



**DISTRIBUTION, VIRULENCE FACTORS AND ANTIMICROBIAL
RESISTANCE OF *Vibrio* SPECIES ISOLATED FROM CULTURED
SHRIMP IN PENINSULAR MALAYSIA**

By

WAN HAIFA HARYANI BINTI WAN OMAR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Philosophy**

January 2024

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fulfilment of the requirement for the degree of Doctor of Philosophy

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January 2024

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Vibrio, the most common bacterium associated with crustacean diseases, poses a significant threat to shrimp farming due to outbreaks of vibriosis. Therefore, the study aims to provide a comprehensive database on the distribution of *Vibrio* spp. as well as virulence genes and antimicrobial resistance profiling in Malaysian shrimp farms. Briefly, a total of 210 cultured shrimp were collected from seven farms in Peninsular Malaysia. The findings revealed that 225 presumptive *Vibrio* isolates were isolated from cultured shrimp which were then subjected to biochemical test and molecular detection using *pyrH* gene analysis. Eventually, 13 different *Vibrio* spp. were successfully isolated and characterized including *Vibrio parahaemolyticus* (55%), *V. communis* (9%), *V. campbellii* (8%), *V. owensii* (7%), *V. rotiferianus* (5%), *Vibrio* spp. (4%), *V. alginolyticus* (3%), *V. brasiliensis* (2%), *V. natriegens* (2%), *V. xuii* (1%), *V. harveyi* (1%), *V. hepatarius* (0.4%) and *P. damsela* (3%). Based on virulence genes assessment, eight of the eleven

virulence genes in this study were detected, including *chiA* (89%), *flaC* (74%), *tlh* (72%), *toxR* (70%), *luxR* (40%), *pirA* (11%), *pirB* (11%) and *hlyA* (0.4%). Other virulence genes, such as *tdh*, *trh* and *ctxA*, were not detected in any isolates (0%). From the virulence gene assessment, three *Vibrio* isolates were chosen for the pathogenicity test based on highest number present in each of the species. The isolates including *V. parahaemolyticus* S12-3, *V. campbellii* S10-4, and *V. rotiferianus* S24-4 were tested by immersion challenge test against postlarvae (PL) *Penaeus monodon* approximately PL-20 with three different concentrations of 1×10^6 CFU/mL, 1×10^7 CFU/mL, and 1×10^8 CFU/mL. The LC₅₀ result revealed that the lowest were *V. parahaemolyticus* S12-3 (1×10^5 CFU/mL) followed by *V. rotiferianus* S24-4 (1×10^{11} CFU/mL) and *V. campbellii* S10-4 (1×10^{13} CFU/mL). Moreover, histopathological analysis showed sloughing of epithelial cells in hepatopancreatic tubule, haemocytic infiltration, massive vacuolation and loss of hepatopancreatic tubule structure. Meanwhile, the majority of the species tested were resistant to penicillin G (100%), but susceptible to norfloxacin (96%). According to the multiple antibiotic resistance (MAR) index, 84% of *Vibrio* spp. exhibited a MAR index value of more than 0.2. Thus, the findings suggested that a large number of *Vibrio* spp. were resistant to several antibiotics and had been exposed to antibiotics on a regular basis at the farms. Plasmid profiling revealed that 125 isolates from a total 225 of *Vibrio* isolates harboured plasmid with the size ranging from 1 kb and above 10 kb, separating the isolates into 23 plasmid profiles. After plasmid curing, *Vibrio*-positive plasmid was lost resistance to antibiotics. The findings revealed that the resistance to antibiotics in isolated *Vibrio* spp. were either chromosomal or plasmid mediated. In conclusion, ongoing

monitoring of antibiotic-resistant microorganisms as well as understanding the genetic basis of resistance are essential to implement a strategy for minimizing AMR strain yet to improve shrimp farming management in Malaysia.

