



**UNIVERSITI PUTRA MALAYSIA**

**ISOLATION, IDENTIFICATION AND MOLECULAR  
CHARACTERISATION OF AEROMONAS SPECIES FROM FISH**

**NOORLIS AHMAD**

**FSMB 2001 17**

**ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERISATION  
OF *AEROMONAS* SPECIES FROM FISH**

**By**

**NOORLIS AHMAD**

**Thesis Submitted in Fulfilment of the Requirement for the  
Degree of Master of Science in the Faculty of Science  
Universiti Putra Malaysia**

**June 2001**



**SPECIALLY DEDICATED TO :**

*My beloved*

*Grandmother, abah, emak, adik,*

*relatives and friends*

*for your support .....*

*Thank You Very Much.....*

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

**ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERISATION  
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**Chairman : Associate Professor Son Radu, Ph. D.**

**Faculty : Food Science and Biotechnology**

A total of 60 isolates of *Aeromonas* species which were originally isolated from various fish samples obtained from wet markets in Selangor were examined and further characterised by both phenotypic (antibiotics resistance and hemolysis assay) and genotypic (plasmid profiling, RAPD pattern and SDS-PAGE) methods. All the isolates examined in this study exhibited multiple antibiotic resistance pattern to antibiotics (ampicillin (98.4%), carbenicillin (93.6%), erythromycin (91.9%), bacitracin (87.1%), streptomycin (74.2%), kanamycin (58.1%), gentamycin (53.2%), tetracycline (46.8%), cephalothin (33.9%), nalidixic acid (25.8%), ceftriaxone (76.1%), cefoperazone (14.5%) and ceftazidime (8.06%) ) tested. Plasmid analysis showed that 38.3%, 20%, 16.7% and 8.3% of isolates from Ikan Tilapia Merah, Ikan Keli, Ikan Terubuk and Ikan Merah respectively contained plasmid DNA bands with sizes ranging from 1.7 to 10.4 megadalton (MDa). Based on their plasmid profiles, the isolates of the *Aeromonas* species isolates were grouped into 18 plasmid patterns. Three 10-mer oligonucleotides primers GEN 1-50-02 (5'-CAATGCGTCT-3'), GEN1-50-06 (5'-CGGATAACTG-5')



and GEN1-50-08 (5'-GGAAGACAAC-3') were used to amplify genomic DNA. The profiles observed after electrophoretic separation for the 3 primers when combined together were able to distinguish the *Aeromonas* species isolates into 4 major clusters, respectively. In haemolysis assays of *Aeromonas* species, 71.7% were observed to be alpha ( $\alpha$ ), 21.7% were beta ( $\beta$ ) and only 6.7% were gamma ( $\gamma$ ) haemolytic. The SDS-PAGE of whole cell protein pattern analysis indicated that the strains of *Aeromonas hydrophila* group have a dominant band of variable molecular weight between 25 to 67 kDa. Thus, the strains of *Aeromonas* species examined from various types of fish are genotypically diverse as shown by RAPD and SDS-PAGE protein pattern, suggesting that different strains have been brought into this geographical region or strains already present have continued to evolve. These results suggest that RAPD-PCR assay and SDS-PAGE whole cell protein pattern are more powerful methods than plasmid profiling and antibiotic resistance technique for discriminating *Aeromonas* species. Thus, RAPD-PCR assay and SDS-PAGE whole cell protein can be used as a valuable tool for epidemiological studies.

