UNIVERSITI PUTRA MALAYSIA

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

NAJIAH MUSA

FPV 2002 10

EXPERIMENTAL INFECTION OF RIVER CATFISH *MYSTUS NEMURUS* WITH *VIBRIO PARAHAEMOLYTICUS* AND MOLECULAR 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_{50} 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. Antibiogram 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 *tox*R and *tox*S genes of isolates F1 and W4 revealed a very high homology (97%). The genetic variations of *tox*R fragment resulted in 59 to 77% amino acid homology. This might have contributed to the different degrees of virulence of the isolates. The *tox*S fragment showed 100% amino acid homology, indicating that this fragment was more conserved than *tox*R gene fragment.

It appeared that not all *V. parahaemolyticus* isolates could induce infection in *M. nemurus*. However, slight genetic variations in *tox*R 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 intraperitonial (IP), intramuskular (IM) dan rendaman. Keputusan LD_{50} 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 dilukakan.

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 resistan 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 *tox*R dan *tox*S pada isolat-isolat F1 dan W4 menunjukkan homologi yang sangat tinggi (97%). Variasi genetik pada fragmen gen *tox*R menyebabkan 59% hingga 77% homologi asid amino. Ini mungkin telah menyumbangkan kepada perbezaan darjah kevirulenan isolat-isolat. Fragmen gen *tox*S menunjukkan 100% homologi asid amino, menandakan fragmen ini adalah lebih terpelihara berbanding fragmen gen *tox*R.

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

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

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious and Most Merciful, I am thankful and grateful to Allah for giving me the strength and capacity to complete this study.

It is my special pleasure to extend my deepest appreciation and gratitude to my main supervisor Dr. Hassan Haji Mohd Daud for his untiring supervision, advices, guidances and kindness, my committee members namely, Prof. Dr. Mohamed Shariff Mohamed Din for his superb supervision, priceless critisms, great opinions and providing emergency fundings, Assoc. Prof. Dr. Mohd Azmi Mohd Lila for his critical thinkings, suggestions, kindness and speedy corrections, and Assoc. Prof. Dr. Mariana Nor Shamsudin for her valuable opinions during the study period.

My sincere thanks to Kolej Universiti Sains dan Teknologi Malaysia (KUSTEM) and Universiti Putra Malaysia (UPM) for providing me the financial support under "Skim Latihan Akademik Bumiputra" (SLAB). Special thanks are also accorded to the Rector of KUSTEM, Prof. Dato' Dr. Mahyuddin Mohamad Dahan, Dean of Faculty of Science and Technology, Assoc. Prof. Dr. Noor Azhar Shazili and previous Dean, Prof. Dr. Haji Mohd Azmi Ambak, Head department of Fisheries and Marine Sciences, Assoc. Prof. Dr. Sakri Ibrahim, previous head of department(s) namely, Prof. Dr. Hajjah Faizah Shaharom, Mr. Aizam Zainal Abidin and Prof. Dr. Lokman Shamsudin for their encouragement and support during my study. I would also like to thank the Dean of Veterinary Medicine Faculty, Prof. Dr. Mohd Zamri Saad and previous Deans, Prof. Dato' Dr. Abdul Salam Abdullah and Prof. Dato' Dr. Sheikh Omar Abd Rahman for their supports and encouragement during my study in this faculty. I would also like to express my gratitudes to Tuan Haji Karim Noh Shah

(Ex-Deputy Registrar of UPM, currently Director of Development of UPM), Mr. Mohamed Adil Muhammad Tajuddin (Deputy Registrar of UPM) and Mr. Johan Abd Rahim (Registrar Department) for giving me the opportunities of attending an international shrimp pathology course in the University of Arizona, USA, a training workshop on applications of molecular techniques on "White Spot Syndrome Virus" (WSSV) detection in shrimp in the University of Can Tho, Vietnam and presenting my paper in The Third World Fisheries Congress, Beijing, China as well as presenting my papers in the local seminars and conferences during my study.

