UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF *AEROMONAS* SPECIES ISOLATED FROM WATER AND SELECTED FOOD

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FSMB 1999 5

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

December 1999

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

MOLECULAR CHARACTERISATION OF *AEROMONAS* SPECIES ISOLATED FROM WATER AND SELECTED FOOD

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Chairman: Son Radu, Ph.D.

Faculty: Food Science and Biotechnology

Three species of *Aeromonas*; *A. hydrophila* (60 isolates), *A. sobria* (34 isolates) and *A. caviae* (113 isolates) were isolated from beef, milk, freshwater prawns, fish, and water from fishpond. *A. hydrophila* was predominant in fish and freshwater prawn, whereas *A. caviae* was predominant in the milk and water samples. *A. sobria* was only found in freshwater prawn, water and fish samples. The results of the study indicated that all the five different sources examined were potential vector for *Aeromonas* in Malaysia. The percentage of antibiotic resistant among *Aeromonas* species was varying according to its origin. *A. hydrophila* was more resistant towards ampicillin, bacitracin, carbenicillin and erythromycin. Most of the *A. sobria* isolates were resistant to ampicillin, bacitracin, carbenicillin and streptomycin and *A. caviae* was resistant to ampicillin, bacitracin and carbenicillin. The multiple antibiotic resistance (MAR) index of *A. hydrophila*, *A. sobria* and *A. caviae* ranged between 0.08 to 0.66, 0.16 to 0.66, and 0 (zero) to 0.5, respectively. Hence, the MAR indexing of the *Aeromonas* strains showed that more than 90% originated from high

risk contaminated environments where antibiotics were often used. Plasmids were detected in 50 of the 60 *A. hydrophila* isolates and 35 plasmid profiles were identified. The plasmid size ranged from 1.4 to 7.0 MDa. Plasmids of 1.4 to 4.1 MDa were detected in 28 of the 34 *A. sobria* isolates tested, while six isolates were found to be plasmidless. Their plasmid patterns were grouped into 18 patterns. Twenty-one plasmid patterns were found from *A. caviae* with plasmid sizes ranging from 1.4 to 8.0 MDa. In general, *Aeromonas* species under study harboured high number of plasmids. The plasmid analysis indicated the presence of more than one clone with the same antibiotic resistance patterns. The data obtained indicated that the samples sources tested form a reservoir for multiple-resistant and plasmid containing *Aeromonas* species in the study area. The RAPD-PCR was performed to characterise the *Aeromonas* spp. by using three random primers (GEN26003, GEN26007, and GEN26010). Among the *Aeromonas* spp., *A. hydrophila* was found to be differentiated by the three primers distinctively. Primer GEN26003 was the most suitable primer to differentiate the isolates. The three primers generated polymorphisms in all 207 strains of *Aeromonas* species tested, producing bands ranging from 0.24 to 4.5 Kb. The RAPD profiles revealed a wide variability and no correlation with the source of isolation. In addition, the RAPD data suggested that the *Aeromonas* strains might have originated from diverse sources.

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

PENCIRIAN MOLIKUL SPESIS *AEROMONAS* DARIPADA AIR DAN MAKANAN TERPILIH

Oleh

MOHD KAMIL RAJAB

Disember 1999

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Tiga spesis *Aeromonas* iaitu *A. hydrophila* (60 isolat), *A. sobria* (34 isolat) dan *A. caviae* (113 isolat) dapat dipencil dari daging, susu, udang air tawar, ikan dan air dari kolam ikan. *A. hydrophila* didapati dominan dalam sampel ikan dan udang air tawar. *A. caviae* pula dominan dalam sampel susu dan air kolam, dan spesis *A. sobria* didapati hanya dalam sampel udang air tawar, air dan ikan. Keputusan kajian menunjukkan lima sumber yang diperiksa berpotensi sebagai vektor kepada *Aeromonas* di Malaysia. Peratus kerentanan isolat *Aeromonas* terhadap antibiotik didapati berbeza berdasarkan kepada sumber sampel. *A. hydrophila* didapati rentan pada ampicillin, bacitracin, carbenicillin dan erythromycin. Kebanyakkan isolat *A. sobria* rentan pada ampicillin, bacitracin, carbenicillin dan streptomycin, dan *A. caviae* pula rentan pada ampicillin, bacitracin dan carbenicillin. Indeks kerentanan antibiotik berbilang *A. hydrophila*, *A. sobria* dan *A. caviae* adalah dalam julat 0.08 - 0.66, 0.16 – 0.66 dan 0 (kosong) - 0.5. Indeks ini menunjukkan lebih 90% daripada strain *Aeromonas* adalah berasal daripada