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

**PEMENCILAN, IDENTIFIKASI DAN PENCIRIAN SECARA MOLEKUL  
SPESIS *AEROMONAS* DARIPADA IKAN**

Oleh

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Sejumlah 60 pencilan spesis *Aeromonas* yang dipencilkan daripada pelbagai jenis ikan yang didapati daripada pasar-pasar di Selangor dikaji dan seterusnya dicirikan dengan kaedah “phenotypic” (antibiotik dan asai hemolisis) dan “genotypic” (profil plasmid, polimorfik menggunakan analisis RAPD dan SDS-PAGE). Semua pencilan yang digunakan di dalam kajian ini didapati memaparkan kepelbagaian corak terhadap kerintangan antibiotik (ampicillin (98.4%), carbenicillin (93.6%), erythromycin (91.9%), bacitracin (87.1%), streptomycin (74.2%), kanamycin (58.1%), gentamycin (53.2%), tetracycline (46.8%), cephalothin (33.9%), nalidixic acid (25.8%), ceftriaxone (76.1%), cefoperazone (14.5%) and ceftazidime (8.06%) ) yang diuji. Profil plasmid yang diperolehi menunjukkan 38.3%, 20%, 16.7% dan 8.3% pencilan untuk Ikan Tilapia Merah, Ikan Keli, Ikan Terubuk dan Ikan Merah masing-masing mengandungi plasmid yang berada pada julat saiz antara 1.7 hingga 10.4 megadalton (MDa). Berdasarkan profil plasmid, pencilan spesis *Aeromonas* dapat dikumpulkan kepada 18 corak plasmid masing-masing. Tiga primer oligonukleotid 10-mer iaitu GEN 1-50-02 (5'-



CAATGCGTCT-3'), GEN1-50-06 (5'-CGGATAACTG-5') dan GEN1-50-08 (5'-GGAAGACAAC-3') digunakan untuk mengamplifikasikan genomik DNA. Penggabungan ketiga-tiga profil plasmid dapat membezakan kesemua spesis *Aeromonas* yang diuji kepada 4 kumpulan utama. Kajian hemolisis yang dijalankan ke atas semua pencilan *Aeromonas* di dalam kajian menunjukkan 71.7% pencilan hemolisis jenis alfa ( $\alpha$ ), sementara 21.7% jenis beta ( $\beta$ ) dan hanya 6.7% jenis gama ( $\gamma$ ) sahaja. Dengan menggunakan teknik analisis SDS-PAGE bagi profil protein sel, spesis *Aeromonas* mempunyai beberapa jalur dominan dengan berat molekul diantara 25 hingga 67 kDa. Pencilan spesis *Aeromonas* yang diperolehi dari pelbagai jenis ikan adalah berbeza seperti yang ditunjukkan oleh corak RAPD dan SDS-PAGE. Keputusan ini mencadangkan bahawa pencilan-pencilan yang sedia ada terus mengalami proses evolusi. Keputusan keseluruhan kajian ini menunjukkan bahawa teknik RAPD-PCR dan SDS-PAGE adalah lebih berkesan dari teknik profil plasmid dan kerintangan terhadap antibiotik untuk mendiskriminasikan spesis *Aeromonas*. Oleh itu RAPD-PCR dan SDS-PAGE boleh digunakan sebagai kaedah atau teknik yang amat berguna di dalam bidang kajian epidemiologi.

## **ACKNOWLEDGEMENTS**

I would particularly like to express my deepest gratitude to my supervisor **Associate Professor Dr. Son Radu** for his continuous support, patience, invaluable advice, and guidance through out the course of this project. My deepest gratitude to my co-supervisors, **Dr. Foo Hooi Ling** and **Dr. Abdul Reezal Abdul Latif**. Without their persistent assistance and exceptional generosity, this work would not have been possible and meet the requirement of the course.

Special thanks are extended to all the postgraduate students of Foodborne Pathogen and Molecular Subtyping Group for their continuous support and assistance through out the whole project. Heart felt thanks to those helpful people of the Department of Biotechnology and Department of Food Science, and Graduate School (GSO) who have assisted and supported me either directly or indirectly through out the whole duration of my project and writing up for the final thesis.

Last but not least of all, a special note of thanks to my family (Abah, Emak and Adik), friends (Yanti, Wai Ling, Kqueen, Sam, Pak Herman, Zaza, Mr. Naseer, Ibu Endang, Kak Zila, Kak Zu, En. Zainuri and Kamil) and last but not least, all of my housemates (Ku Zah, Pura, Julia, Kak Ta, Yeen, Ina, P-Nat, Deeda, Lyna and Ja) who share much of my joy and sorrow. Words just cannot express my gratitude for their love, concern, support and patience that have sustained me throughout the whole course of my postgraduate studies.