Keywords: Antimicrobial resistance, Peninsular Malaysia, shrimp, *Vibrio* spp., virulence

SDG: GOAL 4: Quality Education



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

**TABURAN, FAKTOR VIRULEN DAN KERINTANGAN ANTIMIKROB
TERHADAP SPESIES *Vibrio* YANG DIPENCILKAN DARIPADA TERNAKAN
UDANG DI SEMENANJUNG MALAYSIA**

Oleh

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Vibrio adalah bakteria yang paling kerap dikaitkan dengan penyakit pada krustasea yang akhirnya memberikan ancaman yang serius terhadap penternakan udang disebabkan oleh wabak vibriosis. Oleh itu, kajian ini dijalankan bertujuan untuk menyediakan pangkalan data komprehensif mengenai taburan spesies *Vibrio*, gen virulensi dan profil kerintangan antibiotik terhadap spesies *Vibrio* yang dipencilkan daripada ternakan udang di Semenanjung Malaysia. Secara ringkasnya, sebanyak 210 ekor ternakan udang dari tujuh ladang yang terletak di kawasan geografi yang berbeza di Semenanjung Malaysia. Hasil penemuan menunjukkan sebanyak 225 spesies *Vibrio* telah berjaya dipencilkan daripada ternakan udang yang kemudiannya disaring dengan ujian biokimia dan pengesanan molekular berdasarkan gen *pyrH*. Melalui pengesanan molekular menggunakan gen *pyrH*, sebanyak 13 spesies *Vibrio* telah dipencilkan dan dicirikan iaitu *Vibrio parahaemolyticus* (55%), *V. communis* (9%), *V. campbellii* (8%), *V. owensii* (7%), *V. rotiferianus* (5%), *Vibrio* spp. (4%), *V. alginolyticus* (3%), *V.*

brasiliensis (2%), *V. natriegens* (2%), *V. xuii* (1%), *V. harveyi* (1%), *V. hepatarius* (0.4%) dan *P. damsela* (3%). Kesemua isolat daripada spesies *Vibrio* kemudiannya disaring untuk gen virulensi di mana lapan daripada 11 gen virulensi dikesan termasuk *pirA* (11%), *pirB* (11%), (72%), *flaC* (74%), *toxR* (70%), *chiA* (89%), *luxR* (40%) dan *hlyA* (0.4%). Gen virulensi yang lain seperti *tdh*, *trh*, dan *ctxA* tidak ditemui dalam mana-mana isolate (0%). Daripada pencirian virulensi, sebanyak tiga spesies *Vibrio* telah dipilih untuk ujian patogenisiti berdasarkan bilangan gen virulensi tertinggi yang terdapat dalam setiap isolat. Isolat yang terdiri daripada *V. parahaemolyticus* S12-3, *V. campbellii* S10-4, dan *V. rotiferianus* S24-4 telah diuji dengan kaedah infeksi secara rendaman terhadap benih udang *Penaeus monodon* (PL-20) dengan tiga kepekatan yang berbeza iaitu 1×10^6 CFU/mL, 1×10^7 CFU/mL dan 1×10^8 CFU/mL. Keputusan LC_{50} menunjukkan bacaan yang paling rendah ialah *V. parahaemolyticus* S12-3 (1×10^5 CFU/mL) diikuti oleh *V. rotiferianus* S24-4 (1×10^{11} CFU/mL) dan *V. campbellii* S10-4 (1×10^{13} CFU/mL). Analisis histopatologi pula menunjukkan terdapat penyusutan sel epitelium dalam tubul hepatopankreatik, penyusupan hemositik, pembesaran pada vakuolasi dan kehilangan struktur tubul hepatopankreatik. Sementara itu, kesemua isolat bersifat rintang terhadap penisilin G (100%), tetapi rentan terhadap norfloxacin (96%). Tambahan pula, 84% daripada isolat menunjukkan indeks kerentanan antibiotik berbilang (MAR) lebih daripada 0.2. Manakala 16% menunjukkan indeks kerentanan antibiotik berbilang (MAR) kurang daripada 0.2. Pemprofilan plasmid mendedahkan sebanyak 125 isolat daripada jumlah 225 isolat yang mengandungi plasmid dengan saiz plasmid di antara 1 kb dan lebih daripada 10 kb, yang memisahkan sebanyak 23 profil plasmid. Semasa proses pemulihan

