



**UNIVERSITI PUTRA MALAYSIA**

**EXPERIMENTAL INFECTION OF RIVER CATFISH *MYSTUS NEMURUS* WITH *VIBRIO PARAHAEMOLYTICUS* AND MOLECULAR CHARACTERIZATION OF THE ISOLATES**

**NAJIAH MUSA**

**FPV 2002 10**

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CHARACTERIZATION OF THE ISOLATES**

**By**

**NAJIAH MUSA**

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

**October 2002**



*Dedicated to*

*Ma & Ayah*

*& my late Tok*

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

**EXPERIMENTAL INFECTION OF MALAYSIAN RIVER CATFISH  
*MYSTUS NEMURUS* WITH *VIBRIO PARAHAEMOLYTICUS* AND  
MOLECULAR CHARACTERIZATION OF THE ISOLATES**

By

**NAJIAH MUSA**

**October 2002**

**Chairman: Hassan Hj Mohd Daud, Ph.D.**

**Faculty: Veterinary Medicine**

Declining marine fish resources in Malaysia have led to the innovation of rearing indigenous freshwater river catfish *Mystus nemurus*, locally known as 'baung' in brackishwater. This however will inevitably expose the fish to the pathogen, *Vibrio parahaemolyticus* that is ubiquitous in brackishwater. The present research was undertaken to study the virulence and pathogenicity of clinical and environmental *V. parahaemolyticus* isolates in *M. nemurus*. *Vibrio parahaemolyticus* isolates from various sources and locations in Peninsular Malaysia were identified based on morphological, biochemical and physiological characteristics.

Virulence studies revealed that clinical *V. parahaemolyticus* from clinical cases were more virulent ( $p < 0.05$ ) to *M. nemurus* as compared to environmental isolates. The virulence was categorized as virulent, moderately virulent, weakly virulent and avirulent. The most virulent isolate (F1) was used to infect fish via intraperitoneal (IP), intramuscular (IM) and immersion routes. The LD<sub>50</sub> results revealed that IP exposure was most pathogenic, following by IM and immersion exposures. Intraperitoneal exposure caused toxemia in fish while IM exposure

caused localized lesions at the injection sites, and immersion exposure caused only mild inflammatory responses on the gills and the scraped skin.

Random amplification polymorphic DNA (RAPD) revealed DNA polymorphism in all isolates tested, indicative of high variability among the *V. parahaemolyticus* isolates. Dendrogram revealed a distant genetic relationship between the virulent (F1) and avirulent (W4) isolates. Antibigram showed resistance to intermediate to erythromycin, and 90% of the isolates were intermediate to cephalosporins and cefotaxim. The absence of plasmids in all isolates indicated that antimicrobial resistance of the isolates were chromosomally mediated.

Partial sequence analysis of the *toxR* and *toxS* genes of isolates F1 and W4 revealed a very high homology (97%). The genetic variations of *toxR* fragment resulted in 59 to 77% amino acid homology. This might have contributed to the different degrees of virulence of the isolates. The *toxS* fragment showed 100% amino acid homology, indicating that this fragment was more conserved than *toxR* gene fragment.

It appeared that not all *V. parahaemolyticus* isolates could induce infection in *M. nemurus*. However, slight genetic variations in *toxR* gene fragment of *V. parahaemolyticus* isolates could contribute to different degree of virulence. *Mystus nemurus* was least susceptible to the immersion challenge of a virulent *V. parahaemolyticus* isolate.

