UNIVERSITI PUTRA MALAYSIA

PATHOGENESIS OF ACUTE HEPATOPANCREATIC NECROSIS DISEASE CAUSED BY VIBRIO SPP. IN WHITELEG SHRIMP PENAEUS VANNAMEI (BOONE, 1931)

SARMILA MUTHUKRISHNAN

FP 2021 3

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By

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

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July 2020

Chairman : Associate Professor Natrah Fatin Mohd Ikhsan, PhD
Faculty : Agriculture

Acute hepatopancreatic necrosis disease (AHPND) first emerged as a new shrimp disease in 2009 and has heavily affected the shrimp industry leading to global economic losses. The aetiological agent was previously identified as *Vibrio parahaemolyticus* that carries a pVA1-type plasmid carrying *pirAB*^{vp} toxins. However, previous research revealed that *V. parahaemolyticus* is not the only bacterial species capable of causing AHPND. Hence, the objectives of this study is (1) to isolate, screen, characterise, and identify the AHPND positive bacteria from the whiteleg shrimp *Penaeus vannamei* in Malaysia, (2) to screen and elucidate the involvement of quorum sensing (QS) in AHPND positive bacteria in *in vitro* and (3) *in vivo* and finally, (4) to assess the horizontal gene transfer of AHPND causing genes from AHPND positive bacterium to a non-AHPND causing bacterium.

Sampling of whiteleg shrimp was carried out at iSharp, Terengganu shrimp farm, which located at the east coast of Peninsular Malaysia, to isolate the AHPND causing bacteria. Preliminary pathogenicity study was conducted using brine shrimp, *Artemia franciscana* as a model organism. The most pathogenic bacteria from the brine shrimp challenge test was employed in the whiteleg shrimp challenge test. Histopathology analysis was performed to further study the clinical signs and AHPND's pathology. The AHPND positive isolates were identified using multilocus sequencing analysis (MLSA) and biochemical tests. Three types of quorum sensing (QS) signal molecules, namely, N-acyl-homoserine lactone (AHL), Autoinducer-2 (AI-2), and Cholerae autoinducer-1-like (CAI-1) molecules were then screened in the AHPND positive isolates using *Agrobacterium tumefaciens* KYC55, *Vibrio campbellii* JMH597, and *V. campbellii* JAF375 biosensors respectively. Molecular screening of the QS-related genes, *luxR* and *luxS* was conducted to justify the *in vitro* activity of QS in AHPND positive isolates. The *in vivo* gene expression study of *pirA, pirB, toxR,* and *luxR* genes in whiteleg shrimp using quantitative PCR (qPCR) was conducted to study the expression patterns during

the infection. A superoxide dismutase (SOD) was performed to study the oxidative state during the infection. Finally, a horizontal gene transfer (HGT) study was demonstrated by co-culturing the AHPND positive bacteria and the non-AHPND bacteria to evaluate the conjugation efficiency rate (n°).

Out of the 86 isolates, 12 isolates were screened positive for AHPND using conventional polymerase chain reaction (PCR) method. All the 12 AHPND positive isolates with *pirA* and *pirB* genes demonstrated significant ($P < 0.05$) mortalities (23-97%) of brine shrimp compared to the negative control (2%). Based on 16S rRNA, *RctB*, and *RpoD* sequencing analysis, the 12 isolates belong to the *Harveyi* clade and identified as *V. parahaemolyticus* (7 isolates) and *Vibrio harveyi* (5 isolates). Further test showed that the yellow colony *V. harveyi* BpShHep24 (100% mortalities in 48 h) was found to be more virulent than the green colony *V. parahaemolyticus* BpShHep31 (50% mortalities in 48 h) in the whiteleg shrimp challenge test. The histopathology analysis of the challenged shrimp demonstrated terminal stage characteristics of AHPND pathology.

All the three types of QS signal molecules were detected in AHPND positive *V. parahaemolyticus* and *V. harveyi*. The *luxR* and *luxS* gene screening was positive for all the 12 AHPND positive isolates. The formation of biofilm increased with the increase in AHL concentration (1-100 nmol L^{-1}) in 11 AHPND positive isolates and demonstrated that the QS signal molecules control the formation of biofilm in AHPND positive *Vibrio* isolates.