plasmid, kebanyakan *Vibrio*-positif plasmid telah kehilangan kerentanan terhadap antibiotik. Hasil penemuan mendapati isolat yang rentan terhadap antibiotik ini mungkin disebabkan oleh mediasi-kromosom atau mediasi-plasmid. Secara kesimpulannya, pemantauan yang berterusan terhadap mikroorganisma rintang antibiotik serta pemahaman asas genetik rintangan adalah penting untuk melaksanakan strategi meminimumkan strain AMR dan pada masa yang sama meningkatkan pengurusan ternakan udang di Malaysia.

Kata Kunci: Kerintangan antimikrob, Semenanjung Malaysia, spesies *Vibrio*, udang, virulen

SDG: MATLAMAT 4: Kualiti Pendidikan

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iv
ACKNOWLEDGEMENTS	vii
APPROVAL	ix
DECLARATION	xi
LIST OF TABLES	xvii
LIST OF FIGURES	xix
LIST OF APPENDICES	xxi
LIST OF ABBREVIATIONS	xxii
 CHAPTER	
1 INTRODUCTION	1
1.1 Background of the study	1
1.2 Problem statements	2
1.3 Significance of the study	4
1.4 Objectives of the study	4
1.5 Hypotheses of the study	7
 2 LITERATURE REVIEW	 8
2.1 Shrimp aquaculture	8
2.1.1 Global shrimp aquaculture industry	8
2.1.2 Malaysia shrimp aquaculture industry	11
2.2 Common diseases associated in shrimp aquaculture industry	14
2.3 <i>Vibrio</i> species	22
2.3.1 General overview of <i>Vibrio</i> species	22
2.3.2 <i>Vibrio</i> spp. diversity as aquatic pathogen in shrimp aquaculture	23
2.3.3 Identification of <i>Vibrio</i> spp.	27
2.4 Virulence factors of <i>Vibrio</i> spp. in aquaculture	30
2.4.1 Thermostable direct hemolysin (TDH) and thermostable direct hemolysin (TRH)	31
2.4.2 Thermolabile hemolysin (<i>tlh</i>)	32
2.4.3 Transcriptional regulatory protein (<i>toxR</i>)	32
2.4.4 Chitinase (<i>chi</i>)	33
2.4.5 Cholera toxin (<i>ctx</i>)	33
2.4.6 Hemolysin (<i>hly</i>)	34
2.4.7 Transcriptional activator protein (<i>luxR</i>)	35
2.4.8 Flagella (<i>fla</i>)	35

2.4.9	Other virulence factor	36
2.5	Pathogenesis of <i>Vibrio</i> spp.	37
2.6	The use of antibiotics in aquaculture	39
2.7	Common antibiotics used in shrimp aquaculture	41
2.8	Antibiotics and their modes of action	43
2.8.1	The basis mechanism of antibiotic action	43
2.8.2	The antibiotic resistance mechanisms	46
2.9	Antimicrobial resistance (AMR) issues in aquaculture	48
2.9.1	Regulations and standards of antibiotics use	51
2.10	Plasmid in <i>Vibrio</i> spp.	53
2.10.1	General overview of plasmid	53
2.10.2	Plasmid profiling	55
2.10.3	Plasmid curing	56
3	ISOLATION, CHARACTERIZATION AND DISTRIBUTION OF <i>Vibrio</i> SPECIES ISOLATED FROM CULTURED SHRIMP IN PENINSULAR MALAYSIA	59
3.1	Introduction	59
3.2	Materials and Methods	61
3.2.1	Shrimp sampling	61
3.2.2	<i>In-situ</i> examination	64
3.2.3	Measurement of water quality	66
3.2.4	Isolation of bacteria	66
3.2.5	Glycerol stock and maintaining of bacteria	66
3.2.6	Phenotypic characterization of <i>Vibrio</i> spp.	67
3.2.7	Molecular characterization of <i>Vibrio</i> spp.	68
3.2.8	DNA sequencing and sequence alignment	70
3.2.9	Deposition of the sequences	70
3.2.10	Phylogenetic analysis	70
3.3	Results	71
3.3.1	Clinical examination	71
3.3.2	Water quality analysis	73
3.3.3	Isolation and morphological characterisation of <i>Vibrio</i> isolates	74
3.3.4	Biochemical characterisation of <i>Vibrio</i> spp.	76
3.3.5	Molecular characterisation of <i>Vibrio</i> spp. by <i>pyrH</i> gene	92
3.3.6	Deposition of sequences	92
3.3.7	Nucleotide blast (BLASTn) analysis	92
3.3.8	Phylogenetic analysis	93

