UNIVERSITI PUTRA MALAYSIA

PREVALENCE AND MOLECULAR CHARACTERISTICS OF AEROMONAS SPECIES ISOLATED FROM FISH, SHRIMP AND WATERS

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By

Yousr Abdulhadi Noaman

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2006

DEDICATION

To

my wonderful husband (Adnan), daughter (Roba), son (Ammar)

&

to the loving memory of my late father (who still very much lives in my heart), I dedicate this work

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

PREVALENCE AND MOLECULAR CHARACTERISTICS OF *AEROMONAS* SPECIES ISOLATED FROM FISH, SHRIMP AND WATERS

By

YOUSR ABDULHADI NOAMAN

September 2006

Chairman: Associate Professor Suhaimi Napis, PhD

Faculty : Biotechnology and Biomolecular Sciences

Aeromonas species are ubiquitous aquatic micro-organisms which are opportunistic pathogens that have been associated to wound infections, gastroenteritis, septicemia, and traveler's diarrhea in humans and hemorrhagic septicaemia in fish. The main routes of exposure in humans are ingestion of contaminated foods and drinking water, or direct contact with recreational waters. In this study, a total of 450 samples were obtained from different sources and locations in Malaysia. Two hundred fifty samples of fresh water were obtained from ponds in UPM (n=150) and in tiger prawn farm in Malacca (n=150), respectively. In addition, 100 samples of shrimps *(Penaeus indicus)* were purchased from different wet markets in Selangor state: Seri Kembangan (35), Kajang (35) and Bangi (30). Hundred samples of fish (*Clarias batrachus*) (n=50) and Tiger prawn (*Penaeus monodon*) (n=50) were obtained from UPM and tiger prawn farm in Malacca, respectively. 238 (52.8%) of the 450 samples were tested positive for the isolation of *Aeromonas* species, 49 (10.8%), 43 (9.5%),

33 (7.3%), 37 (8.4%), 25 (5.5%), 23 (5.1%), 19 (4.2%), 15 (3.3%) and 2% harbored *A. veronii, A. allosacharophila, A. hydrophila, A. caviae, A. enterpelogenes, A. encheleia, A. trota, A. media* like DNA and *A. veronii* biovar *sobria*, respectively. All the strains of *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* were tested for resistance to 15 antibiotics and 98.8% and 94.1% of the strains were resistant to amoxcillin and penicillin, respectively, followed by teicoplanin (89.4%), penicillin (81.1%) and 12.9% of the strains were found resistant to chloramphenicol. The Multiple Antibiotic Resistance Indexing (MAR) and the bionumeric analysis of *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria*, showed that all of them originated from high-risk sources. Two molecular typing methods were used in this study to examine the intra/inter-specific genetic relatedness among the *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* strains. In the analysis by RAPD-PCR and ERIC-PCR, the size for RAPD and ERIC fragments ranged from 0.25 to 10.0 kb with an average number of sixteen and eight bands, respectively. Eighty five genotypes among the 85 *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates were generated using RAPD and ERIC-PCR which indicated that the strains were very diverse. The PCR analysis for detection of aerolysin (*aer*) and hemolysin (*hly*) showed that 50.5% of the isolates carried hemolysin (*hly*) gene and 45.9% of the isolates carried aerolysin (*aer*) gene. The nucleotide blast results of aerolysin gene sequences representative *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* revealed a high homology of 94%, 95% and 95% for *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* published sequences, respectively. The protein blast also showed homology (97%, 94% and 96%) as compared with the Genbank database of National Centre for Biotechnology Information (NCBI). The present study demonstrates the high intra/inter-specific diversity within the *Aeromonas* species and

reveals a clear differentiation of strains according to their ecological origin. Increasing presence of haemolysin-producing multiple antibiotic resistant *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* in food and environment may become a potential human health hazard. In conclusion, the research undertaken has contributed significantly to our knowledge of *Aeromonas* by providing new information on its distribution, its specific detection by PCR and inter/intra-species relationship.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KEJADIAN DAN SIFAT-SIFAT MOLEKULAR SPESIS *AEROMONAS* DIPENCILKAN DARIPADA SUMBER IKAN, UDANG DAN AIR YANG DIPEROLEHI