I am indebted to all my wonderful friends, especially to my best pals Dr. Lee Kok Leong and Ms Nadirah Musa, my colleagues, Ms Abeer Al-Sahtout, Dr. Tan Lee Tung, Dr. Wang Ying Geng and Chen Xia, Dr. Sanjoy Banarjee, Dr. Devaraja, Mrs Fennie Fong, Dr. Samson Soon, Ms Nor Azizah Mohd Taib, Ms Norazila Kassim Shaari, Ms Wan Somarny, Mrs Adnalizawati, Mr Mohd Zulkamal Radzi and Asst. Prof. Dr. Ahmed Jalal Khan Chowdry for their marvelous friendships.

To my parents, Ma and Ayah, whom I owe most of the gratitude, thank you very much for your love, prayers, affections, moral and financial supports throughout these years. My great and best sisters and brothers, Dee, Aid, Cah, Jang and Eee, thank you all for the encouragement, helps, concerns and understandings. Finally, a very special thank you to my BB whom I'm indebted for his patience, understandings, assistance and good heart throughout this project. Most of all, I thank God for making all these possible.

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		viii
APPROVAL SHEETS		x
DECLARATION FORM		xii
TABLE OF CONTENTS		xiii
LIST OF TABLES		xvii
LIST OF FIGURES		xviii
LIST OF ABBREVIATIONS		xxiv

CHAPTER

I	INTRODUCTION	1.1
	1.1 Fisheries Profile In Malaysia	1.1
	1.2 Aquaculture	1.3
	1.3 Fingerlings Production	1.4
	1.4 Recent Scenario In Fisheries Industry	1.4
	1.5 Solution and Innovation	1.5
	1.6 Fish Diseases In Malaysia	1.6
	1.7 Vibriosis In Malaysia	1.7
	1.8 Diagnosis of Vibriosis	1.8
	1.9 Statement of Problem and Significance of Study	1.9
	1.10 Hypotheses of Study	1.10
	1.11 Objectives of Study	1.10
2	LITERATURE REVIEW	2.1
	2.1 History of Vibriosis	2.1
	2.2 Occurrence of Vibriosis	2.2
	2.3 *Vibrionaceae*	2.3
	2.4 Biochemical Characteristics of *V. parahaemolyticus*	2.3
	2.5 Life Patterns and Morphologies of *V. parahaemolyticus*	2.4
	2.6 Clinical Signs	2.5
	2.7 Pathogenicity	2.6

	2.8	Diagnosis	2.10
	2.9	Rapid Identification System	2.10
	2.10	Virulence Factors	2.11
	2.11	*Tox*RS Gene	2.13
	2.12	Nucleotide Sequence Variation	2.13
	2.13	Prophylaxis and Therapy	2.14
	2.14	Genetic Basis of Bacterial Resistance to Antibiotics	2.15
	2.15	Plasmid	2.16
	2.16	Antibiotic Resistance	2.18
	2.17	Random Amplified Polymorphic DNA (RAPD)	2.19
	2.18	Polymerase Chain Reaction (PCR)	2.20
	2.19	Genomic Informatics	2.21
	2.20	Importance of Baung	2.22
	2.21	Performances and Diseases In Freshwater Fish Reared in Brackishwater	2.22
3		CHARACTERIZATION OF *VIBRIO PARAHAEMOLYTICUS* ISOLATES FROM DISEASED FISH AND BRACKISHWATER SHRIMP CULTURE PONDS	3.1
	3.1	Introduction	3.1
	3.2	Materials and Methods	3.2
		3.2.1 Sampling areas and types of samples	3.2
		3.2.2 Bacterial identification	3.3
		3.2.3 Conventional test	3.3
		3.2.4 Confirmatory test	3.4
		3.2.5 Storage	3.4
	3.3	Results	3.5
		3.3.1 *V. parahaemolyticus's* description	3.7
	3.4	Discussion	3.8
4		VIRULENCE OF CLINICAL AND ENVIRONMENTAL *VIBRIO PARAHAEMOLYTICUS* ISOLATES IN AN INDIGENOUS RIVER CATFISH *MYSTUS NEMURUS*	4.1
	4.1	Introduction	4.1
	4.2	Materials and Methods	4.2
		4.2.1 Bacterial preparation	4.2
		4.2.2 Experimental fish	4.2
		4.2.3 Experimental design	4.3
		4.2.4 Experimental A: Comparison of virulence between clinical and environmental *V. parahaemolyticus* isolates	4.3
		4.2.5 Experimental B: Susceptibility of *Mystus nemurus* to different invasion challenges	4.4
	4.3	Results	4.4
		4.3.1 Experiment A: Comparison of virulence between clinical and environmental *V. parahaemolyticus* isolates	4.4
		4.3.2 Experiment B: Susceptibility of *Mystus nemurus* to different invasion challenges	4.7
	4.4	Discussion	4.9