3.3.9	Taxonomy composition and the clades of the <i>Vibrio</i> spp.	95
3.3.10	Distribution of <i>Vibrio</i> spp. from cultured shrimp in Peninsular Malaysia	96
3.4	Discussion	100
3.6	Conclusion	105
4	VIRULENCE GENES ASSESSMENT AND PATHOGENICITY OF SELECTED <i>Vibrio</i> SPECIES ISOLATED FROM CULTURED SHRIMP IN PENINSULAR MALAYSIA	106
4.1	Introduction	106
4.2	Materials and Methods	107
4.2.1	Collection of <i>Vibrio</i> spp.	107
4.2.2	Virulence gene assessment	108
4.2.3	Pathogenicity of selected <i>Vibrio</i> isolates in cultured shrimp	113
4.2.4	Histopathology analysis	117
4.2.5	Bacterial re-isolation	117
4.2.6	DNA extraction of <i>Vibrio</i> spp.	118
4.2.7	Detection using <i>pyrH</i> gene of <i>Vibrio</i> spp.	118
4.2.8	Gel electrophoresis of <i>Vibrio</i> spp.	118
4.2.9	Statistical analysis	118
4.3	Results	119
4.3.1	Virulence genes assessment	119
4.3.2	Pathogenicity study	139
4.3.3	Histopathology study	144
4.4	Discussion	147
4.5	Conclusion	156
5	ANTIBIOTICS SUSCEPTIBILITY TEST AND PLASMID PROFILING OF <i>Vibrio</i> SPECIES ISOLATED FROM CULTURED SHRIMP IN PENINSULAR MALAYSIA	157
5.1	Introduction	157
5.2	Materials and Methods	159
5.2.1	Collection of <i>Vibrio</i> spp.	159
5.2.2	Antibiotics susceptibility test	159
5.2.3	Calculation of multiple antibiotic resistance (MAR) index	161
5.2.4	Determination of plasmid profiling and plasmid curing	162
5.2.5	Plasmid purification	163
5.2.6	Detection and visualization of plasmid	164
5.2.7	Antibiotics susceptibility test after plasmid curing	164
5.3	Results	164
5.3.1	Antibiotics susceptibility profiling of <i>Vibrio</i> spp. isolated from cultured shrimp	164

5.3.2	Multiple antibiotic resistance (MAR) index of <i>Vibrio</i> spp.	169
5.3.3	Analysis of plasmid profiling	170
5.3.4	Analysis of plasmid curing	185
5.4	Discussion	190
5.5	Conclusion	195
6	SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	196
6.1	Summary	196
6.2	Conclusion	199
6.3	Recommendations for future research	200
	REFERENCES	202
	APPENDICES	231
	BIODATA OF STUDENT	259
	LIST OF PUBLICATIONS	260

