isolation and acid-fast staining	67
2	Biochemical tests of mycobacteria isolated from Siamese fighting fish and six reference strains, <i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. fortuitum</i> , <i>M. marinum</i> <i>M. parafortuitum</i> , <i>M. vaccae</i> and "M. piscicida"	68
3	Melting point of precipitates of mycolic acid of <i>Mycobacterium</i> sp. isolated from Siamese fighting fish	72
4	Mol% G+C of <i>Mycobacterium</i> sp. isolated from Siamese fighting fish	72
5	Average growth rate of <i>Mycobacterium</i> sp. isolated from Siamese fighting fish	82
6	Sensitivity of <i>Mycobacterium</i> sp. to antimicrobial agents	99
7	Minimal inhibitory concentration and minimal bactericidal concentration of kanamycin sulphate on <i>Mycobacterium</i> sp. isolated from Siamese fighting fish	99
8	Sensitivity of antituberculosis drugs of humans medicine to <i>Mycobacterium</i> sp	100



LIST OF FIGURES

Figure		Page
1	Growth of <i>Mycobacterium</i> sp. isolated from Siamese fighting fish at different temperature	81

LIST OF PLATES

Plate		Page
1	The normal Siamese fighting fish <i>Betta splendens</i> Regan	1
2	After injection: Muscle necrosis (MN) and mild haemorrhagic lesion at the site of injection. (H&E) x 264	111
3	Day 1: Injection site with focal muscle necrosis (MN). Note the infiltration of red blood cell. (H&E) x 264	112
4	Day 2: Large number of macrophages (arrowhead) and a few lymphocyte (arrow) present between the muscle bundles in the infected area. (H&E) x 528	112
5	Day 3: Fibroblasts (arrowhead) were seen spreading throughout the affected area. (H&E) x 528	113
6	Day 4: Epithelioid cells (EC) start to form a layer surrounding the macrophages (arrowhead). (H&E) x 528	114
7	Day 5: Initiation of granulomas (G) at the site of inflammation, with the presence of a single layer of epithelioid cells. Note the large number of macrophages dispersed throughout the site of inflammation. (H&E) x 268	115
8	Day 5: Foreign type giant cells (GC) present in the infected area seen only on day 5. (H&E) x 536	115



	Page
9	Day 6: Increased numbers of granulomas (G) associated with more fibroblasts observed at the site of injection. (H&E) x 264 117
10	Day 7: Prominence of muscle regeneration (arrowhead). Note the reduction of fibrosis. (H&E) x 536 118
11	Day 10: Increase in the number and size of the granulomas (G) in the spleen. (H&E) x 264 118
12	Day 10: Increase in number and size of granulomas (G) in the kidney (a) and mesentary (b). (H&E) x 268 119
13	Day 14: Dilation and hyperaemia of the glomerulus capillaries (arrow) were observed. (H&E) x 528 120
14	Day 21: Granulomas with caseous necrosis (arrowhead) in the centre were seen in the muscle. (H&E) x 536 121
15	Day 28: Melanin deposits (arrowhead) among the connective tissues of granulomas. (H&E) x 264 122
16	Day 28: Granuloma (G) formation with the infiltration of fibrinous exudate seen in the kidney. (H&E) x 264 122
17	Naturally infected fish with (a) exophthalmia, (b) abdominal swelling, fin rot, tail rot, scale defect and white nodules (arrowhead) 124



	Page
18	Granulomas in various organs of naturally infected fish (a) lips x 40 (b) oesophagus x 80 (c) stomach x 99 125
19	Granulomas of (a) liver and pancreas x 201 (b) spleen x 201 (c) kidney x 99 in naturally infected fish (H&E) 126
20	Granulomas of (a) eye x 40 (b) heart x 99 (c) gills x 99 in naturally infected fish (H&E) 127
21	Granulomas of (a) epithelial layer of skin x 99 (b) mesentary x 201 (c) ovary x 201 in naturally infected fish (H&E) 128



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 Schiff
pH	=	potential of hydrogen
ppm	=	parts per million
RNA	=	ribonucleic acid
SDS	=	sodium dodecyl sulfate
SSC	=	Standard saline citrate
T _m	=	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

Temdoung Pungkachonboon

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 MIKOBACTERIOSIS KE ATAS IKAN PELAGA SIAM
Betta splendens REGAN**

Oleh

Temdoung Pungkachonboon

Februari 1994

Pengerusi : Prof. Dr. Mohamed Shariff Mohamed Din

Fakulti : Perikanan dan Sains Samudera

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. Kekekapan hadirnya mikobakteria di dalam ikan pelaga Siam yang dikultur berjangkit 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.

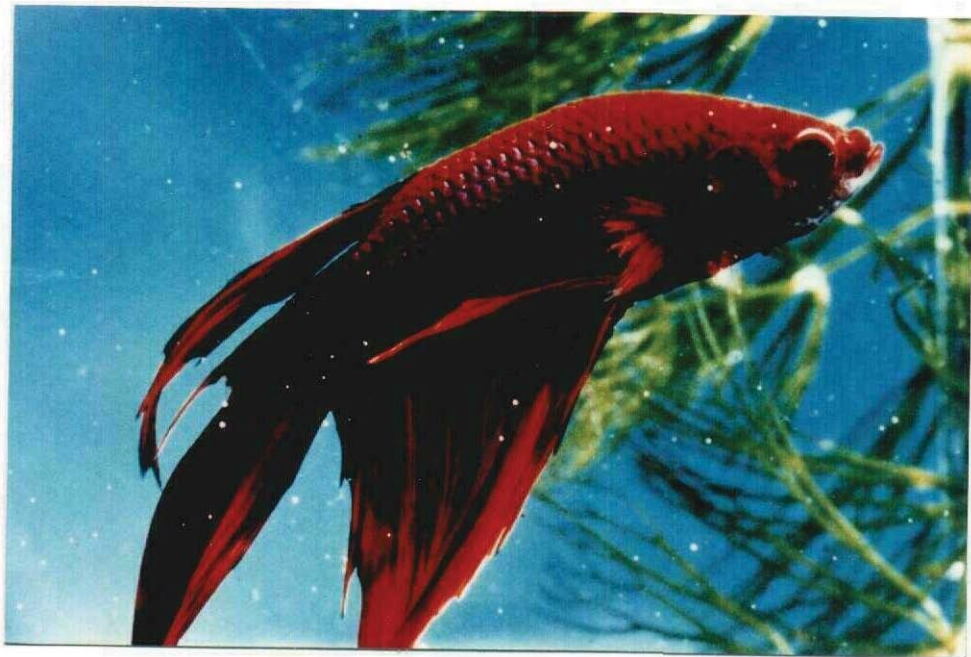


Plate 1 The normal Siamese fighting fish *Betta splendens* Regan

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 chelonae, *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.