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

**EKSPERIMEN INFEKSI KE ATAS IKAN BAUNG *MYSTUS NEMURUS*  
DENGAN *VIBRIO PARAHAEMOLYTICUS* DAN PENCIRIAN MOLEKULAR  
ISOLAT**

Oleh

**NAJIAH MUSA**

**Oktober 2002**

**Pengerusi : Hassan Hj Mohd Daud, Ph.D.**

**Fakulti : Perubatan Veterinar**

Kekurangan sumber ikan marin dari laut di Malaysia telah membawa kepada inovasi menternak ikan sungai tempatan, *Mystus nemurus*, nama tempatannya 'baung' di dalam air payau. Bagaimanapun langkah ini akan menyebabkan ikan ini terdedah patogen, *Vibrio parahaemolyticus* yang sentiasa ada di dalam air payau. Justeru, kajian ini telah dijalankan bagi mengkaji kevirulenan dan patogenisiti isolat-isolat klinikal dan persekitaran *V. parahaemolyticus* ke atas *M. nemurus*. Isolat-isolat *V. parahaemolyticus* ini yang diambil dari pelbagai sumber dan lokasi di Semenanjung Malaysia telah dikenalpasti berdasarkan sifat-sifat morfologi, biokimia dan fisiologi.

Kajian kevirulenan menunjukkan bahawa isolat-isolat klinikal *V. parahaemolyticus* adalah lebih virulen ( $p < 0.05$ ) kepada *M. nemurus* berbanding dengan isolat-isolat persekitaran. Kevirulenan telah dikategorikan sebagai virulen, virulen sederhana, virulen lemah dan tidak virulen. Isolat yang paling virulen (F1) telah digunakan untuk menjangkiti ikan melalui pendedahan secara intraperitoneal (IP), intramuskular (IM) dan rendaman. Keputusan LD<sub>50</sub> menunjukkan pendedahan

IP adalah paling patogenik, diikuti oleh IM dan rendaman. Pendedahan IP menyebabkan toksemia pada ikan manakala pendedahan IM menyebabkan lesi setempat pada kawasan suntikan dan pendedahan rendaman hanya menyebabkan respon inflamatori ringan pada insang dan kulit ikan yang telah dilakukan.

Amplifikasi secara rawak DNA polimorfik (RAPD) menunjukkan polimorfik DNA dalam semua isolat yang diuji, menandakan variasi yang tinggi pada isolat-isolat *V. parahaemolyticus*. Dendrogram menunjukkan hubungan genetik yang jauh di antara isolat yang virulen (F1) dan tidak virulen (W4). Keputusan kerentanan antibiotik menunjukkan adanya resisten hingga sederhana terhadap eritromycin di kalangan isolat dan didapati 90% daripada isolat-isolat adalah sederhana terhadap cephalosporins dan cefotaxim. Ketiadaan plasmid pada kesemua isolat menunjukkan kerentanan antibiotik adalah bermediasikan kromosom.

Analisa jujukan sebahagian gen *toxR* dan *toxS* pada isolat-isolat F1 dan W4 menunjukkan homologi yang sangat tinggi (97%). Variasi genetik pada fragmen gen *toxR* menyebabkan 59% hingga 77% homologi asid amino. Ini mungkin telah menyumbang kepada perbezaan darjah kevirulenan isolat-isolat. Fragmen gen *toxS* menunjukkan 100% homologi asid amino, menandakan fragmen ini adalah lebih terpelihara berbanding fragmen gen *toxR*.

Oleh yang demikian, didapati bukan semua isolat *V. parahaemolyticus* boleh menyebabkan infeksi pada *M. nemurus*. Bagaimanapun, variasi genetik yang sedikit berbeza pada fragmen gen *toxR* boleh menyumbang kepada perbezaan darjah

kevirulenan. *Mystus nemurus* tidak mudah dihindangi jangkitan isolat *V. parahaemolyticus* yang virulen melalui pendedahan secara rendaman.



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I certify that an Examination Committee met on 11<sup>th</sup> October 2002 to conduct the final examination of Najiah Musa on her Doctor of Philosophy thesis entitled “Experimental Infection of River Catfish *Mystus nemurus* with *Vibrio parahaemolyticus* and Molecular Characterization of The Isolates ” 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:

**Abdul Rani Bahaman, Ph.D.**

Professor  
Institute of Bioscience  
Universiti Putra Malaysia  
(Chairman)

**Hassan Hj Mohd Daud, Ph.D.**

Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

**Mohamed Shariff Mohamed Din, Ph.D.**

Professor  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia  
(Member)

