UNIVERSITI PUTRA MALAYSIA

BIOHAZARDS OF VIBRIO CHOLERAE AND VIBRIO PARAHAEMOLYTICUS IN FRESHWATER FISH AND THEIR DECONTAMINATION IN SELANGOR MARKETS, MALAYSIA

NOORLIS AHMAD

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By

NOORLIS AHMAD

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

December 2012

Specially dedicated to my parents (Mak & Abah) and my sister for their unconditional love and endless support throughout my study.

Not forgetting my lovely husband (Nordin Noor) and my 2 lovely sons (Muhammad Iman Naim & Muhammad Ikmal Naim), who have always been by my side and given me the encouragement and support that carries me through my study period.

Dedicated to my friends for the wonderful friendship, love and joy.

Dedicated to everyone whom has invested their loves in my life.

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

BIOHAZARDS OF *VIBRIO CHOLERAE AND VIBRIO PARAHAEMOLYTICUS* IN FRESHWATER FISH AND THEIR DECONTAMINATION IN SELANGOR MARKETS, MALAYSIA

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December 2012

Chairman: Professor Son Radu, PhD

Faculty: Food Science and Technology

Bacteria of the genus *Vibrio* are capable of causing epidemics of cholera and human intestinal diseases. However, little is known on the biosafety level of *Vibrio* spp. in freshwater fish in Malaysia. The purpose of this study was to investigate the biohazard of *Vibrio cholerae* and *Vibrio parahaemolyticus* in freshwater fish at retail level in Malaysia. A combination technique of most probable number and polymerase chain reaction (MPN-PCR) method was used to quantify the prevalence and number of total *Vibrio* spp., pathogenic *Vibrio cholerae* harboring *ctx* genes and pathogenic *Vibrio parahaemolyticus* harboring *tdh* and *trh* genes, and to enumerate their density in the fish samples. The biohazard of vibrios was also carried out by phenotypic

(antibiotic resistance) and genotypic (virulence genes detection and RAPD-PCR) characterization of the isolates from freshwater fish. A kitchen simulation study was also conducted to provide decontamination and cross-contamination data and information for the estimation of the risk of acquiring vibriosis from consumption of freshwater fish and when handled in the local domestic kitchen.

At retail level, 300 samples of two types of freshwater fish, *Pangasius hypopthalmus* (Catfish) and *Oreochromis* sp. (Red tilapia) normally available at the wet markets and hypermarkets were collected over a one year period (June 2009 to June 2010). The vibrios were isolated from the flesh, intestinal tracts and gills of the freshwater fish. By using MPN-PCR method, the prevalences of *Vibrio* spp., *Vibrio cholerae*, and *Vibrio parahaemolyticus* were found to be 100%, 2.67% and 25%, respectively. *Vibrio cholera*e (*OmpW*) was mostly detected from the gills of Red tilapia sampled in hypermarkets at 14% followed by 2% in Catfish intestinal tracts. All of the *Vibrio cholerae* isolates in this study were the non-01 and non-0139 *Vibrio cholerae.* However, *Vibrio parahemolyticus* (*toxR*) were detected in samples from both types of markets, 28% from Red tilapia gills followed by the intestinal tracts and flesh at 26%. *Vibrio parahemolyticus* was frequently found in Catfish gills (30%), followed by flesh (22%) and intestinal tract (18%).

Using the MPN-PCR method, the occurrence of total *Vibrio cholerae* harboring *ctx* virulent genes were 0.67% and *Vibrio parahemolyticus* harboring *tdh* and *trh* were 4% and 0%, respectively. Even though, the detection of the virulent gene was relatively low but the concentration of total vibrios in the samples ranged from 3 X 10^4 MPN/g to 1.1X10^7 MPN/g.

A total of 57 isolates (*Vibrio cholerae* = 8 isolates, *Vibrio parahemolyticus* = 49 isolates) were recovered by plating method and were confirmed by PCR. All of the isolates showed multi-resistance to as many as 15 antibiotics tested with high resistance to Bacitracin (98%), Furazolidone (88%) and Tetracycline (83%) and were mostly susceptible to Imipenem with only 11% showed resistance. The multiple antibiotic resistance (MAR) indices detected in the study, ranged from 0.13 to 0.93. RAPD-PCR was used to generate polymorphic genomic fingerprints to determine genetic relatedness among *Vibrio cholerae* and *Vibrio parahaemolyticus* isolates under study. Two primers (OPA10; 5'-GTGATCGCAG-3' and OPAR8; 5'-TGGGGCTGTC-3'), out of the 10 primers used showed the best results and were selected for further study. It was found that all isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* could be grouped into two major clusters for each primer used. The clustering isolates based on RAPD-PCR profiles suggested that

overall, most of the isolates were from both types of markets and clustered together into the same group although some isolates from other types of markets were also found clustered in the same group.

