UNIVERSITI PUTRA MALAYSIA

STUDIES ON MOTILE *AEROMONAS SPP*. ASSOCIATED WITH HEALTHY AND EPIZOOTIC ULCERATIVE SYNDROME-POSITIVE FISH

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STUDIES ON MOTILE *AEROMONAS* SPP. ASSOCIATED WITH HEALTHY AND EPIZOOTIC ULCERATIVE SYNDROME-POSITIVE FISH

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ATCC	- American Type Culture Collection
ECP	- Extracellular product
EUS	- Epizootic ulcerative syndrome
GC (=G+C)	- Guanine + Cytosine
LPS	- Lipopolysaccharide
MR	- Methyl red
NB	- Nutrient broth
OD (=O.D.)	- Optical density
RS	- Rimler - Shotts medium
SEAFDEC-AQD	- Southeast Asean Fisheries Development Center - Aquaculture Department
SIM	- Sulfide indol motility
SLS	- Sodium lauryl sulfate
SSC	- Standard saline citrate
S_{value}	- Similarity value
TSA	- Trypticase soy agar
UPV-BAC	- University of the Philippines in the Visayas-Brackishwater Aquaculture Center
VP	- Voges-Proskauer

Abstract of the thesis presented to the Senate of the Universiti Pertanian Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy.

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by

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August, 1990

Supervisor: Assoc. Prof. Mohamed Shariff, Ph.D.

Faculty: Fisheries and Marine Science

Aeromonas hydrophila was reported to be associated with epizootic ulcerative syndrome (EUS) outbreaks in the Asia-Pacific region since the 1980s. However, the precise role of *A. hydrophila* in EUS is not clear because its taxonomy is confusing, with reports of wide phenotypic, serological and genotypic characteristics of the organism. *Aeromonas hydrophila* is normally present in freshwater environments and in fish intestines.

This study was, therefore, undertaken to examine the relationships among motile *Aeromonas spp.* associated with healthy and EUS-positive fish.

A total of 54 motile *Aeromonas* strains were studied. Twenty-nine strains were from EUS-positive fish, 19 from healthy fish, and six were reference strains. Out of the 54 strains, 24 were identified as *A. hydrophila*, 17 were *A. hydrophila-like*, three *A. sobria*, five *A. sobria-like*, one *A. caviae*, and four unclassified *Aeromonas spp*. The identification took into consideration the biochemical characteristics of the two type strains, ATCC 7966 (*A. hydrophila*) and ATCC 9071 (*A. sobria*).

Nine of the strains were categorized highly virulent, 12 weakly virulent, and 27 avirulent. The reference strains were not screened for virulence. The LD_{50} of representative highly virulent strains ranged from 1 to 2 x 10^4 cells/fish. Crude ECP resulted in oedematous reaction upon intramuscular injection. In addition, all strains studied, except one, caused hemolysis to bovine blood cells.

Numerical taxonomy analysis of the strains resulted to eight phenetic groups. Phenons I and II were identified as *A. hydrophila*. Phenon I, on one hand, comprised the highly virulent *A. hydrophila*, the majority of which were isolated from EUS-positive fish. Phenon II, on the other hand, were avirulent, the majority of which were isolated from healthy fish. The type strain ATCC 7966 (*A. hydrophila*) fell in phenon

VII, while type strain ATCC 9071 (*A. sobria*) did not cluster with any of the phenons.

Serological studies of the strains revealed that isolates in Phenon I shared common antigens with nos. 5 and 45 which were members of the same phenon. Phenon I was, therefore, designated *A. hydrophila* serotype I. All other *A. hydrophila* which were serologically heterogeneous were designated under *A. hydrophila* serotype II.

Subsequent DNA-DNA hybridization of representative strains from the phenons revealed that phenons I (*A. hydrophila*), II (*A. hydrophila*), and III (*A. hydrophila* and *A. hydrophila-like*) were genetically related with the type strain ATCC 7966 [*A. hydrophila* (phenon VII)] with homology values of more than 70%. Phenons V, VI and VIII were, however, genetically distant against the type strain. Phenon V was proposed as a new species given the name *A. pastoria* because of its distinct phenotypic characters against the other three recognized motile aeromonads and its distant genetic relationship with ATCC 7966. *Aeromonas pastoria*, however, clearly belonged to the genus *Aeromonas* in the family Vibrionaceae.

Based on virulence, numerical taxonomy and serological studies, it was suggested that *A. hydrophila* serotype I was the causative agent of epizootic ulcerative syndrome.

Abstrak tesis yang diserahkan kepada Senat Universiti Pertanian Malaysia sebagai syarat bagi memenuhi keperluan Ijazah Doktor Falsafah

KAJIAN KEATAS SPESIES-SPESIES *AEROMONAS* MOTIL YANG BERKAITAN DENGAN IKAN SIHAT DAN IKAN BERSINDROM EPIZOOTIK POSITIF

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Aeromonas hydrophila telah dilaporkan terbabit dengan wabak sindrom epizootic ulseratif (SEU) dalam kawasan Asia-Pasifik semenjak tahun 1980an. Tetapi peranan sebenar *A. hydrophila* dalam SEU tidak berapa jelas kerana taksonominya adalah mengelirukan, dengan laporan fenotipik yang luas, ciri-ciri serologikal dan genotipik organisma tersebut. *Aeromonas hydrophila* biasanya berdapat dalan perseketaran air dan usus ikan.