**Mohd Azmi Mohd Lila, Ph.D.**


Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

**Mariana Nor Shamsudin, Ph.D.**

Associate Professor  
Faculty of Medicine and Health Sciences,  
Universiti Putra Malaysia  
(Member)

**Ansary Ahmed, Ph.D.**

Professor  
Senior President  
Open University Malaysia  
(Independent Examiner)

  
\_\_\_\_\_  
**SHAMSHEER MOHAMAD RAMADILI, Ph.D.**  
Professor/Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 26 NOV 2002



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

**Hassan Hj Mohd Daud, Ph.D.**

Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

**Mohamed Shariff Mohamed Din, Ph.D.**

Professor  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia  
(Member)

**Mohd Azmi Mohd Lila, Ph.D.**

Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

**Mariana Nor Shamsudin, Ph.D.**

Associate Professor  
Faculty of Medicine and Health Sciences,  
Universiti Putra Malaysia  
(Member)



---

**AINI IDERIS, Ph.D.**  
Professor/Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: **13** FEB 2003

## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully 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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NAJIAH MUSA

Date: 25.11.2002

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- 29 Normal histology of muscle of the lateral body wall showing the epidermis (E) and dermis (D). The hypodermis (H) attaches to the underlying muscle. Goblet cells (G) and club cells (CC) are unicellular glands located in the epidermis. H & E (x350) 5.44
- 30 Hemorrhages at the exposed muscle at 24 hpi. Note in the foreground, normal muscle tissue (N). H & E (x700) 5.45
- 31 Colonies of *V. parahaemolyticus* (B) were seen between the muscle layer. Massive numbers of inflammatory cells (IC) were found intermingled with erythrocytes (E) and exudates adjacent to the site at 48 hpi. H & E (x700). 5.46
- 32A Scraped skin area of infected fish showing muscle bundles with presence of large numbers of *V. parahaemolyticus* (B) at 3 hpi (scale bar = 10  $\mu$ m) 5.47
- 32B Necrotised tissues (NT) in fish were noted at 3 hpi (scale bar = 1  $\mu$ m) 5.47

32C	Abundance of <i>V. parahaemolyticus</i> (B) on muscle fibers of infected fish at 36 hpi (scale bar = 1µm)	5.48
32D	Abundance of <i>V. parahaemolyticus</i> (B) from 36 to 48 hpi on fish scraped skin surface. Note the presence of fibrous stroma (FS) restructuring the damaged area. Four erythrocytes (E) could be seen on the fibrous stroma (scale bar = 10µm)	5.48
33A	Normal gills (scale bar = 10µm)	5.49
33B	The surfaces of distal lamellae (L) were slightly eroded at 72 hpi (scale bar = 10µm)	5.49
33C	Presence of PMN leucocytes (L), macrophage (M) and very few cells of <i>V. parahaemolyticus</i> (B) on the lamellae of gills at 12 hpi (scale bar = 10µm)	5.50
33D	Absence of <i>V. parahaemolyticus</i> on gills of fish after 12 hpi. Note the presence of abnormal erythrocytes (AE) having spike-projections on the surface and flatten morphology (scale bar = 10µm)	5.50
34	<i>Vibrio parahaemolyticus</i> plaques (B) were seen on the scraped lesion at 3 hpi. H & E (x700)	5.51
35	Increased inflammatory cells (IC) were below the scraped tissue at 6 hpi. Bacterial plaques (B) were also observed. H & E (x175)	5.52
36	Massive inflammatory cells (IC) and hemorrhages (E) were found at the site of scraped tissue at 24 hpi. H & E (x175)	5.53
37	The site below the scraped tissue undergone degenerative changes and necrosis (N). At the same time fibrosis (F) took place at the area at 96 hpi. H & E (x700)	5.54
38	An epidermal layer had covered the open wound. Note the increase of fibrous tissues (FT) indicating the healing process at 144 hpi. B & B (x175)	5.55
39	Massive fibroplasia (F) was seen at 120 hpi. H & E (x700)	5.56
40	Regeneration of new epithelial cells began where spongiosis (S) of the epithelial cells occurred at 144 hpi. Note the presence of spongiosis (S) of the epithelial cells and club cells (CC). Uneven layer of new epidermis were found on the previously scraped area. B & B (x700)	5.57
41	RAPD banding profiles of <i>V. parahaemolyticus</i> isolates obtained with primer Gen1-50-01. Lane 1: 100-bp DNA molecular mass marker; lane 2: 1-kb DNA molecular mass marker; lane 3: isolate	6.6