The expression of AHPND virulence factors, quorum sensing regulator *luxR*, and virulence regulator *toxR* in whiteleg shrimp challenged with *V. parahaemolyticus* BpShHep31 and *V. harveyi* BpShHep24 demonstrated a significant ($P < 0.05$) increase of the quorum sensing master regulator *luxR* when compared with the control shrimp (unchallenged group). There was also a substantial difference in *pirA*, *pirB*, and *toxR* expressions in the challenged shrimp compared to the unchallenged shrimp. However, shrimp challenged with *V. harveyi* BpShHep24 demonstrated 8.7-, 17.4-, 13.3-, and 21.8- fold higher expression of *pirA*, *pirB*, *toxR*, and *luxR* respectively when compared to shrimp challenged with *V. parahaemolyticus* BpShHep31.

The superoxide dismutase (SOD) study in the challenged shrimp has shown an expression peak of oxidative stress at 24 h post challenged and followed by a reduced expression level. Furthermore, the protein content in the challenged group decreases, suggesting poor growth performance due to the stress induced by the pathogens. In general, the *in vivo* gene expression study, SOD study, and protein content analyses demonstrated a clear difference between the challenged (with AHPND positive isolates) and unchallenged shrimp.

The present study also demonstrated occurrence of HGT from AHPND positive *V. parahaemolyticus* to a non-AHPND and non-*Vibrio* species identified as *Algoriphagus* sp. strain NBP. The HGT of *pirA* and *pirB* genes from the AHPND positive *V. parahaemolyticus* to *Algoriphagus* sp. strain NBP was found to occur at three different

temperatures (20°C, 30°C, and 40°C). The conjugation efficiency rate (n°) of *pirAB* from *V. parahaemolyticus* to *Algoriphagus* sp. strain NBP at 30°C and 40°C showed 80% to 91% efficiency. Shrimp challenged with the *pirA* and *pirB* positive *Algoriphagus* sp. strain NBP also demonstrated typical pathognomonic AHPND lesions during the histopathologic examination.

In conclusion, this study documented the types of *Vibrio* spp. involved in AHPND outbreak in Malaysia. All the 12 AHPND positive isolates were positive for QS screening and positively correlates with AHPND virulence genes (*pirA* and *pirB*). Virulence factors produced by the pathogen play a vital role in spreading the infection in aquaculture farms. Therefore, the foremost aspect is to understand the virulence mechanisms involved during the infection which may aid in developing proper mitigation and sustainable method to control the disease outbreak in aquaculture farms.

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

KAJIAN PATOGENIK PENYAKIT AKUT NEKROSIS HEPATOPANKREAS YANG DISEBABKAN OLEH *Vibrio* spp. DALAM UDANG KAKI PUTIH *Penaeus vannamei* (BOONE, 1931)

Oleh

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Julai 2020

Pengerusi : Profesor Madya Natrah Fatin Mohd Ikhsan, PhD
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Penyakit nekrosis akut hepatopankreas (AHPND) mula muncul sebagai penyakit udang yang baharu pada tahun 2009 dan telah menjejaskan industri udang dengan teruk yang mengakibatkan kerugian ekonomi global. Agen penyebab sebelum ini dikenal pasti sebagai *Vibrio parahaemolyticus* yang membawa plasmid jenis pVA1 yang membawa toksin *pirAB*^{vp}. Walau bagaimanapun, kajian dahulu mendedahkan bahawa bukan *V. parahaemolyticus* sahaja spesis bakteria yang berkeupayaan menyebabkan AHPND. Maka, objektif kajian ini adalah (1) untuk mengasing, menyaring, menciri, dan mengenal pasti bakteria positif AHPND dalam udang putih, *Penaeus vannamei*, di Malaysia, (2) untuk menyaring dan menjelaskan penglibatan pengesan kuorum (QS) dalam bakteria positif AHPND secara *in vitro* dan (3) *in vivo* dan akhirnya, (4) untuk menilai pemindahan gen melintang AHPND daripada bakteria positif AHPND kepada bakteria bukan penyebab AHPND (objektif 4).