sumber berisiko tinggi yang tercemar di mana antibiotik kerap digunakan. Plasmid dapat dikesan dalam 50 daripada 60 isolat *A. hydrophila* dengan saiz plasmid dalam julat 1.4 - 7.0 MDa dan 35 profil plasmid telah dikenalpasti. Dua puluh lapan isolat *A. sobria* mengandungi plasmid dalam julat 1.4 - 4.1 MDa dan 6 isolat didapati tiada berplasmid. Isolat *A. caviae* menghasilkan plasmid dalam julat 1.4 - 8.0 MDa dan 20 paten plasmid dihasilkan. Secara amnya, spesis-spesis *Aeromonas* dalam kajian menghasilkan banyak plasmid. Analisis plasmid menunjukkan kehadiran lebih daripada satu klon tunggal dengan paten kerentanan antibiotik yang sama. Data yang diperolehi menunjukkan sumber-sumber sampel yang diuji membentuk takungan untuk kerentanan berbilang dan spesis *Aeromonas* berplasmid. Kaedah RAPD-PCR dijalankan untuk mencirikan spesis-spesis *Aeromonas* dengan menggunakan tiga primer rawak (GEN26003, GEN26007 dan GEN26010). Antara spesis *Aeromonas*, *A. hydrophila* didapati senang dibezakan menggunakan tiga primer tersebut dengan jelas. Primer GEN26003 merupakan primer paling sesuai untuk membezakan isolat-isolat *Aeromonas*. Tiga primer yang diguna menghasilkan polimofisma dalam 207 strain *Aeromonas* yang diuji dan menghasilkan fragmen DNA dalam julat 0.24 - 0.45 Kb. Profil RAPD menunjukkan variasi yang luas dan tiada korelasi dengan sumber pemencilan. Strain *Aeromonas* mungkin berasal dari berbagai-bagai sumber berdasarkan data RAPD yang diperolehi.

ACKNOWLEDGEMENTS

My deepest gratitude to my supervisor, Dr. Son Radu for his continuous support, patient and advice given to me throughout the course of this project. I also wish to thank my co-supervisors, Professor Dr. Gulam Rusul Rahmat Ali and Professor Dr. Mohamed Ismail Abdul Karim for spending their valuable time on my thesis.

I would like to extent my heartfelt thanks to all members of the Laboratory of Foodborne Pathogen and Molecular Subtyping; Razali, Syamir, Wai ling, Zainuri, Rozila, Samuel and others for their friendship, helps and understanding.

Lastly, I would like to thank my parents, sisters and brothers for their guidance, support and love.

I certify that an Examination Committee met on 30 December, 1999 to conduct the final examination of Mohd Kamil bin Rajab, on his Master of Science thesis entitled "Molecular Characterisation of *Aeromonas* Species from Water and Selected Food" 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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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.