LIST OF TABLES

Table	Page
2.1 Major viral, bacterial, parasite and fungi diseases in shrimp aquaculture.	17
2.2 Typical morphology of <i>Vibrio</i> colonies on TCBS agar.	28
2.3 The mechanisms involved in the mode of action in antibiotics.	45
3.1 Shrimp sampling data from seven shrimp farms in different geographical regions of Peninsular Malaysia.	63
3.2 Classification of shrimps' length, weight and types according to sampling sites.	71
3.3 The percentage of healthy and unhealthy shrimp from seven farms in Peninsular Malaysia.	72
3.4 Total number of <i>Vibrio</i> spp. from the cultured shrimp based on the colour of the colony on TCBS agar.	75
3.5 Biochemical properties of <i>Vibrio</i> spp. isolated from cultured shrimp in Peninsular Malaysia.	78
3.6 Distribution of <i>Vibrio</i> spp. isolated from cultured shrimp from seven sampling sites in Peninsular Malaysia.	98
4.1 Summary of the functions each of the virulence factors.	109
4.2 List of primer used in this study.	110
4.3 The experimental setup for challenge experiment.	115
4.4 List of virulence genes possessed by each of the <i>Vibrio</i> spp.	121
4.5 The frequency of virulence genes presents in each <i>Vibrio</i> spp.	137
4.6 Percentage of mortality observed from the pathogenicity test after 168 hours.	140
5.1 Guidelines chart of zone of inhibition based on Clinical Laboratory Standards Institute (version M45-A2).	161
5.2 The percentage of antibiotics resistance profiles of different types of <i>Vibrio</i> spp. towards the antibiotics tested.	168

5.3	The MAR index value and the percentage of the <i>Vibrio</i> spp.	170
5.4	Antibiograms and the presence of plasmid of different <i>Vibrio</i> spp.	171
5.5	Plasmid profiles of the resistance <i>Vibrio</i> spp. (n = 125) in this study.	185



LIST OF FIGURES

Figure		Page
1.1	Research framework of this study based on the objectives throughout Chapter 3, 4 and 5	6
2.1	Map of main shrimp producer.	9
2.2	Shrimp aquaculture production in major farming nations in Asia.	10
2.3	The distribution patterns among leading producers of shrimp production.	12
2.4	Common shrimp cultivated in Malaysia.	13
2.5	Development of shrimp life cycle.	15
2.6	General overview of pathogenesis process of <i>Vibrio</i> spp. in shrimp.	37
2.7	The consumption of antibiotics in aquaculture in 2017 and 2030.	40
2.8	Common class of antibiotics used in aquaculture.	41
2.9	The structure of bacteria cell envelope.	43
2.10	General antimicrobial resistance mechanisms.	46
2.11	Illustration of the major route of antimicrobial resistance (AMR).	50
2.12	The structure of plasmid.	54
3.1	A map of Peninsular Malaysia showing seven different spots that were involved in the sampling of cultured shrimp.	62
3.2	The view of ponds at the selected sampling sites.	64
3.3	The sampling process at the sampling sites.	65
3.4	Gross signs of the shrimp between healthy and unhealthy shrimp.	73
3.5	Colony growth on TCBS agar of presumptive <i>Vibrio</i> spp.	74
3.6	Biochemical tests performed in this study for identification of <i>Vibrio</i> spp.	76
3.7	Gel electrophoresis showing <i>pyrH</i> gene amplification by presumptive <i>Vibrio</i> spp. isolated from cultured shrimp.	92
3.8	Maximum likelihood phylogenetic tree (Kimura-2-parameter model) of concatenated partial <i>pyrH</i> gene sequences from <i>Vibrio</i> spp. isolated from cultured shrimp.	94

3.9	The classification of <i>Vibrio</i> clades isolated based on <i>pyrH</i> gene.	95
3.10	The distribution of <i>Vibrio</i> spp. isolated from cultured shrimp in Peninsular Malaysia.	96
3.11	Overview distribution different types of <i>Vibrio</i> spp. isolated from cultured shrimp in Peninsular Malaysia.	99
4.1	Overview of experimental design for pathogenicity study using <i>P. monodon</i> .	116
4.2	The presence of virulence genes of <i>Vibrio</i> spp.	119
4.3	Risk classification of virulence genes.	134
4.4	Overall distribution pattern of virulence genes in the <i>Vibrio</i> spp.	135
4.5	The distribution of virulence genes of <i>Vibrio</i> spp.	138
4.6	Clinical signs in <i>P. monodon</i> resulted from immersion challenge test of three <i>Vibrio</i> spp.	143
4.7	Transversal view of hepatopancreas sections of shrimp by haematoxylin and eosin (H & E) staining.	146
5.1	Standard disc diffusion method of <i>Vibrio</i> spp. against antibiotics.	164
5.2	The overview of the antibiotic susceptibility profiles of the 225 <i>Vibrio</i> spp. from cultured shrimp in Peninsular Malaysia.	166
5.3	The overview of MAR index percentage of <i>Vibrio</i> spp.	169
5.4	A standard of disc diffusion method was used to measure the antibiotic susceptibility of <i>Vibrio</i> spp. before (left) and after (right) plasmid curing.	186
5.5	Antimicrobial susceptibility profiles of 125 <i>Vibrio</i> spp. against antibiotics tested.	188