A simulation study was conducted to imitate the real situation in domestic kitchens as much as possible to give a realistic quantitative data on how vibrios could be reduced by washing and soaking procedures. The eight procedures of washing and soaking were applied in this simulation study. It was found that the overall mean percent transfer rate for procedure 1 (without washing or soaking), procedure 2 (washed under running tap water), procedure 3 (soaked in 100 ml of sterile distilled water), procedure 4 (soaked in 100 ml of lime juice) and procedure 5 (soaked in 100 ml of tamarind juice) ranged from 0% to >100%, followed by procedure 6 (soaked in 1% NaCl solution) from 3.8% to > 100% and procedure 7 (soaked in 3% NaCl solution) from 80.6% to >100%. It was found that washing by rinsing and soaking the flesh of freshwater fish with tap and distilled water showed a 0 log reduction. By soaking the fish flesh in the lime juice, tamarind juice, 1%, 3% and 10% of NaCl solution could decrease the number of vibrios up to 2.9 log reduction.

In conclusion, the freshwater fish could act as a transmission route for *Vibrio cholera* and *Vibrio parahaemolyticus* and thus pose a risk for consumers. Further studies on a bigger scale are recommended for a better understanding on the presence of *Vibrio cholera* and *Vibrio parahaemolyticus* in freshwater fish and the risks when handling and consuming such contaminated freshwater fish.

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

KEMUDARATAN BIO *VIBRIO CHOLERAE* DAN *VIBRIO PARAHAEMOLYTICUS* PADA IKAN AIR TAWAR DAN DEKONTAMINASI DI PASARAN SELANGOR, MALAYSIA

Oleh

NOORLIS AHMAD

Disember 2012

Pengerusi : Profesor Son Radu, PhD

Fakulti : Sains dan Teknologi Makanan

Bakteria dari genus *Vibrio* berupaya memulakan wabak taun dan jangkitan usus pada manusia. Walau bagaimanapun, tidak banyak yang diketahui mengenai tahap kemudaratan bio *Vibrio* spp. pada ikan air tawar di Malaysia. Tujuan kajian ini adalah untuk mengkaji kemudaratan bio *Vibrio cholerae* dan *Vibrio parahaemolyticus* pada ikan air tawar di peringkat runcit di Malaysia. Dengan menggunakan satu kombinasi kaedah Jumlah Paling Mungkin – Reaksi Polimer Berantai (MPN-PCR) untuk mengukur kehadiran dan jumlah *Vibrio* spp., patogen *Vibrio cholerae* yang mengandungi gen *ctx* dan patogen *Vibrio parahaemolyticus* yang mengandungi gen *tdh* dan *trh* serta mengira kepekatan pada ikan. Kemudaratan bio *Vibrio* juga dinilai dengan

menggunakan pencirian fenotip (kerintangan antibiotik) dan genotip (pengesanan gen virulen dan RAPD-PCR) terhadap pencilan daripada ikan air tawar. Kajian simulasi dapur domestik juga dijalankan untuk mendapatkan data dan maklumat berkaitan penganggaran risiko untuk dijangkiti Vibriosis dengan memakan ikan air tawar tempatan yang di kendalikan di dapur domestik.

Pada peringkat peruncitan, 300 sampel ikan air tawar yang terdiri daripada dua jenis ikan air tawar yang biasa terdapat di pasar basah dan pasaraya iaitu *Pangasius hypopthalmus* (Patin) dan *Oreochromis* spp. (Tilapia merah) dipilih dalam tempoh pensampelan selama satu tahun (Jun 2009 hingga Jun 2010). Sampel yang digunakan ialah bahagian isi, perut dan insang ikan. Dengan menggunakan kaedah MPN-PCR, kehadiran *Vibrio* spp., *Vibrio cholerae* dan *Vibrio parahaemolyticus* adalah masing-masing 100%, 2.67% dan 25%. *Vibrio cholerae* (*OmpW*) dapat dikesan pada insang ikan Tilapia merah yang dibeli di pasaraya dengan jumlah keseluruhan 14%, diikuti dengan perut ikan Patin dengan kehadiran hanya 2%. Kesemua *Vibrio cholerae* yang dikaji adalah dari jenis *Vibrio cholerae* bukan 01 dan *Vibrio cholerae* bukan 0139. Walau bagaimanapun *Vibrio parahaemolyticus* (*toxR*) telah dikesan pada sampel daripada kedua-dua jenis pasar dengan 28% daripada insang ikan Tilapia merah dan diikuti dengan 26% pada perut ikan. *Vibrio*

parahaemolyticus sering dijumpai pada insang ikan Patin (30%) diikuti dengan isi ikan (22%) dan perut ikan (18%).