I certify that an Examination Committee met on 19<sup>th</sup> June 2001 to conduct the final examination of Noorlis Ahmad on her Master of Science thesis entitle “Isolation, Identification and Molecular Characterisation of *Aeromonas* species Isolated from Fish” 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 Examination Committee are as follows :

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## **DECLARATION**

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



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(NOORLIS AHMAD)

Date : 20 June 2001

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## LIST OF ABBREVIATIONS

%	percentage
β	beta
α	alpha
γ	gamma
μg	microgram
μl	microlitre
Am	ampicillin
AMP	adenosine 3', 5'- monophosphate
AP-PCR	arbitrarily primed- polymerase chain reaction
APS	ammonium persulphate
APW	alkaline peptone water
B	bacitracin
BAA	blood ampicillin agar
BHIA	brain heart infusion agar
BSA	Bovine serum albumin
C	chloramphenicol
°C	degree Celsius
Caz	ceftazidime
Cb	carbenicillin
Cf	ceftriaxone



Cfu	colony forming unit
Cfp	cefoperazone
CO <sub>2</sub>	carbon dioxide
Cro	ceftriaxone
DNA	deoxyribonucleic acid
dNTP	deoxynucleic triphosphate
E	erythromycin
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	for example
EDTA	ethylenediamine tetraacetic acid
EPEC	enteropathogenic <i>Escherichia coli</i>
EtBr	ethidium bromide
ETEC	enterotoxigenic <i>Escherichia coli</i>
F	fertility
g	gram
G+C	guanine + cytosine
GET	glucose-EDTA-tris buffer
Gm	gentamicin
GSP	glutamate starch phenol-red agar
H <sub>2</sub>	hydrogen
HCl	Hydrochloric acid
HGs	hybridisation groups

<b>K</b>	<b>kanamycin</b>
<b>Kb</b>	<b>kilobase</b>
<b>kDa</b>	<b>kilodalton</b>
<b>LB</b>	<b>Luria Bertani</b>
<b>LT</b>	<b>Heat-labile toxin</b>
<b>M</b>	<b>molar</b>
<b>MDa</b>	<b>megadalton</b>
<b>Mg</b>	<b>miligram</b>
<b>MgCl<sub>2</sub></b>	<b>magnesium chloride</b>
<b>ml</b>	<b>mililitre</b>
<b>Mol</b>	<b>mole</b>
<b>Na</b>	<b>nalidixic acid</b>
<b>NaCl</b>	<b>sodium chloride</b>
<b>ND</b>	<b>non detected</b>
<b>ng</b>	<b>nanogram</b>
<b>no.</b>	<b>number</b>
<b>Nor</b>	<b>norfloxacin</b>
<b>PBS</b>	<b>phosphate buffer saline</b>
<b>PCI</b>	<b>phenol chloroform isoamylalcohol</b>
<b>PCR</b>	<b>polymerase chain reaction</b>
<b>PFGE</b>	<b>pulsed field gel electrophoresis</b>
<b>BIBG</b>	<b>bile-salts-irgasan-brilliant green agar</b>
<b>POR</b>	<b>plasmid occurrence rate</b>

<b>pmol</b>	<b>picomole</b>
<b>R</b>	<b>resistant</b>
<b>RNA</b>	<b>ribonucleic acid</b>
<b>rpm</b>	<b>revolution per minute</b>
<b>S</b>	<b>streptomycin</b>
<b>SAA</b>	<b>starch ampicillin agar</b>
<b>SDS-PAGE</b>	<b>sodium dodecyl sulphate – polyacrylamide gel electrophoresis</b>
<b>sp.</b>	<b>species</b>
<b><i>Taq</i></b>	<b><i>Thermus aquaticus</i> DNA (polymerase)</b>
<b>TBE</b>	<b>tris-boric-acid-EDTA</b>
<b>Te</b>	<b>tetracycline</b>
<b>TEMED</b>	<b>N,N,N,N, tetramethylethylene-diamine</b>
<b>Tris</b>	<b>tris (hydroxymethyl methylamine)</b>
<b>TSBA</b>	<b>typticase soy broth agar</b>
<b>UV</b>	<b>ultra violet</b>
<b>V</b>	<b>volts</b>
<b>v/v</b>	<b>volume per volume</b>
<b>w/v</b>	<b>weight per volume</b>