Pensampelan udang putih dijalankan di ladang udang iSharp, Terengganu yang terletak di Pantai Timur Semenanjung Malaysia, untuk mengasingkan bakteria penyebab AHPND. Kajian patogenik awal dijalankan dengan menggunakan udang air garam, *Artemia franciscana* sebagai organisma model. Bakteria yang paling patogenik daripada ujian cabaran udang air garam digunakan untuk ujian cabaran udang putih. Analisis hispatologi dilaksanakan untuk mengkaji dengan lebih lanjut tanda-tanda klinikal dan patologi AHPND. Asingan positif AHPND dikenal pasti dengan menggunakan analisis penjujukan multilokus (MLSA) dan ujian biokimia. Tiga jenis molekul isyarat kuorum pengesan (QS) yang dinamakan sebagai molekul N-acyl-homoserine lactone (AHL), Autoinducer-2 (AI-2), dan Cholerae autoinducer-1-like (CAI-1) ditapis dalam asingan positif AHPND dengan masing-masing menggunakan biosensor *Agrobacterium tumefaciens* KYC55, *Vibrio campbellii* JMH597, dan *V. campbellii* JAF375. Penapisan molekul gen yang berkaitan dengan QS, *luxR* dan *luxS* dijalankan untuk menjustifikasi aktiviti *in vitro* QS dalam asingan positif AHPND. Kajian ungkapan gen *in vivo* kepada

gen *pirA*, *pirB*, *toxR*, dan *luxR* dalam udang putih yang menggunakan PCR (qPCR) kunatitatif dijalankan untuk mengkaji corak ungkapan sewaktu jangkitan. Superoksida dismutase (SOD) digunakan untuk mengkaji keadaan pengoksidaan sewaktu jangkitan. Akhirnya, kajian pemindahan gen melintang dijalankan dengan mengkulturkan bakteria positif AHPND dan bakteria bukan AHPND untuk menilai kadar kecekapan konjugasi (n°).

Daripada 86 asingan, 12 asingan yang ditapis didapati positif AHPND. Kesemua 12 asingan positif AHPND dengan gen *pirA* dan *pirB* menunjukkan kematian signifikan ($P < 0.05$) udang air garam berbanding kawalan negatif. Berdasarkan analisis penjujukan 16S rRNA, *RctB*, dan *RpoD*, 12 asingan dimiliki oleh klad *Harveyi* dan dikenal pasti sebagai *V. parahaemolyticus* (7 asingan) dan *Vibrio harveyi* (5 asingan). Kajian lanjut menunjukkan koloni kuning *V. harveyi* BpShHep24 didapati lebih virulen berbanding koloni hijau *V. parahaemolyticus* BpShHep31 dalam ujian cabaran udang putih. Analisis hispatologi udang yang dicabar menunjukkan ciri-ciri peringkat terminal patologi AHPND.

Kesemua tiga jenis molekul isyarat QS dikesan dalam AHPND positif *V. parahaemolyticus* dan *V. harveyi*. Penapisan gen *luxR* and *luxS* didapati positif untuk kesemua 12 asingan positif. Pembentukan biofilem meningkat dengan peningkatan kepekatan AHL (1-100 nmol L^{-1}) dalam 11 asingan positif AHPND dan ini menunjukkan bahawa molekul isyarat QS mengawal pembentukan biofilem dalam asingan *Vibrio* positif AHPND.

Ungkapan faktor virulen AHPND, pengawal selia pengesan kuorum *luxR*, dan pengawal selia virulen *toxR* dalam udang putih yang dicabar dengan *V. parahaemolyticus* BpShHep31 dan *V. harveyi* BpShHep24 menunjukkan peningkatan signifikan ($P < 0.05$) pengawal selia induk pengesan kuorum *luxR* apabila dibandingkan dengan udang kawalan (kumpulan tidak dicabar). Terdapat juga perbezaan yang besar dalam ungkapan *pirA*, *pirB*, dan *toxR* dalam udang yang dicabar berbanding udang yang tidak dicabar. Walau bagaimanapun, udang yang dicabar dengan *V. harveyi* BpShHep24 menunjukkan masing-masing mempunyai ungkapan 8.7-, 17.4-, 13.3-, dan 21.8- kali ganda lebih tinggi *pirA*, *pirB*, *toxR*, dan *luxR* apabila dibandingkan dengan udang yang dicabar dengan *V. parahaemolyticus* BpShHep31.