LIST OF APPENDICES

Appendix		Page
A	Letter of approval by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia.	231
B	List of media used.	232
C	Protocols involved in this study.	236
D	Gene ruler.	242
E	Information of <i>Vibrio</i> spp. found in this study.	243
F	The water parameter from each sampling sites.	255
G	Preparation for pathogenicity test.	256
H	Probit analysis of all isolates.	257

LIST OF ABBREVIATIONS

α	alpha
β	beta
$^{\circ}\text{C}$	degree celsius
%	percentage
μL	microliter
μm	micrometre
μM	micromolar
AMP	ampicillin
AMR	antimicrobial resistance
AO	acridine orange
ARG	antimicrobial resistance genes
BLASTn	nucleotide basic local alignment search tool
bp	base pair
C	chloramphenicol
CaCl_2	calcium chloride
CAZ	ceftazidime
CFU/mL	colony forming units per millilitre
<i>chiA</i>	chitinase a
CIP	ciprofloxacin
CN	gentamicin
CTX	cefotaxime
<i>ctxA</i>	cholera toxin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DO	dissolved oxygen
DOF	Department of Fisheries
E	erythromycin
EB	ethidium bromide
F	nitrofurantoin
FAO	Food and Agriculture Organization of the United Nations

FDA	Food and Drug Administration
FEP	cefepime
<i>fla</i>	flagella
g	gram
g/L	gram per litre
H&E	haematoxylin and eosin
HGT	horizontal gene transfer
<i>hly</i>	hemolysin
IACUC	Institutional Animal Care and Use Committee
K	kanamycin
kb	kilobase
KF	cephalothin
kg	kilogram
L	litre
LC ₅₀	lethal concentration of 50
LDC	lysine decarboxylase
LPS	lipopolysaccharide
<i>luxR</i>	transcriptional activator protein
M	molar
MAR	multiple antibiotic resistance
mg	milligram
mL	millilitre
MgCl ₂	magnesium chloride
MHA	Muller-Hinton agar
ML	Maximum Likelihood
mM	millimolar
MT	metric tonnes
Na ₂ HPO ₄	sodium hydrogen phosphate
NaCl	sodium chloride
NaH ₂ PO ₄	sodium dihydrogen phosphate
NOR	norfloxacin
OMP	outer membrane protein
ONPG	ortho-nitrophenyl-β-galactoside

P	penicillin G
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
<i>pirA/pirB</i>	photorhabdus insect-related toxin
<i>pyrH</i>	uridylylate kinase
ppt	part per thousand
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
spp.	species
SXT	sulfomethiazole-trimethoprim
TAE	tris-acetic EDTA
TCBS	thiosulfate citrate bile salts sucrose
<i>tdh</i>	thermostable direct hemolysin
TET	tetracycline
<i>tlh</i>	thermolabile hemolysin
<i>toxR</i>	transcriptional regulatory protein
<i>trh</i>	thermostable direct hemolysin
tRNA	transfer ribonucleic acid
TSA	tryptic soy agar
TSB	tryptic soy broth
TSI	triple sugar iron
U/mL	units per millilitre
UV	ultra violet
V	voltan
v/v	volume per volume
VA	vancomycin

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Aquaculture is one of the fastest growing industries in the world. According to FAO (2022), marine shrimp was among the highest demand in aquaculture, approximately USD 34.2 billion accounting for 5.51 metric tonnes (MT) followed by freshwater crustaceans at USD 24.3 billion accounting for 2.53 MT. However, the shrimp industry faces numerous challenges such as disease outbreak caused by bacterial, viruses and fungal diseases in the aquaculture system (El- Saadony et al., 2022).