Dengan kaedah MPN-PCR, jumlah *Vibrio cholerae* yang mengandungi gen virulen *ctx* adalah 0.67% dan *Vibrio parahaemolyticus* yang mengandungi gen *tdh* dan *trh* adalah masing-masing 4% dan 0%. Walaupun pengesanan gen virulen adalah rendah tetapi kepekatan jumlah *Vibrio* adalah diantara 3 X 10^4 MPN/g hingga 1.1 X 10^7 MPN/g.

Sejumlah 57 pencilan (*Vibrio cholerae* = 8 pencilan, *Vibrio parahaemolyticus* = 49 pencilan) diperoleh dengan mengguna kaedah plat dan dikenalpasti dengan PCR. Kesemua pencilan menunjukkan kerintangan berganda terhadap 15 jenis antibiotic yang diuji dengan tahap kerintangan yang tinggi terhadap Bacitracin (98%), Furazolidone (88%) dan Tetracycline (83%) dan kebanyakkan adalah peka (lemah) terhadap Imipenem dengan kerintangan 11%. Indeks kerintangan antibiotik berganda (MAR) yang tinggi dikesan dalam kajian ini, dari 0.13 hingga 0.93. RAPD-PCR digunakan bagi mendapatkan perkaitan genetik diantara pencilan *Vibrio cholerae* dan *Vibrio parahaemolyticus* dalam kajian ini. Dua primer (OPA10 : 5′-GTGATCGCAG-3′ dan OPAR8 : 5′-TGGGGCTGTC-3′ daripada 10 primer yang disaring memberikan keputusan terbaik, telah dipilih untuk kajian seterusnya.

Didapati kesemua pencilan *Vibrio cholerae* dan *Vibrio parahaemolyticus* boleh dikumpulkan kepada dua kluster utama untuk setiap primer. Penklusteran adalah berdasarkan profil RAPD-PCR, memandangkan kebanyakan pencilan dari sampel yang sama adalah berada dalam kumpulan kluster yang sama walaupun beberapa pencilan dari lokasi sampel yang berlainan didapati berada dalam kumpulan kluster yang sama.

Kajian simulasi dijalankan bagi meniru keadaan sebenar di dapur domestik setempat yang mungkin untuk memberikan data kuantitatif yang lebih realistik tentang bagaimana vibrios boleh dikurangkan dengan kaedah membasuh dan merendam. Lapan prosedur basuhan dan rendaman digunakan dalam kajian simulasi ini. Oleh itu purata peratusan kadar perpindahan untuk prosedur 1 (tanpa basuhan dan rendaman), prosedur 2 (membasuh dibawah laluan air paip), prosedur 3 (rendam dalam 100 ml air suling yang disterilkan), prosedur 4 (rendam dalam 100 ml air limau nipis) hingga prosedur 5 (rendaman dalam 100 ml air asam jawa) didapati berada dalam julat 0% hingga >100% diikuti oleh prosedur 6 (rendaman dalam 100 ml larutan garam berkepekatan 1%) dalam julat 3.8% hingga >100%, prosedur 7 (rendaman dalam 100 ml larutan garam berkepekatan 3%) dalam julat 80.6% hingga >100% dan yang terakhir ialah prosedur 8 (rendaman dalam 100 ml larutan garam berkepekatan 10%) dalam julat 2.3% hingga >

100%. Kaedah mencuci dengan membilas dan merendam isi ikan air tawar dengan air paip dan air suling tidak menunjukkan pengurangan iaitu pada tahap 0 log. Hanya dengan kaedah merendam dalam larutan air limau nipis, larutan air asam jawa, larutan garam (NaCl) berkepekatan 1%, 3% dan 10% ada menunjukkan angka pengurangan sehingga ke 2.9 log.

Sebagai kesimpulan, ikan air tawar boleh bertindak sebagai perantara *Vibrio cholera* dan *Vibrio parahaemolyticus* yang berisiko kepada pengguna. Adalah disyorkan untuk menjalankan penyelidikan yang lebih lanjut untuk pemahaman yang lebih baik mengenai kehadiran *Vibrio cholera* dan *Vibrio parahaemolyticus* pada ikan air tawar dan risiko apabila mengendalikan dan memakan ikan air tawar yang tercemar.

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Finally, I offer my regards and blessing to all of those who supported me in respect during the completion of the project…..

Thank you all and May Allah SWT bless you always….

I certify that a Thesis Examination Committee has met on _____ to conduct the final examination of **Noorlis Ahmad** on her thesis entitled **"Biohazard of *Vibrio cholera* and *Vibrio parahaemolyticus* in Freshwater Fish in Selangor Markets and the Decontamination Methods"** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Examination Committee were as follows:

(Chairman)

(Internal Examiner)

(Internal Examiner)

(External Examiner)

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NORITAH OMAR, PhD
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Date:

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

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BUJANG BIN KIM HUAT, PhD
Professor and Dean
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Date:

DECLARATION

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

_____

NOORLIS BINTI AHMAD

Date: 19 December 2012

TABLE OF CONTENTS