Kajian superoksida dismutase (SOD) dalam udang yang dicabar menunjukkan kemuncak ungkapan tekanan oksidatif pada 24 j pasca cabaran dan diikuti dengan penurunan tahap ungkapan. Tambahan pula, kandungan protien dalam kumpulan yang dicabar menurun, menunjukkan prestasi pertumbuhan yang buruk akibat tekanan yang didorong oleh patogen. Secara amnya, kajian ungkapan gen *in vivo*, kajian SOD, dan analisis kandungan protein menunjukkan perbezaan yang jelas antara udang yang dicabar (dengan asingan positif AHPND) dan tidak dicabar.

Kajian ini juga menunjukkan kejadian HGT daripada *V. parahaemolyticus* positif AHPND kepada spesis bukan AHPND dan bukan *Vibrio* yang dikenal pasti sebagai strain *Algoriphagus* sp. NBP. HGT gen *pirA* dan *pirB* daripada *V. parahaemolyticus* positif AHPND kepada strain *Algoriphagus* sp. NBP didapati berada pada tiga suhu yang berbeza (20°C, 30°C, dan 40°C). Kadar kecekapan konjugasi (n°) *pirAB* daripada *V. parahaemolyticus* kepada strain *Algoriphagus* sp. NBP pada 30°C dan 40°C menunjukkan 80% hingga 91% kecekapan. Udang yang dicabar dengan *pirA* dan *pirB* positif strain *Algoriphagus* sp. NBP juga menunjukkan luka AHPND patagnomonik biasa sewaktu pemeriksaan hispatologik.

Kesimpulannya, kajian ini mendokumentasi jenis *Vibrio* spp. yang terlibat dalam wabak AHPND di Malaysia. Kesemua 12 asingan positif AHPND didapati positif untuk penapisan QS dan berkolerasi secara positif dengan gen virulen AHPND (*pirA* dan *pirB*). Faktor virulen yang dihasilkan patogen memainkan peranan penting dalam penyebaran jangkitan di ladang akuakultur. Maka, aspek yang paling utama ialah memahami mekanisma virulen yang terlibat sewaktu jangkitan yang mungkin boleh membantu dalam menghasilkan kaedah pengurangan yang sesuai dan lestari untuk mengawal wabak penyakit di ladang akuakultur.

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This thesis was submitted to the Senate of the 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 were as follows:

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%	percent
°C	degree Celcius
AHL	N-acyl Homoserine Lactones
AI-2	Autoinducer-2
AHPND	Acute Hepatopancreatic Necrosis Disease
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	carbon
CAI-1	Cholerae autoinducer-1
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
DMSO	dimethyl sulfoxide
DNA	dioxyribonucleic acid
DOM	dissolved organic matter
EHP	Enterocytozoon hepatopenaei
ELISA	enzyme-linked immunosorbent assay
EMS	Early mortality syndrome
H	hour
HDPE	high density polyethylene
HGT	horizontal gene transfer
HP	hepatopancreas
hpi	hour post-infection
HSL	homoserine lactone
IM	intramuscular injection
kDA	kilo Dalton
L	litre

LB	Luria-Bertani medium
LAMP	Loop-Mediated Isothermal Amplification
MA	marine agar
MB	marine broth
MCC	Maximum Clade Credibility
MGE	mobile genetic element
mg	milligrams
min	minute/s
MLSA	Multi Locus Sequence Analysis
mRNA	messenger RNA
MT	metric tonnes
NCBI	National Centre for Biotechnology Information
PCR	polymerase chain reaction
Pir	Photorhabdus insect-related
PL	post-larvae
PP	posterior probability
QQ	quorum quenching
QS	quorum sensing
QSI	quorum sensing inhibitor
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
Rpm	revolutions per minute
RPS	relative percentage of survival
SEM	scanning electron microscope
spp.	species
SPF	specific pathogen free
SOD	superoxide dismutase
TAAPs	transboundary aquatic animal pathogens
TAADs	transboundary aquatic animal diseases

TCBS	Thiosulphate-Citrate-Bile-Salts-Sucrose
TSB	tryptic soy broth
TSV	Taura syndrome virus
V	voltan/volt
w/v	weight per volume
WSSV	white spot syndrome virus
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microliter
ng	nanogram

CHAPTER 1

INTRODUCTION

1.1 Background

Increasing number of people globally increases the rate of expansion and intensification of food production. Seafood demand increases in the recent years as more people learn that seafood is an essential source of protein. Seafood consumption rose from 9 kg (live weight) in 1961 to 20.3 kg in 2017 in terms of per capita (FAO, 2020). Thus, the aquaculture sector plays a vital role in meeting the demand for seafood and making it cheaper and more accessible. Moreover, one of the goals by United Nations' 2030 Agenda for Sustainable Development and its 17 Sustainable Development Goals (SDGs) is to support developing nations to increase sustainable aquaculture production for better development of economic, social and environment (FAO, 2018).