(MOHD KAMIL BIN RAJAB)

Date: 25/2/2000

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CHAPTER I

GENERAL INTRODUCTION

Genus *Aeromonas* are rod shape, motile or nonmotile and gram negative bacteria. Motile *Aeromonas* species consist of *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae* which are ubiquitous bacteria in continental aquatic environment (Hazen *et al.,* 1978). They are recognised as pathogens in fish, reptiles and amphibians (Holt *et al.,* 1994). These bacteria can be found in both polluted and unpolluted fresh water, in sewage and drinking water (Monfort and Baleux, 1990). The bacteria have also been isolated from food and clinical specimens (Fiorentini *et al.,* 1998). *Aeromonas hydrophila* has received particular attention because of its association with soft tissue and disseminated, infectious and acute or chronic gastro-enteritis following ingestion of contaminated food or water (Son *et al.,* 1997). Antibiotic resistant bacteria have been isolated from raw sewage, sewage effluent receiving water, fresh and marine recreation water and marine shellfish, coastal sediments and soil (Altherr and Kasweck, 1982). The extensive use of antibiotics and other chemotherapeutics in fish farms as feed additives to prevent and treat fish diseases has resulted in and increase of drug-resistant bacteria as well as R plasmid (Toranzo *et* *al.,*1984). The ability of bacteria to resist to the antibiotics may vary from year to year, so epidemiological study must be carried out to obtain the latest information. Plasmid profiling is the other method used to study the characteristics of the *Aeromonas* according to the different plasmid profiles produce by each bacterium. Polymerase chain reaction is the latest technique that can be 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 organisms, sets of random primers have been used to generate randomly amplified polymorphic DNA (RAPD)-PCR products, which produce banding patterns, when separated on agarose gels, that are characteristics of in-between species or strains (Smith *et al.,* 1998). The objectives of this project are to isolate *Aeromonas hydrophila, Aeromonas sobria* and *Aeromonas caviae* from various sources and to characterise them by antibiotics susceptibility test, plasmid profiling and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR).

CHAPTER II

LITERATURE REVIEW

Microbiology of *Aeromonas* species

Aeromonas means gas producing monad, are rod shaped with rounded ends to coccoid, cells straight and gram negative bacteria, generally motile by single polar flagellum and facultative anaerobes. *Aeromonas* metabolised glucose in both respiratory and fermentative also oxidase-positive. Psychrophilic and nonmotile aeromonads are clustered in the first group, named *Aeromonas salmonicida*. The second group consists of mesophilic and motile bacteria; this group can be divided into three species: *Aeromonas hydrophila*, *Aeromonas sobria*, *and Aeromonas caviae*. The optimum growth temperature for motile *Aeromonas* species is 28°C. Some strain can grow at 5°C. The maximum temperature at which growth occurs is usually 38 to 41°C. On nutrient agar, colonies of motile aeromonads are round, raised, with an entire edge and having a smooth surface (Popoff, 1984).

Growth of Aeromonads at Low Temperature

With consumer-driven demand for less processed and more natural foods containing fewer additives, there is growing emphasis on refrigeration as the primary means of restricting the growth of pathogens and spoilage microorganisms. Although most *Aeromonas* strains in food appear to have optimum and maximum temperatures for growth characteristic of mesophiles, many (including exotoxin-producing strains) also have the ability to grow at chill temperatures (Kirov *et al.,* 1990; Beuchat, 1991). Populations of *Aeromonas* species naturally present in foods of all types show a 10 - 1000-fold increased during 7 - 10 days storage at 5°C (Palumbo *et al.,* 1985a; Callister and Agger, 1987; Berrang *et al.,* 1989).

The sources of microorganisms in foods may influence the rate at which they grow at low temperatures. Knochel (1990) found that microbial strain from cold (< 15°C) environments (chilled food, springs and cold water aquaculture), grew less frequently at 37°C than those from warm (> 25°C) environments (human diarrhoea, septicaemia, warm water aquaculture), whereas for strains isolated from the high temperature environments, the opposite was true. Hudson (1992) reported that strain (*A. hydrophila*) from ready-to-eat flesh foods grew better at low temperature than those from clinical or meat-processing sources. However, most clinical strains can grow at 5°C, and the growth of many is rapid at 10°C (Palumbo and Buchanan, 1988; Kirov *et al.,* 1990; Eley *et al.,* 1993).