5	SCANNING ELECTRON MICROSCOPY AND HISTOPAHOLOGY OF RIVER CATFISH *MYSTUS NEMURUS* FINGERLINGS EXPERIMENTALLY INFECTED WITH *VIBRIO PARAHAEMOLYTICUS*	5.1
	5.1 Introduction	5.1
	5.2 Materials and Methods	5.2
	5.2.1 Experimental design	5.2
	5.2.2 Bacterial isolate	5.2
	5.2.3 Bacterial preparation	5.2
	5.2.4 Experimental fish	5.3
	5.2.5 Intraperitoneal (IP) and Intramuscular (IM) exposures	5.3
	5.2.6 Immersion exposure	5.3
	5.2.7 Scanning electron microscopy (SEM)	5.4
	5.2.8 Histology	5.4
	5.3 Results	5.5
	5.3.1 SEM: IP exposure	5.5
	5.3.2 Histopathology: IP exposure	5.5
	5.3.3 SEM: IM exposure	5.7
	5.3.4 Histopathology: IM exposure	5.7
	5.3.5 SEM: Immersion exposure	5.8
	5.3.6 Histopathology: Immersion exposure	5.8
	5.4 Discussion	5.9
6	RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD), ANTIMICROBIAL SUSCEPTIBILITY AND PLASMID PROFILE OF CLINICAL AND ENVIRONMENTAL *VIBRIO PARAHAEMOLYTICUS* ISOLATES	6.1
	6.1 Introduction	6.1
	6.2 Materials and Methods	6.3
	6.2.1 Bacterial isolation and identification	6.3
	6.2.2. DNA extraction and plasmid profiling	6.3
	6.2.3 RAPD amplification	6.3
	6.2.4 RAPD profile analysis	6.4
	6.2.5 Antimicrobial susceptibility tests	6.4
	6.3 Results	6.5
	6.3.1 Bacterial isolation and identification	6.5
	6.3.2 RAPD amplification and analysis	6.5
	6.3.3 Antimicrobial susceptibility tests	6.9
	6.3.4 Plasmid profiles	6.9
	6.4 Discussion	6.10
7	COMPARISON OF PARTIAL NUCLEOTIDE SEQUENCE OF *TOX*RS GENE FRAGMENTS OF MALAYSIAN VIRULENT AND AVIRULENT *VIBRIO PARAHAEMOLYTICUS* ISOLATES WITH A PUBLISHED JAPANESE ISOLATE (11929)	7.1
	7.1 Introduction	7.1
	7.2 Materials and Methods	7.3
	7.2.1 Bacteria isolates	7.3
	7.2.2 DNA preparation, PCR amplification and analysis	7.3
	7.2.3 DNA cloning, colony PCR and restriction enzyme	7.5

	7.3	Results	7.7
		7.3.1 Amplification of *Tox*RS gene fragments	7.7
		7.3.2 Cloning of *Tox*RS gene fragments, colony PCR, RE	7.7
		7.3.3 Partial *Tox*RS gene fragments sequence analysis	7.7
	7.4	Discussion	7.11
8		GENERAL DISCUSSION AND CONCLUSION	8.1
REFERENCES			R.1
APPENDICES			
		Appendix A: Solutions and media	A.1
		Appendix B: Published Japanese *V. parahaemolyticus* sequence data L1929	A.3
		Appendix C: Electropherogram of cloned *tox*RS gene fragment of Malaysian virulent *V. parahaemolyticus* isolate (F1)	A.4
		Appendix D: Electropherogram of cloned toxRS gene of Malaysian avirulent *V. parahaemolyticus* isolate (W4)	A.9
BIODATA			B.1