Vibriosis caused by *Vibrio* spp. has gained attention as a causative agent of diseases in aquatic animals, and it is recognised as significant contributor to the diseases with huge economic impacts on global aquaculture industry including shrimp aquaculture industry (Hossain et al., 2024). This *Vibrio*-related disease causes significant losses in yield and causing substantial economic damage every year (Li et al., 2020). In order to solve the problem, antibiotics are commonly used to promote growth, prevent and treat bacterial infections. However, the widespread and inappropriate usage of antibiotics are leading for emergence and spread of antimicrobial resistance (AMR). This alarming issue threatens public health by diminishing the effectiveness of antibiotics in treating diseases (Tang et al., 2023).

In addition, the presence or absence of virulence factors can influence how severe an infection is and how easily it can be treated (Li et al., 2019). There

is some evidence to suggest that certain mechanisms of AMR can also contribute to the virulence. This means that when the bacteria become resistant to antibiotics, it may also increase virulency (De Nies et al., 2021). Some bacteria may acquire resistance genes on mobile genetic elements such as plasmid, that also carry genes involved in virulence (Dionisio et al., 2019). The plasmid plays a crucial role in facilitating the transfer of antibiotic-resistant genes, which can be transmitted to the next generation via vertical gene transfer or exchanged with other bacteria via horizontal gene transfer (Liu et al., 2022).

Overall, the surveillance of antimicrobial resistance and monitoring of antibiotic use in aquaculture should be encouraged to improve antibiotics management. In addition, aquaculture producers must adhere to the regulations and guidelines set by their local governments to minimize the risk of antibiotic resistance. Furthermore, it is also important to develop alternative methods for preventing and controlling disease to eliminate shrimp health risks.

1.2 Problem statements

Although there were several studies related to distribution, virulence and antimicrobial resistance on *Vibrio* spp. isolated from shrimp across the world, however in Malaysia, there were limited information regarding on these studies. In fact, if it covered *Vibrio* spp., it was just one or two species, and some of the studies only covered a limited number of geographical areas.

Therefore, the epidemiology of vibriosis in shrimp Malaysia's aquaculture remains poorly understood.

According to Lulijwa et al. (2020), there is a lack of data and information on antibiotic use in Malaysia. This causes difficulty in making an accurate comparison with other studies. Moreover, the research revealed that only 5.9% studies on antibiotic resistance in aquaculture and associated environments have been performed. The species involved were catfish (*Clarias gariepinus*), tilapia (*Tilapia mossambica*), banana prawn (*Penaeus indicus*), red prawn (*Solenocera subnuda*), groupers (*Epinephelus* spp.), Asian seabass (*Lates calcarifer*) and red snapper (*Lutjanus* sp.) (Budiati et al., 2013; Kathleen et al. 2016; Laith et al. 2017; Letchumanan et al. 2019; Amalina et al., 2019; Mohamad et al., 2019). With increasing concern over AMR, a threat to both aquaculture industries and public health, it is critical to monitor and prevent AMR among *Vibrio* species.

In addition, the virulence factors associated with *Vibrio* spp. pathogenicity in Malaysian cultured shrimp are yet unknown. As a result, some knowledge on the distribution and expression of virulence genes in *Vibrio* spp. might provide essential insights into environmentally friendly techniques to treating *Vibrio* infection in shrimp farming. Thus, a better understanding of vibriosis epidemiology will allow for the development of more effective curative and preventative methods to fight the disease.