Factors Influencing the Growth and Survival of Aeromonads in Foods Stored at Low Temperature

Salt concentration, pH, atmosphere and the background microflora interact to influence the growth and survival of aeromonads in foods at low temperature (Palumbo, 1988; Palumbo and Buchanan, 1988; Knochel, 1990; Beuchat, 1991; Palumbo *et al.,* 1992). Although some isolates of *Aeromonas* spp. may tolerate NaCl levels greater than 4% (w/w) and many strains have the ability to grow over a wide pH range (between pH 4.0 and 10.0) at their optimal temperatures (~ 28°C), tolerance to salt and pH is reduced at lower temperatures (Knochel, 1990). It has been reported that *Aeromonas* spp. are unlikely to present problems in foods with more than 3 to 3.5%(w/w) in the water phase and pH values below 6.0 stored at low temperature (Palumbo, 1988; Palumbo and Buchanan, 1988).

There is conflicting evidence as to whether strains of *Aeromonas* spp. can compete sufficiently at low temperature with the background microbiota of foods to reach high levels before food spoilage (Palumbo and Buchanan, 1988; Kirov *et al.,* 1990; Erickson and Jenkins, 1992). Further investigations into the growth characteristics of aeromonads in foods under storage condition are required. Decreasing oxygen levels surrounding refrigerated meats is reported favour the growth of *Aeromonas*, possibly by retarding the growth of aerobic competitors (Buchanan and Palumbo, 1985). Several publications have cautioned the used of modified atmospheres to extend the shelf-life of vacuum packaged meats and fresh vegetables, as this may lead to the consumption of foods containing high levels of aeromonads (Enfors *et* *al.,* 1979; Berrang *et al.,* 1989). Thus, the behaviour of *Aeromonas* on foods which have been packaged under modified atmospheres requires particular investigation (Beuchat, 1991). Variables such as pH, salt level, nitrite level, may all need to be manipulate to decrease the growth of some *Aeromonas* species at low temperature (Palumbo *et al.,* 1991,1992).

Mediums for Isolation

Aeromonas hydrophila does not require enriched media and can be isolated on the media used for Enterobacteriaceae, for example Salmonella-Shigella agar (Difco) or MacConkey's agar. On blood agar most of the *Aeromonas* cultures can be recognised because they form greyish colonies surrounded by a zone of haemolysis. Unfortunately blood agar is not suitable for stool sample because of spreading of *Proteus* often hinders the examination of *Aeromonas* (Rogol *et al.,* 1979). They proposed Pril-Xylose-Ampicillin agar (PXA agar) for isolation of *Aeromonas* from stool specimen consists of nutrient agar containing xylose 1% (w/v), phenol red 25 mg/L as indicator, ampicillin 30 mg/L and Pril 0.02% (w/v). Ampicillin was added to eliminate most of the Enterobacteria. 'Pril' is a quaternary ammonium detergent consisting of a mixture of primary alkyl sulphate, alkyl-benzyl sulphonate and salts. This preparation was recommended for inhibition of swarming of *Proteus.*

von Graevenitz and Bucher (1983) studied nine solid and two liquid media for their suitability to isolate *Aeromonas* and *Plesiomonas* spp. from human stools (Table 1). They reported that addition of ampicillin inhibited 4 of 12 *Aeromonas sobria* strains and 9 of 10 *Plesiomonas shigelloides* strains. Addition of 0.005% toluidine blue to Dnase test agar did not inhibit any of the *Aeromonas* strains. Compared with MacConkey agar, DFS and RS were not inhibitory, whereas RS and DNTA reduced growth of one third to one-half of the *Aeromonas* strains. They recommended APW as *Aeromonas* enrichment medium, IBB, DFS, XDC and PXA as *Aeromonas* plate media, and APW and IBB as *Plesiomonas* media (Table 1 and 2).

Table 1: Selective or differential media (or both) for Isolation *Aeromonas* and Plesiomonas spp.

