UNIVERSITI PUTRA MALAYSIA

AETIOPATHOGENICITY OF ULCERATIVE DISEASE IN KOI CARP, *CYPRINUS CARPIO* LINNAEUS

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By

SUREERAT BUTPROM

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

November 2004

Dedicated to

My parent: Charat & Varee Butprom

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

AETIOPATHOGENICITY OF ULCERATIVE DISEASE IN KOI CARP, *CYPRINUS CARPIO* LINNAEUS

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November 2004

Chairman: **Associate Professor Dr. Hassan Hj Mohd Daud, Ph.D.**

Faculty: **Veterinary Medicine**

A chronic persistent ulcerative lesion of the skin in Koi carp (*Cyprinus carprio* L.) was reported in several commercial aquarium shops in Klang Valley. A study was conducted to chart the epizootiology and pathogenicity of the disease and its aetiological agents which included histopathological, bacteriological, virological and experimental infection.

Thirty-six diseased Koi carps with skin ulcer were examined. Findings showed that the ulcerative lesion development involved the deep muscle layers showing tissue necrosis and inflammation in chronic lesions. The fish abdomen was filled with a clear or red-tinged ascitic fluid. The liver and kidneys were pale in colour, swollen and friable. In the histopathological study, lesions were found in the skin, gill, kidneys, liver, spleen and intestine. The changes were characterized by diffuse haemorrhages, cell degeneration and necrosis in the skin, liver and kidneys. Lamellar epithelial cells showed hyperplasia and hypertrophy at the base of gill lamellae. Depositions of haemosiderin were seen in kidneys, liver, hepatopancreas and spleen. In the intestine, haemorrhage in the tunica propria and atrophy of mucosal epithelium

were seen. Electron microscopy revealed two type of virus like particles which were associated with the histopathological changes in the kidneys. The virus-like particles were presumably coronavirus and reovirus based on their morphology.

Morphological and biochemical characteristics of bacteria isolated from the diseased Koi were determined by routine biochemical tests in combination with the BBL Crystal Kit™. In the present study, Gram-negative non-lactose fermenting rods were isolated from the skin lesions and kidneys. In total, 11 bacteria species were identified and *Aeromonas hydrophila* was the dominant species isolated from the fish in this study. The other species of the ulcer-associated bacteria were (i) *Shewanella putrefaciens*, (ii) *Vibrio cholerae*, (iii) *Pseudomonas diminuta*, (iv) *Chryseobacterium meningosepticum*, (v) *Empedobacter brevis*, (vi) *Pseudomonas aeruginosa*, (vii) *Pantoea agglomerans*, (viii) *Enterobacter sakazakii*, (viiii) *Morganella morganii* and (x) *Aeromonas veronii*.

Primary cell cultures were initiated from the brain, gonad and kidneys of goldfish (*Carassius auratus*) by using trypsinization technique. The cells were cultured in Leibovitz-15 (L-15) medium supplemented with 10 to 20% fetal calf serum (FCS) and L-glutamine. The primary cell cultures from brain and gonad tissues were successfully established and reached monolayer confluence within 15 to 30 days. The attachment efficiency was serum-dependent though increasing FCS concentration did not stimulate further growth of cells. Virological examinations of ulcerated tissues on the primary cell cultures and established cell line, FHM, were negative for cytopathic effect (CPE) even after three blind passages of 10 days interval.

Aeromonas hydrophila, *S. putrefaciens* and *V. cholerae* isolated from natural Koi carp with skin ulcer were used in experimental infection. The fish were injected 0.1 ml bacteria suspension at $1x10^7$ cfu/ml. Bacteria were reisolated from ulcerative lesion and kidneys and had the same biochemical characteristics as those isolated from naturally infected fish. Ulcers began to appear three post infection as small and flat lesions. No apparent mortality was observed in all groups during the 30 days of the experiment. Histopathological studies revealed that *A. hydrophila* individually or in combination with other bacteria could have caused the small superficial ulcerative lesions. Haemorrhages and inflammation were seen in spleen, adipose tissue and kidneys. While individually or in combination, injection of *S. putrefaciens* and *V. cholerae* displayed localized lesion which was restricted only to the injection site.

In conclusion, ulcerative lesion in Koi carp was primarily caused by multiple infection of several bacteria species, although the possibility of viral involvement must not be ruled out.