## CHAPTER 1

### INTRODUCTION

Foodborne disease has become a topic of much recent attention as reported incidence of gastrointestinal disease worldwide has increased dramatically during the 1990s. Various organisms such as *E. coli*, *Shigella*, *Vibrio* and *Aeromonas* have been isolated. The genus *Aeromonas* was proposed first by Kluver and Van Niel in 1936 (Popoff, 1984). The genera *Aeromonas*, *Vibrio*, *Photobacterium* and *Pleisomonas* are included in the family *Vibrionaceae*. On the basis of molecular genetic evidence, proposals have been made to divide the genus *Aeromonas* in a new family, *Aeromonadaceae* (Kuijper *et al.*, 1989).

The genus *Aeromonas* consists of two groups of organisms ; (1) a single nonmotile species (*Aeromonas salmonicida*) that is pathogenic to fish but not human, and (2) several motile species (the *Aeromonas hydrophila* group) that are associated with human illness. Based on biochemical characteristics and DNA relatedness, *Aeromonas hydrophila* group has been divided into 3 species ; *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae*. Bacteria of the *A. hydrophila* group occur widely in aquatic environments, belong to the flora of reptiles, amphibian and fish, and have been implicated in the aetiology of a variety of systematic and localised diseases in fish and reptiles (Burke *et al.*, 1984; Palumbo and Buchanan, 1988; Palumbo *et al.*, 1989; Kirov *et al.*, 1990; Ibrahim and Mac Rae, 1991; Walker and Brooks, 1993; Son *et al.*, 1997).



*A. hydrophila* group has received particular attention because of its association with soft tissue and disseminated infectious and acute or chronic gastroenteritis following ingestion of contaminated food or water (Son *et al.*, 1997). This group of organism is also pathogenic to many aquatic species and causes hemorrhagic septicaemia (red sore disease) in many fresh water pond-cultured and wild native fish (Abeyta *et al.*, 1986). Other spectrum of infections by *Aeromonas* species including otitis, eye infections, tonsillitis, pneumonia, urinary tract infections, osteomyelitis and meningitis. This broad spectrum of infections is paralleled by a range of virulence factors including adhesins, cytotoxins, hemolysis, and various enzymes (Donna and Lindsey, 1988).

Drug resistant in *Aeromonas* species is well known. Animals reared in aquaculture facilities are susceptible to numerous bacterial diseases, which can be treated with a variety of antimicrobial compounds. The extensive use of antibiotics and other chemotherapeutics in fish farms as feed additives or the direct administration thereof into fishpond water to prevent and treat fish diseases, has resulted in an increase of drug-resistant bacteria as well as R plasmids. Increased incidence of bacterial resistance to standard antibiotic treatments has been recognised, particularly in fish shipped from Asia (Son *et al.*, 1997). More over, there remains the possibility that resistance may be transmitted from antibiotic-resistant bacteria to the susceptible ones (Imzilin *et al.*, 1996).



For the identification of the sources and monitoring the spread of *Aeromonas* species, a number of epidemiology markers, including various molecular characterisation techniques such as antibiotype, plasmid profile, polymerase chain reaction, pulsed field gel electrophoresis (PFGE), protein profile, phage typing and classical electrophoresis of DNA-restricted digests are useful to determine the genetic relatedness among the determined isolates under study. Nowadays, polymerase chain reaction (PCR) is the most common technique used to study the characteristics of bacteria. The PCR reaction shows differences in-between species or strains by analysing the size of the DNA products amplified from genomic DNA templates by a variety of primers. In higher organism, sets of random primers have been used to generate random amplified polymorphic DNA (RAPD)-PCR products, which produce banding patterns, when separated on agarose gels, that are characteristics of in-between species or isolates (Smith *et al.*, 1998).

In this study, *A. hydrophila*, *A. veronii* biovar *sobria* and *A. caviae* isolated from fish are used as the *Aeromonas* species of interest.