Medium	inhibitor(s)	differential substance(s)	original purpose[a]
Dextrin-fuchsin-Sulfite agar (DFS)	Sodium sulfite, fuchsin	Dextrin	water (A)
Dnase-toluidine blue-Ampicillin agar (DNTA)	Ampicillin (30mg/L)	Dnase (toluidine blue)	stool(A)
Inositol-brilliant green-Bile salts agar (IBB)	Brilliant green bile salts	Inositol	stool(P)
Peptone-beef extract-Glycogen agar (PBG)	Sodium lauryl sulfate	Glycogen	multipurpose (A)
Pril-xylose-ampicillin Agar (PXA)	Pril, ampicillin (30 mg/L)	Xylose	stool (A)
Rimler-Shotts agar (RS)	Citrate, sodium desoxycholate, novobiocin (5 mg/l)	Lysine, ornithine, maltose	multipurpose(A)
Rippey-Cabelli agar (RC)	Sodium desoxy-cholate, ethanol, ampicillin (20 mg/L)	Trehalose	water (A)
Salt-starch-xylose-Lysine-sodium de-soxycholate agar (SSXLD)	Sodium desoxycholate, citrate,NaCL (1.5 %)	Lysine, starch xylose	stool (V)
Xylose-sodium de-soxycholate-citrate agar (XDC)	Citrate, sodium desoxycholate	Xylose	stool (A)
Alkaline peptone-water (APW)	pH 8.6		stool (A)
Trypticase soy-Ampicillin broth (TSBA)	Ampicillin (30 mg/L)		stool (A)

[a]A, *Aeromonas* spp.; P, *Plesiomonas* spp.; V, *Vibrio* spp.
** Reference from von Graevenitz and Bucher, 1983.

Table 2: Colony appearances on various media

Medium	*Aeromonas* strains	*Plesiomonas* strains	Coliforms
DFS	Dark red, large,turbid halo	Very small, bright red, light halo	Bright red with light halo or colourless
DNTA	Halo of decolourisation	No halo[a]	No halo
IBB	Colourless	Whitish to pinkish	Greenish or pink
PBG	Yellow, typical	Yellow, atypical	Yellow, atypical
PXA	Colourless	Colourless[a]	Yellow
RS	Yellow	Greenish yellow	Greenish yellow
RC	Yellow	Yellow	Yellow
SSXLD	Yellow, halo	Yellow	Yellow
XDC	Colourless	Colourless	Red

[a]; Unless inhibited by ampicillin

Incidence of *Aeromonas* spp. in Food and Food Products

In the last decade, mesophilic *Aeromonas* spp. have received increased recognition as international pathogens (Altwegg and Geiss, 1989) with water and food as the possible sources of infection (Abeyta *et al*., 1986). A variety of food products were shown to harbour *Aeromonas* spp. In raw meat, up to 80% positive samples were found (Fricker and Tompsett, 1989; Knochel and Jeppesen, 1990; Ibrahim and MacRae, 1991), while ready-to-eat flesh food products showed 5 to 25% positive samples (Hudson *et al.*, 1992; Knochel and Jeppesen, 1990). Altwegg *et al*. (1991) described a shrimp cocktail responsible for *Aeromonas* gastro-enteritis.

Gobat and Jemmi (1993), examined qualitatively as well as quantitatively for mesophilic *Aeromonas* spp. in meat, poultry, fish and shellfish products commonly consumed in Switzerland. Their investigation found that, out of 829 samples, 200 (24.1%) yielded mesophilic *Aeromonas* spp.; 158 *A. hydrophila* strains (61.2% of the isolates); 42 *A. sobria* strains (16.3%); and 58 *A. caviae* strains (22.5%). *A. sobria* was significantly ($P < 0.05$) more often encountered in raw foods, whereas *A. hydrophila* was always the most encountered species.

Hudson and De Lacy (1991), investigated the incidence of motile aeromonads in New Zealand retail foods. They found no motile aeromonads in the baked confectionery products (most of which contained cream or mock cream). The percentage of salads and coleslaws (vegetable-containing