- F1; lane 4: isolate F2; lane 5: isolate F3; lane 6: isolate F4; lane 7 : isolate W1; lane 8: isolate W2; lane 9: isolate W3; lane 10: isolate W4; lane 11: isolate W5; lane 12: isolate W6 and lane 13 : negative control
- 42 RAPD banding profiles of *V. parahaemolyticus* isolates obtained with primer Gen1-50-02. Lane 1: 100-bp DNA molecular mass marker; lane 2: 1-kb DNA molecular mass marker; lane 3: isolate F1; lane 4: isolate F2; lane 5: isolate F3; lane 6: isolate F4; lane 7 : isolate W1; lane 8: isolate W2; lane 9: isolate W3; lane 10: isolate W4; lane 11: isolate W5; lane 12 : isolate W6 and lane 13 : negative control 6.7
- 43 Dendrogram based on RAPD profiles of 10 *V. parahaemolyticus* isolates revealed by primers Gen1-50-01 and Gen1-50-02. Branch length represents the genetic distance between isolates in each cluster. Genetic distances are indicated on each arm of the tree 6.8
- 44 PCR results of partial *toxRS* gene in both virulent (F1) and avirulent (W4) *V. parahaemolyticus* isolates. Lane 1: negative control; lane 2: 1 kb DNA molecular mass marker; lane 3 to 6: replicates of virulent isolates (Isolate F1) and lane 7 to 10: replicates of avirulent isolates (Isolate W4) 7.17
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- 46 Verification of the insert (1,171 bp) in the plasmid (3.9 kbp) using restriction analysis (digestion with *EcoRI*). Lane 1: virulent isolate (Isolate F1); lane 2: avirulent isolate (Isolate W4); lane 3: virulent isolate (Isolate F1) and lane 4: 1 kb DNA molecular mass marker 7.18
- 47 Homology of three DNA sequence of *toxR* fragments of Malaysian virulent (V) isolate (upper sequence), reference (R) L11929 (middle sequence) and Malaysian avirulent (A) isolate (lower sequence). Those positions of sequences that have same compositions are shown as “; dash (-) signifies that no base occurs at those positions; plus (+) signifies there is an additional base at those position. Reference L11929 is used as the standard reference in the study 7.19
- 48 Homology of two *toxR* amino acid sequences deduced from the nucleotide sequences of reference L11929 (upper sequence) and Malaysian virulent isolate (lower sequence). Identical residues are indicated by vertical lines 7.21

- 49 Homology of two *toxR* amino acid sequences deduced from the nucleotide sequences of reference L11929 (upper sequence) and Malaysian avirulent isolate (lower sequence). Identical residues are indicated by vertical lines 7.22
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- 52 Homology of amino acid deduced from *toxS* gene fragment of Malaysian (V) isolate (upper sequence), reference L11929 (middle sequence) and Malaysian avirulent (A) isolate (lower sequence) 7.24



**LIST OF ABBREVIATIONS**

Anova	Analysis of variance
cfu	Colony forming unit
H & E	Hematoxylin and eosin
IP	Intraperitoneal
IM	Intramuscular
pi	Post infection
NaCl	Sodium chloride
RM	Ringgit Malaysia
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TCBS	Thiosulphate citrate bile salt sucrose agar
ppt	Parts per thousand
OD	Optical density
bp	Base pair
°C	Degree celcius
Dh <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
LD <sub>50</sub>	Lethal dose 50%
kb	Kilobase
M	Molar
μl	Microlitre
min	Minute
PCR	Polymerase chain reaction