One of the dominant species produced in world aquaculture production is whiteleg shrimp, *Penaeus vannamei*. About 53% of overall shrimp aquaculture production depends on whiteleg shrimp (FAO, 2018) (Figure 1.1). This species is also one of the target species in the Malaysian agriculture and listed under the National Key Economic Areas. The Malaysia Economic Transformation Programme (2010-2020) has framed initiative to increase shrimp production and productivity. However, for the past decade, whiteleg shrimp and other shrimp species farms, particularly in Asia, Mexico, South America and USA, suffered from emergent shrimp disease known as Acute Hepatopancreatic Necrosis Disease (AHPND) (FAO, 2014; Nunan et al., 2014).

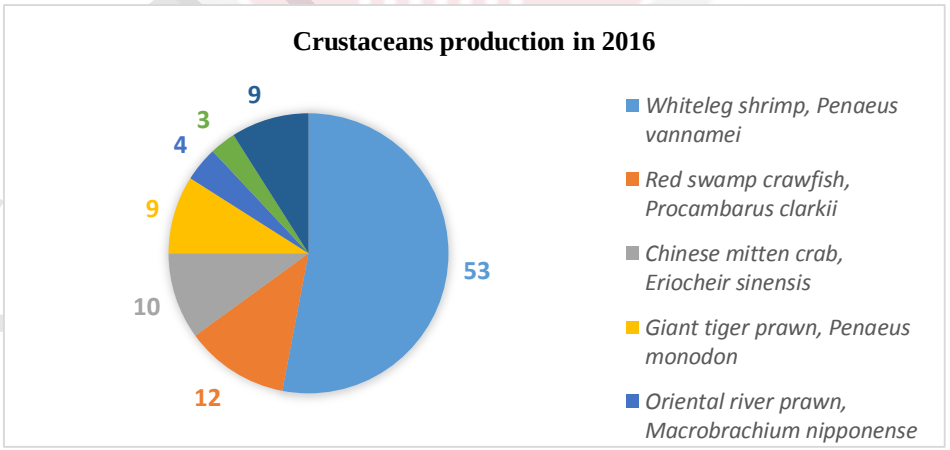

Figure 1.1 : Crustaceans production based on species in percentage (%) for the year of 2016
(Source: FAO, 2018)

This disease has first emerged in China in the year 2009 (FAO, 2013), and then spread to Vietnam in year 2010 (Tran et al., 2013), Malaysia in year 2011 (Kua et al., 2016), Thailand in year 2012 (Lightner et al., 2012), Mexico in year 2013 (Nunan et al., 2014), Philippines in year 2015 (Dabu et al., 2015), South America in year 2016 (Restrepo et al., 2016) and Bangladesh in year 2016 (Ahmmed et al., 2019) (Figure 1.2). Mortalities are often observed within 30 days of stocking shrimp ponds with postlarvae (PL) (Leano and Mohan, 2012).