LIST OF TABLES

Table		Page
1	The fisheries sectors and their production (tones) in 1998	1.2
2	Differentiation of the arginine-negative, lysine-positive of *Vibrio* spp.	2.4
3	The advantages and disadvantages, yields and wholesale prices among the catfish family	2.22
4	Sampling areas and types of samples from October to December 1998	3.3
5	Morphological, biochemical and physiological characteristics of 10 *Vibrio parahaemolyticus* isolated from diseased fish (clinical) and brackishwater (environmental) samples using conventional tests and BBL Crystal kit	3.6
6	*Vibrio parahaemolyticus* origin, sampling sites of diseased fish and brackishwater in Peninsular Malaysia	3.8
7	Daily mean mortality in three consecutive days post infection (p.i) and cumulative mortality of fingerlings in seven days post infection (p.i) observed in rivercatfish injected intraperitoneally (IP) with 1.0 x 10^7 cfu/ml of *V. parahaemolyticus* isolates	4.5
8	Classification of virulence of *V. parahaemolyticus* isolates based on daily mortality by Tukey's test	4.6
9	LD_{50} values of fish challenged with *V. parahaemolyticus* via intraperitoneal (IP), intramuscular (IM) and immersion routes	4.7
10	Antibiogram of 10 *V. parahaemolyticus* isolates	6.9
11	Designed PCR (TRG 4(1)- sense) primer using Primer Premiere®	7.4
12	Designed PCR (TRG 4(2)- antisense) primer using Primer Premiere®	7.5
13	Analysis of *tox*R gene fragments on the reference (L11929), virulent and avirulent isolates. 628 delC* indicates the deletion in avirulent isolate	7.10
14	Analysis of *tox*S gene fragments on reference (L11929), virulent and avirulent isolates	7.10

LIST OF FIGURES

Figure		Page
1	Cumulative mortality via intraperitoneal (IP) exposure using *V. parahaemolyticus* isolates	4.6
2	Daily mean mortality via intraperitoneal (IP) exposure using *V. parahemolyticus* isolates	4.7
3	The relationship between cumulative mortality of fish (replicates) and the doses given to the fish via intraperitoneal (IP), intramuscular (IM) and immersion exposures	4.9
4A	*Vibrio parahaemolyticus* (B) were abundant in the peritoneal cavity. Polymorphonuclear leucocyte (PMN) was found amongst bacteria (B) at 24 hpi (scale bar = 10 μm)	5.17
4B	Aggregation of degenerated *V. parahaemolyticus* (B) at necrotized tissues (NT) and the presence of unidentified inflammatory cells (I) at 6 hpi (scale bar = 1μm)	5.17
4C	Massive fibrin (F) networks, lymphocytes (L), abnormal erythrocytes with spikes on their surfaces (AE) and *V. parahaemolyticus* (B) were seen at 72 hpi (scale bar = 10 μm)	5.18
4D	Necrotic cells were found sticking to the macrophage (M) at the peritoneal wall at 96 hpi (scale bar = 10 μm)	5.18
5	Normal architecture of liver parenchyma which is composed of laminae of hepatocytes separated by blood sinusoids (BS) draining to the central vein (CV). H & E (x350)	5.19
6	At 24 hpi, inflammatory cells (IC) were observed near the distrupted vein. H & E (x700)	5.20
7	Inflammatory cells like PMN leucocytes (P) as well as macrophage (M) and erythrocytes (E) were seen near the hepatic vein at 24 hpi. H & E (x1750)	5.21
8	Severely infected hepatocytes showed distruption of hepatic architecture leaving empty spaces in between hepatocytes as well as necrotic hepatocytes (NH) with marked inflammatory cells (IC) response at 24 hpi. H & E (x175)	5.22
9	Engorged melanomacrophage center (MM) was seen at 7 dpi within hepatic parenchyma. H & E (x700)	5.23