Oleh

YOUSR ABDULHADI NOAMAN

September 2006

Pengerusi : Professor Madya Suhaimi Napis, PhD

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Spesis *Aeromonas* adalah mikroorganisma akuatik yang umum dan patogen berpotensi yang dikaitkan dengan infeksi luka, *gastroenteritis, septicemia* dan *traveler's diarrhea* di dalam manusia dan *hemorrhagic septicemia* di dalam ikan. Pendedahan-pendedahan utama di dalam manusia kepada bacteria ini adalah penghadaman makanan dan minuman yang tercemar, atau pendedahan langsung kepada air rekreasi. Di dalam kajian ini, sejumlah 450 sampel telah diperolehi daripada sumber-sumber dan tempat-tempat yang berbeza di Malaysia. Dua ratus lima puluh sampel air tawar telah diperolehi daripada kolam-kolam di UPM (n=150) dan kolam udang harimau di Melaka (n=150), masing-masing. Selain itu, 100 sampel udang kecil (*Penaeus indicus*) telah diperolehi daripada pasar malam yang berbeza di Selangor: Seri Kembangan (35), Kajang (35) dan Bangi (30). Seratus sampel ikan

(*Penaeus indicus*) (n=50) dan udang kecil (udang harimau) (n=50) diperolehi daripada UPM dan udang harimau di Melaka. Dua ratus tiga puluh lapan (52.8%) daripada 450 sampel adalah positif untuk ujian bagi kehadiran *Aeromonas* spesis, 49 (10.8%), 43 (9.5%), 36 (7.3%), 38 (8.4%), 25 (5.5%), 23 (5.1%), 19 (4.2%), 15 (3.3%) dan 2% membawa *A. veronii, A. allosacharophila, A. hydrophila, A. caviae, A. encheleia , A. trota, A. media* like DNA dan *A. veronii* biovar *sobria*. Kesemua pencilan *A. hydrophila*, *A. caviae* dan *A. veronii* biovar *sobria* diuji untuk kerintangan bagi 15 antibiotik dan didapati 98.8% dan 94.1% pencilan yang rintang kepada amoksilin dan penisilin, diikuti dengan teikoplanin (89.4%), penisilin (81.1%). 12.9% daripada pencilan didapati rintang kepada chloramphenicol. Multiple Antibiotic Resistance Indexing (MAR) dan analisis bionumerik untuk *A. hydrophila, A. caviae* dan *A. veronii* biovar *sobria* menunjukkan yang kesemua spesis ini berasal daripada sumber yang berisiko tinggi (*high-risk sources*). Dua metod *molecular typing* digunakan di dalam kajian ini untuk melihat kaitan intra/inter-specific genetik di antara pencilan-pencilan *A. hydrophila, A. caviae* dan *A. veronii* biovar *sobria.* Di dalam analisis RAPD-PCR dan ERIC-PCR, saiz *fragment* bagi RAPD dan ERIC adalah di antara 0.25 hingga 10.0 kb dengan purata bilangan *bands* di antara enam belas dan lapan *bands*. Lapan puluh lima *genotype* bagi 85 pencilan *A. hydrophila, A. caviae* dan *A. veronii* biovar *sobria* diperolehi menggunakan kaedah RAPD dan ERIC-PCR yang menunjukkan bahawa pencilan sangat pelbagai. Analisis PCR untuk penentuan gen *aerolysin* (*aer*) dan *hemolysin* (*hly*) menunjukkan 50.5% pencilan membawa gen *hemolysin* (*hly*) dan 45.9% pencilan membawa gen *aerolysin*. Keputusan *nucleotide blast* bagi turutan gen *aerolysin representative A. hydrophila, A. caviae* dan *A. veronii* biovar *sobria* menunjukkan homologi yang tinggi untuk turutan *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* yang sebenarnya.

Keputusan *protein blast* juga menunjukkan homologi (97%, 94% dan 96%) merujuk kepada *Genbank database of National Centre for Biotechnology Information (NCBI)*. Kajian ini menyatakan kepelbagaian *intra/inter-specific* yang tinggi di kalangan spesis *Aeromonas* dan menunjukkan perbezaan yang jelas bagi pencilan merujuk kepada ekologi asal mereka. Kehadiran ***haemolysin-producing multiple antibiotic resistant A. hydrophila, A. caviae*** dan *A. veronii* biovar *sobria* yang meningkat di dalam makanan dan persekitaran akan menjadi *hazard* yang berpotensi bagi manusia. Di dalam kesimpulan, kajian ini menyumbangkan kepada kami pengetahuan *Aeromonas* yang penting di dalam menyediakan informasi baru tentang kejadian, penentuan spesis menggunakan PCR dan hubungan inter/intra-spesis.

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If I remove what I owe to all of you, there wouldn't be much left of me….

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I certify that an Examination Committee met on to conduct the final examination of Yousr Abdulhadi Noaman on her Doctor of Philosophy thesis entitled "Occurrence and Molecular Characterization of *Aeromonas* Species Isolated From Different Sources" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act of 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommended that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Chairman, PhD
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Examiner 1, PhD
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HASANAH GHAZALI, PhD
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Date:

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

Suhaimi Napis, PhD
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AINI IDERIS, Ph.D.
Professor/Dean
Dean of Graduate School
Universiti Putra Malaysia.