1.3 Significance of study

In Malaysia, there is currently limited documentation regarding the presence of *Vibrio* spp. in cultured shrimp. Therefore, by investigating the distribution, virulence factors and antimicrobial resistance of *Vibrio* spp. in cultured shrimp, the valuable information for shrimp farmers, aquaculture researchers, and policymakers will be provided. In addition, the knowledge of the prevalence and distribution of diseases may assist farmers, researchers, and policymakers in the development of control measures for diseases in shrimp farming.

Moreover, the information is useful for regulations to control practices on the farms and minimize the magnitude of the antibiotic resistance among *Vibrio* spp. For this reason, information regarding on distribution of *Vibrio* spp. in the farms around Peninsular Malaysia particularly was important in order to provide greater knowledge of the epidemiology of *Vibrio* infection throughout a geographical region. The findings can contribute to the development of effective management strategies to mitigate the risk of disease outbreaks and promote sustainable shrimp farming practices in Peninsular Malaysia.

1.4 Objectives of the study

The research framework of the study was summarized in Figure 1.1. The study was carried out with the following objectives:

1. To isolate and characterize of *Vibrio* spp. from cultured shrimp collected from various geographical region in Peninsular Malaysia.

2. To assess virulence genes profiles of *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia.
3. To evaluate the most virulent strain of isolated *Vibrio* spp. by experimental infection study in shrimp.
4. To determine the distribution and patterns of antimicrobial resistance in *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia and their ability to transfer the resistance plasmid.



Distribution, virulence factors and antimicrobial resistance of <i>Vibrio</i> species isolated from cultured shrimp in Peninsular Malaysia				
	Experimental Chapter 3	Experimental Chapter 4	Experimental Chapter 5	
Objective	To isolate and characterize of <i>Vibrio</i> spp. from cultured shrimp collected from various geographical region in Peninsular Malaysia	To assess virulence genes profiles of <i>Vibrio</i> spp. isolated from cultured shrimp in Peninsular Malaysia and evaluate the most virulent strain of isolated <i>Vibrio</i> spp. by experimental infection study in shrimp.	To determine the distribution and patterns of antimicrobial resistance in <i>Vibrio</i> spp. isolated from cultured shrimp in Peninsular Malaysia and their ability to transfer the resistance plasmid.	
Experiment	- Shrimp sampling from seven selected farms in Peninsular Malaysia - Biochemical test and Gram staining - Molecular identification using <i>pyrH</i> gene.	- Virulence-related gene profiling analysis - Challenge experiment - Histopathology analysis	- Antibiotic profiling - Plasmid profiling and curing	
Output	Distribution of <i>Vibrio</i> spp. in selected shrimp farms in Malaysia were identified.	The multiple of virulence genes, percentage of mortality and histology changes of <i>Vibrio</i> spp. were determined.	Antibiotics and plasmid profiling were determined.	
Outcome	The data provided will help to enhance the understanding of bacterial pathogens' dynamic in shrimp farming and develop target approaches to mitigate the risk of disease outbreak in shrimp farming.			

Figure 1.1: Research framework of this study based on the objectives throughout Chapter 3, 4 and 5. The other chapters included in this study were Chapter 1, Chapter 2 and Chapter 6.

1.5 Hypotheses of the study

The hypotheses of this study are as below:

Hypothesis 1:

H₀: The *Vibrio* spp. was not present in the cultured shrimp from the selected farms in Peninsular Malaysia.

H₁: The *Vibrio* spp. was present in the cultured shrimp from the selected farms in Peninsular Malaysia.

Hypothesis 2:

H₀: There are no difference in the presence of virulence gene profiling of *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia.

H₁: There are difference in the presence of virulence gene profiling of *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia.

Hypothesis 3:

H₀: There are no difference in the pathogenicity of selected *Vibrio* spp. as determined by the experimental infection study.

H₁: There are difference in the pathogenicity of selected *Vibrio* spp. as determined by the experimental infection study.

Hypothesis 4:

H₀: There are no difference in the presence of antibiotic resistance and plasmid profiling of *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia.

H₁: There are difference in the presence of antibiotic resistance and plasmid profiling of *Vibrio* spp. isolated from cultured shrimp in Peninsular Malaysia.

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