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

ETIOPATOGENISITI PENYAKIT ULSER PADA IKAN KOI, *CYPRINUS CARPIO* LINNAEUS

Oleh

SUREERAT BUTPROM

November 2004

Pengerusi: **Profesor Madya Dr. Hassan Hj Mohd Daud, Ph.D.**

Fakulti: **Perubatan Veterinar**

Penyakit ulser yang kronik pada ikan Koi carp (*Cyprinus caprio* L.) telah dilaporkan di beberapa pusat akuarium komersil dan pusat pengumpulan ikan di sekitar Lembah Klang. Satu penyelidikan telah dijalankan untuk mengetahui agen-agen etiologin penyakit ini dan pathogenisitinya yang mana merangkumi kajian histopatologi, bakteriological, virologi dan ujian jangkitan

Sebanyak tiga puluh enam ekor ikan kap Koi yang berpenyakit ulser telah diperiksa. Hasil pemerhatian telah menunjukkan bahawa pertumbuhan ulser adalah melibatkan lapisan otot dalam yang menunjukkan nekrosis tisu dan inflamasi bagi lesi-lesi yang kronik. Bahagian abdomen ikan telah dipenuhi oleh cecair asites yang berwarna merah atau jernih. Bahagian hati dan ginjal pula bewarna pucat, membengkak dan mudah hancur. Di dalam kajian histopatologi, lesi-lesi dapat dilihat di bahagian kulit, insang, ginjal, hati, limpa dan usus. Perubahan-perubahan ini telah dicirikan mengikut hemoraj resap, degenerasi sel dan nekrosis di dalam kulit, hati dan ginjal. Sel epithelial lamella telah menunjukkan hyperplasia dan hipertrofi pada bahagian lamella insang. Deposit haemosiderin telah dilihat di dalam ginjal, hati, pankreas dan

limpa. Di dalam usus kecil hemoraj di dalam tunika propria dan atrofi mucosa epithelium juga telah dilihat. Mikroskopi elektron pula telah menunjukkan dua partikel seperti virus yang mana dikaitkan dengan perubahan-perubahan histopatologikal di ginjal. Partikel seperti virus ini berkemungkinan adalah koronavirus atau reovirus berdasarkan ciri-ciri morfologinya.

Ciri-ciri morfologi dan biokimia bagi isolat bakteria daripada ikan kap Koi yang berpenyakit telah ditentukan dengan cara ujian kimia berserta dengan BBL Crystals Kit™. Di dalam kajian ini, rod gram negatif fermentasi bukan laktas telah diisolat daripada lesi di bahagian kulit dan ginjal. Secara keseluruhannya,11 spesis bakteria telah dikenalpasti di mana *Aeromonas hydrophila* merupakan spesis yang paling dominan diisolat bagi keseluruhan persampelan yang telah dibuat dalam kajian ini. Spesis-spesis bakteria lain yang juga menyebabkan ulser adalah seperti (i) *Shewanella putrefaciens*, (ii) *Vibrio cholerae*, (iii) *Pseudomonas diminuta*, (iv) *Chryseobacterium meningosepticum*, (v) *Empedobacter brevis*, (vi) *Pseudomonas aeruginosa*, (vii) *Pantoea agglomerans*, (viii) *Enterobacter sakazakii*, (viiii) *Morganella morganii* dan (x) *Aeromonas veronii*.

Kultur-kultur sel primer telah dihasilkan daripada tisu otak, gonad dan ginjal ikan emas (*Carassius auratus*) melalui teknik pentripsinin. Sel-sel ini dikultur di dalam media L-15 yang tambah dengan 10-20% serum anak lembu (FCS) dan L-glutamine. Kultur-kultur sel primer daripada tisu otak dan gonad telah berjaya ditumbuhkan serta mencapai konfluen ekalapis dalam masa 15-30 hari. Keupayaan perlekatan sel adalah bergantung kepada kandungan serum walaupun peningkatan kepekatan FCS tidak seterusnya merangsang pertumbuhan sel yang lebih menggalakkan. Kajian

virus ke atas tisu-tisu ulser ke di dalam kultur sel primer dan sel kekal, FHM pula adalah negatif bagi kesan sitopatiknya (CPE) walaupun selepas tiga peringkat pasaj buta yang berselang 10 hari bagi setiap pasaj.