Kajian ini, oleh ito, dijalankan untuk memeriksa hubungan di antara spesies-spesies *Aeromonas* yang terlibat dengan ikan sihat dan SEU positif.

Sejumlah 54 strain *Aeromonas* motil telah dikaji. Dua puluh sembilan strain adalah dari ikan SEU positif, 19 dari ikan sihat dan enam dari strain rujukan. Dari 54 strain, 24 dikenalpasti sebagai *A. hydrophila*, 17 adalah seakan *A. hydrophila*, tiga sebagai *A. sobria*, lima seakan *A. sobria*, satu *A. caviae*, dan empat spesies *Aeromonas* tanpa kelas. Pengenalpastian mengambil kira ciri-ciri biokimia dua jenis strain tip iaitu ATCC 7966 (*A. hydrophila*) dan ATCC 9071 (*A. sobria*).

Sembelan dari strain, dikategorikan sebagai amat virulen, 12 virulen lemah, dan 27 avirulen. Strain rujukan tidak dinilai untuk potensi virulens. LD_{50} bagi wakil strain amat virulen berjulat dari 1 hingga 2 x 104 sel/ikan. ECP kasar menyebabkan reaksi edema selepas suntikan ke dalam otot. Selain daripada itu, kesemua strain, melainkan satu, adalah hemolitik keatas sel darah bovina.

Analisa taksonomi numerikal strain telah mengahasikan lapan kumpulan fenetik. Fenon I dan II dikenalpasti sebagai *A. hydrophila*. Fenon I adalah berkaitan dengan *A. hydrophila* yang amat virulen, yangmana sebahagian besarnya diasing dari ikan yang dijangkiti SEU. Fenon II, di sebaliknya, avirulen, dimana kebanyakanya dipencil dari ikan sihat. Strain tip ATCC 7966 (*A. hydrophila*) jatuh ke dalam fenon VII, sementara strain tip ATCC 9071 (*A. sobria*) tidak dapat ditempatkan ke dalam mana-

mana fenon.

Kajian serologi terhadap strain menunjukan fenon I mempunyai antigen sepunya bersama nos. 5 dan 45 yangmerupakan ahli dari fenon yang sama. Fenon I, dinamakan *A. hydrophila* serotip I. Kesemua *A. hydrophila* yang lain secara serologi adalah heterogen dan diletakkan dibawah *A. hydrophila* serotip II bagi memisahkan mereka dari fenon I.

Hibridisi DNA-DNA strain adalah wakil dari fenon-fenon selajutnya yang menunjukan bahawa fenon I (*A. hydrophila*), II (*A. hydrophila*), dan III (*A. hydrophila* dan seakan *A. hydrophila*) adalah secara genetik berhubung rapat dengan strain tip ATCC 7966 [*A. hydrophila* (fenon VII)] dengan nilai homologi lebih dari 70%. Fenon-fenon V, VI dan VIII adalah, walau bagaimanapun, secara genetik yang jauh berbeza daripada strain tip. Fenon V disyorkan sebagai satu spesies baru yang dinamakan *A. pastoria* kerana ciri-ciri fenotipnya jauh berbeza dari tiga aeromonad motil yang dikenali dan pertalian genetiknya dengan ATCC 7966. *Aeromonas pastoria*, walau bagaimanapun, jelas terletak dalam genus *Aeromonas* dibawah keluarga Vibrionaceae.

Berdasarkan atas virulensi, taksonomi numerikal dan serological, adalah disyorkan bahawa *A. hydrophila* serotip I adalah agen etiologi sindrom epizootik ulseratif.

CHAPTER I

GENERAL INTRODUCTION

Aeromonas hydrophila is a gram-negative bacterium considered autochthonous inhabitant of aquatic environments (Kaper *et al.*, 1981). It is distributed worldwide and has been isolated from both polluted and unpolluted water. Though considered a freshwater species, it is also known to survive in saltwater of low salinity (Williams and LaRock, 1985). *Aeromonas hydrophila* is said to comprise a portion of normal microflora of fishes, as well as other aquatic animals and plants (Simidu *et al.*, 1971; Trust and Sparrow, 1974). It is considered an opportunistic pathogen in fish with immune deficiency but others have considered them a primary pathogen (Austin and Austin, 1987).

Studies have also shown that *A. hydrophila* were pathogenic to fish compared with the other two recognized motile aeromonads: *Aeromonas caviae* and *Aeromonas sobria* (Boulanger *et al.*, 1977; Olivier *et al.*, 1980; Popoff, 1984). However, new findings have shown that *A. sobria* was more pathogenic than *A. hydrophila* and *A. caviae* (Janda *et al.*, 1985). The mechanisms of pathogenesis of motile aeromomads are, however, not fully understood (Brenden and Huizinga, 1986b).