10	Regeneration of liver parenchyma at 14 dpi. Note the presence of mitotic cells (MC), fibroblast (F) networks. H & E (x 700)	5.24
11	Liver parenchyma at 14 dpi showing reorganization of liver architecture. Presence of erythrocytes (E) in the sinusoid indicating formation of damaged sinusoid. Inadequate nutrition during experiment caused the hepatocytes having centrally located nuclei (N). H & E (x350)	5.25
12	Normal histology of kidney showing the hematopoeitic tissue (H) and renal tubules (RT). H & E (x700)	5.26
13	The renal corpuscle which is composed of a glomerulus (G), and Bowman's capsule (BC). They are separated by Bowman's space (S). The wall of the Bowman's capsule is comprised of squamus cell epithelium (SE). The first proximal segment (Fs) of renal tubule has thicker brush border than the second proximal segment (PS). H & E (x700)	5.27
14	Note extensive necrosis of renal tubules (RT) at 96 hpi. H & E (x350)	5.28
15	Extensive hemorrhages in renal parenchyma as well as degenerative changes in tubules and disappearance of tubules were observed at 120 hpi. H & E (x350)	5.29
16	Vacuolation of renal tubular (RT) cells and infiltration of inflammatory cells (IC) were seen at 120 hpi. H & E (x700)	5.30
17	Shrinkage and necrosis of glomeruli (G) showing large space between Bowman's'capsule (BC) and glomerulus as well as extensive necrosis of the renal parenchyma at 144 hpi. H & E (x350)	5.31
18	Normal section of spleen showing spleenic vein (SV), red pulp (RP) and white pulp (WP). H & E (x175)	5.32
19	Marked area of red pulp (RP) and white pulp (WP) at 144 hpi. H & E (x175)	5.33
20A	Presence of hemosiderin pits (H) and increment in red pulp (RP) area and congestion in sinusoid at 168 hpi. H & E (x700)	5.34
20B	Presence of hemosiderin pits (H) and increment in red pulp area and sinusoid filled with erythrocytes (E) at 168 hpi. H & E (x1750)	5.35
21	The spleen recovering to normal. Note red pulp (RP) and white pulp (WP) interspersed with each other at 14 dpi. H & E (x350)	5.36

22	Normal histology of heart which consists of compact (C) and spongy (S) layers. H & E (x350)	5.37
23	Spongy (S) layer is widely spaced with erythrocytes (E) flowing between them. H & E (x700)	5.38
24	The myocardium was edematous, swollen and thickened at 24 hpi. H & E (x350)	5.39
25	Increased number of inflammatory cells (IC) were observed within the myocardial tissues especially PMN leucocytes at 24 hpi. H & E (x700)	5.40
26	Presence of chloride cells (CC) indicated by acidophilic cells, blood vessels (BV), erythrocytes (E) and pillar cell (PC). Note the separation of epithelial layer from secondary lamellae (S) at 72 hpi. H & E (x350)	5.41
27	Hyperplasia (H) in the interlamellar spaces of gills and oedema causing separation of the epitheliod cells lining at 72 hpi. H & E (x700)	5.42
28A	Abnormal erythrocytes (AE) with spike-projections on the surface were found at the injection site (scale bar = 1μm)	5.43
28B	Transverse section of the exposed site showed necrotic muscle bundles in absence of *V. parahaemolyticus* cells at 6 hpi (scale bar = 1μm)	5.43
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 fore ground, 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 = 10μm)	5.47
32B	Necrotised tissues (NT) in fish were noted at 3 hpi (scale bar = 1μm)	5.47

32C	Abundance of *V. parahaemolyticus* (B) on muscle fibers of infected fish at 36 hpi (scale bar = 1μm)	5.48
32D	Abundance of *V. parahaemolyticus* (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 *V. parahaemolyticus* (B) on the lamellae of gills at 12 hpi (scale bar = 10μm)	5.50
33D	Absence of *V. parahaemolyticus* 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	*Vibrio parahaemolyticus* 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 *V. parahaemolyticus* 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 *tox*RS 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
45	Positive clones (white colonies) show heavier molecular weight indicated by increased in base pair size using colony PCR. Lanes 1 to 4: virulent isolates (Isolate F1); lanes 5 to 8: avirulent isolates (Isolates W4); lane 9: 1 kb DNA molecular mass marker and lane 10: negative control	7.17
46	Verification of the insert (1,171 bp) in the plasmid (3.9 kbp) using restriction analysis (digestion with *Eco*RI). 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 *tox*R 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 *tox*R 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 *tox*R 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
50	Homology of two *tox*R 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	7.23
51	Homology of nucleotide sequences of the *tox*S gene fragment of Malaysian virulent (V) isolate (upper sequence), reference L11929 (middle sequence) and Malaysian avirulent (A) isolate (lower sequence). Those positions of sequences that have same compositions are shown as ". Reference L11929 is used as the standard reference in the study	7.24
52	Homology of amino acid deduced from *tox*S 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_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucloside triphosphate
LD_{50}	Lethal dose 50%
kb	Kilobase
M	Molar
µl	Microlitre
min	Minute
PCR	Polymerase chain reaction