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duty 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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YOUSR ABDULHADI NOAMAN

Date:

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CHAPTER I

INTRODUCTION

Aeromonads were first described as pathogens for warm- and cold-blooded animals in 1891 and since then, their presence in drinking water has long been known (Gavriel *et al*., 1998). However, it was not until the 1960s that the Aeromonad was shown to be involved in the human infections. With the knowledge that these environmental microorganisms are responsible for human infections, there is a desire to better understand Aeromonads.

Aeromonads are facultative anaerobic, Gram-negative bacilli that are ubiquitous to aquatic environment (Janda and Abobott, 1998; Escarpulli *et al*., 2002). They have been isolated from all virtually known surfaces, fresh and marine aquatic environment including lakes, rivers, reservoirs and even from treated drinking water (Brandi *et al*., 1999; Sen and Rodgers, 2004). The only water source in which they are not often found is the well-protected underground water. Their presence in most sources of water is due to their ability to grow in a wide range of temperatures, mainly in the optimal temperature between 22 to 28°C, and to their requirement of only minimal amount of nutrients (Newcombe, 2005).

Aeromonas strains are both pathogenic and fairly innocuous, in which most of these usually lead to gastrointestinal problems (Vila *et al*., 2002). Extreme cases in very young or old and immuno-compromised patients, they can lead to diarrhea. *Aeromonas*- associated infections are most common in the summer months or in the warm climate countries; the environment that is optimal for the maximal levels of

bacteria in the water sources. In the same vein, Aeromonad strains have also been linked to wound and enteric extra-intestinal infections (Ferran *et al*., 2004; Newcombe, 2005).

Not all Aeromonads are pathogenic. Of the 17 characterized species of *Aeromonas*, nine are clinical specimens, while the rest have only been found in the environmental settings (Ko *et al*., 1996; Rabaan *et al*., 2001; Vila *et al*., 2002). *Aeromonas hydrophila*, *A. veronii* bv. *sobria*, and *A. caviae* pose the greatest public health risk, accounting for greater than 80% of the clinical isolates (Patrick, 2003). The *A. veronii* bv. *sobria* is the most common species found in lakes, reservoirs and treated drinking water, while the *Aeromonas veronii* bv. *sobria* and *A. caviae* are the most common species found in intestinal infections, and *A. veronii* bv. *sobria* and *A. hydrophila* are the most common species found in extra-intestinal sources (Kelly *et al*., 1993; Swift *et al*., 1999; Guadalupe *et al*., 2005)

Antibiotic resistance is a significant human health issue and there have been many papers reporting a link between the use of antibiotics in the food producing animals, emergence of antibiotic resistance in *Salmonella*, *Escherichia coli*, *Aeromonas*, *Enterococci* and *Campylobacter* in treated animals, and transfer of these resistant organisms to humans (or their resistance genes to human pathogens) via the food chain (Barton and Pace, 2000; Angulo *et al*., 2004) in recent years. In addition to transfer the resistant organisms through the consumption of the contaminated fish and shellfish, there is a substantial risk of the environmental contamination due to the practice of using medicated feeds to treat the whole pens or cages. Alderman and Hastings (1998) noted that the controls on the use of antibiotics in aquaculture vary

widely from country to country. In the developed countries such as members of the EU, USA, Canada and Norway, there is limited number of products, regulatory control is strong and the use of antibiotics is declining because of improvement in the management and development of effective vaccines (Burka *et al*. 1997; WHO 2002; Lillehaug *et al*. 2003). However, according to Bondad-Reantaso *et al*., (2005), 90% of the aquaculture production occurs in the developing countries where the regulatory controls are weak and use of antibiotics appears to be widespread.

The *Aeromonas* spp. is listed by the USEPA on the Candidate Contaminant List. Due to the fact that most waterborne *Aeromonas* lacks of the virulence determinants necessary to make them important from a public health perspective, simply collecting *Aeromonas* counts, without providing a characterization of the isolates, will result in an over-estimation of the public health significance of the isolates. A significant correlation between the *Aeromonas*-induced diarrhea and the presence of various enterotoxins from the *Aeromonas* spp. has been recently demonstrated, indicating that these enterotoxin genes can be used as a marker for the virulence potential of the organism. Currently, the number of species recognized within the genus has increased to 17 (Janda and Abbott, 1998). Despite the increase in the number of genospecies, only nine are currently recognized as human pathogens (Carnahan *et al*., 1991b). Aerokey II is a reliable and accurate system to identify most of the currently recognized Aeromonas species isolated from the clinical specimens (Carnahan *et al*., 1991b). A significant number of the virulence genes have been described among the *Aeromonas* species, including aerolysin, haemolysin, enterotoxins, proteases and haemagglutinins (Thornley *et al*., 1997). These virulence