Aeromonas hydrophila, *S. putrefaciens* dan *V. cholerae* yang diisolat daripada ikan kap Koi yang mempunyai ulser telah digunakan di dalam jangkitan eksperimen. Ikan kap telah disuntik dengan 0.1 ml bakteria pada kepekatan $1x10^7$ cfu/ml. Bakteria ini boleh diisolat semula daripada lesi ulser dan ginjal dan mempunyai ciri-ciri biokimia yang sama seperti bakteria yang diisolat daripada ikan yang mempunyai infeksi semulajadi. Tanda-tanda penyakit mula dilihat tiga hari selepas jangkitan dibuat yang mana berupa lesi ulseratif yang kecil dan rata. Tiada sebarang kematian diperhatikan bagi semua kumpulan selama 30 hari ujikaji dijalankan. Histopatologi telah menunjukkan, sama ada secara individu atau kombinasi dengan bakteria lain, *A. hydrophila* boleh menyebabkan lesi permukaan berulser yang kecil. Hemoraj dan inflamasi telah dikesan pada bahagian limpa, tisu adipos dan ginjal. Walaubagaimanapun, sama ada secara individu atau kombinasi, suntikan *S. putrefaciens* dan *V. cholerae* telah menunjukkan lesi setempat di mana ianya hanya terdapat di bahagian yang disuntik sahaja.

Pada kesimpulanya penyakil ulseratif pada ikan kap Koi pada asasnya disebabkan oleh jangkitan majmuk beberapa spesis bakteria, tetapi kemungkinan penglibatan virus tidak boleh ditolak.

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I certify that an Examination Committee met on 15 September 2004 to conduct the final examination of Sureerat Butprom on her Master of Science thesis entitled "Aetiopathogenicity of Ulcerative Disease in Koi Carp, *Cyprinus carpio* Linnaeus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

SUREERAT BUTPROM

Date:

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AS	Atlantic salmon gonad cell line
BB	brown bullhead trunk cell line
BF-2	bluegill fry
CCO	channel catfish ovary
CCVD	channel catfish virus disease
cfu	colony forming unit
CHSE-214	Chinook salmon embryo
CPE	cytopathic effect
CRV	channel catfish reovirus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetatic acid
ELISA	enzyme-linked-immunosorbent assay
EPC	epithelioma papulosum cyprini
EUS	epizootic ulcerative syndrome
FBS	fetal bovine serum
FHM	fathead minnow peduncle
HCl	hydrochloride acid
HEPES	N-[2-hydroxyethyl] piperazine N^1- [2-ethanesulfonic acid]
H&E	hematoxylin and eosin
H_2 S	hydrogen sulphide
IHNV	infectious haemopoietic necrosis virus
IM	intramuscular
IPNV	infectious pancreatic necrosis virus

IU/ml	international unit per milliliter
MEM	minimum essential medium
MEM-4	minimum essential medium with 4% serum
MEM-10	minimum essential medium with 10% serum
mM	millimolar
$NaHCO_3$	sodium bicarbonate
NEAA	non-essential amino acids
OD	optical density
O/F	oxidative-fermentative
PCR	polymerase chain reaction
PTA	phosphotungstic acid
RNA	ribonucleic acid
rpm	revolution per minute
RTG-2	rainbow trout gonad
SEM	scanning electron microscopy
SGV	sand goby virus
SHRV	snakehead rhabdovirus
SVCV	spring viraemia of carp virus
TCBS	thiosulphate citrate bilesalt sucrose agar
TEM	transmission electron microscopy
TSA	tryptic soy agar
TSI	triple sugar iron agar
UDRV	ulcerative disease rhabdovirus
UV	ultra violet
VHSV	viral hemorrhagic septicaemia virus

CHAPTER 1

INTRODUCTION

Ornamental fish keeping is a world-wide hobby. In Malaysia, aquarium fish trade started in the 1950's within the southern state. Initially, the activity was mainly limited to the collection of fish from the wild for subsequent distribution to Singapore, which began to export fish to Europe in the late 1940's (Tan, 2002). Significant development of aquarium fish farms only began in the 1980's. Today, there are more than 400 farms, 90% of these produce ornamental fishes, and about 10% produce natural feed and aquatic plants (Anon, 2003a). The potential for further expansion of the industry in Malaysia is enormous.

In tandem with the development of ornamental fish farms, there has been a significant increase in the export of ornamental fish from Malaysia in the last few years. Within the five year period, from 1991 to 1995, export had significantly increased from 101.4 million tails valued at RM14.8 million to 175.6 million tails in 1994 valued at RM25.9 million, an increase of more than 150%. Statistical data from 1995 to 2001 showed that in terms of production, this has increased from about 100 million tails in 1995 to 340 million tails in 2001 giving an increase of 240%. Ornamental fish farming is largely an export oriented industry. It was estimated that about 96% of ornamental fish produced in this country are exported. This made the ornamental fish industry the fastest growing component in the fisheries sector and a significant source of foreign currency earner (Tan, 2002; Anon, 2003a; 2003b)