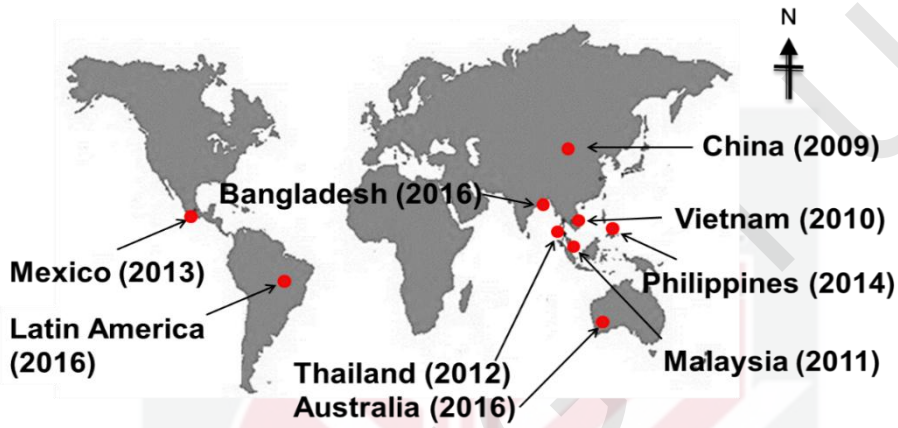

Figure 1.2 : Geographical distribution of AHPND

In Malaysia, AHPND was first detected in the east coast states of Pahang and Johor, Peninsular Malaysia. In the year of 2010, total production of cultured shrimp was 87,000 metric tonnes (MT) and 90% from the total production was contributed by whiteleg shrimp in Malaysia (Kua et al., 2016). Decreased in shrimp production from 70 000 MT in 2010 to 40 000 MT in the year of 2011 was observed (Leano and Mohan, 2012). AHPND is currently among the most threatening non-viral disease in shrimp culture system other than the *Enterocytozoon hepatopenaei* (EHP) (Thitamadee et al., 2016).

Initially, only *Vibrio parahaemolyticus* was reported to be the causative agent of this disease (Tran et al., 2013). However, recent studies reported that other *Vibrio* spp. also causes AHPND. For instance, *V. campbellii* KC13.17.5 from Vietnam (Kondo et al., 2015), *V. owensii* SH-14 from China (Xiao et al., 2017), *V. campbellii* 20130629003S01 from China (Dong et al., 2017a) and *V. punensis* from South American (Restrepo et al., 2018) possess the *pirAB* genes (AHPND toxin genes) which demonstrated similar pathology and clinical signs of AHPND in infected shrimp.

Vibrionaceae are ubiquitous in the marine environment and some of them are capable of causing disease to aquatic animals (Austin and Austin, 2007). Some species in the family of Vibrionaceae are reported to coordinates the expression of certain genes using quorum sensing communication circuits (Milton, 2006). Quorum sensing (QS) is cell-to-cell

communication by using small, secreted signalling molecules known as autoinducers (AIs) (LaSarre et al., 2013). However, to date, there is no detailed study on the relationship between QS and AHPND toxins. Moreover, only countable research and studies were reported on the mechanisms of horizontal gene transfer (HGT) of the AHPND toxin genes (*pirAB*) from one species to another species. It is pivotal to understand the mechanisms and role of the AHPND, causing bacteria to develop a novel or effective biocontrol solution.

1.2 Problem statements

In aquaculture, emerging diseases contributes to severe economic loses annually. Approximately USD 6 billion annual loss of revenues recorded due to diseases in aquaculture (Assefa and Abunna, 2018). Shrimp aquaculture is one of the main sectors of food produced by the world aquaculture. Asia is the top producer of shrimp in the world by producing 705 5000 tonnes out of the total 786 2000 tonnes produced, while about 53% of the total production relies on whiteleg shrimp (FAO, 2018).

However, disease outbreak remains to be the major constraint to the shrimp aquaculture. AHPND causes close to USD 23 billion loss in Asia shrimp aquaculture (Shinn et al., 2018). Even though AHPND has been detected in Malaysia in the year of 2011, there is no intensive studies and proper database on this disease outbreak recorded from the local farms. Furthermore, not many studies were published by Malaysian researchers regarding this disease and its prevalence in Malaysia at the moment. Although, AHPND outbreak was first reported in Malaysia in the year of 2011, the first scientific paper was published by Kua et al. (2016) after 5 years. The long retention time taken for the research and development (R&D) sector to deliver appropriate piece of information and solution from the year of disease outbreak first occurred is the prime factors which leads to losses in aquaculture industry.

Apart from that, other *Vibrio* spp. were also reported to cause AHPND outbreak in China (Dong et al., 2017), Vietnam (Kondo et al., 2015) and South America (Restrepo et al., 2018). The conjugative transfer of the pathogenic plasmid pVA-1 carrying the $pirAB^{vp}$ genes from AHPND positive *V. parahaemolyticus* to a non-AHPND *V. campbellii* was demonstrated by Dong et al. (2019). The horizontal transfer efficiency from the AHPND positive bacterium to a non-AHPND *V. campbellii* was at 2.6×10^{-8} transconjugant/recipient. This scenario could have also taken place in our local farms where we could find other *Vibrio* spp. causing AHPND instead of *V. parahaemolyticus* only. However, to date there is no single report documented on other species causing AHPND outbreak in Malaysia.

1.3 Hypotheses

1. Objective 1:
   H_0 : AHPND causing *Vibrio* spp. could not be isolated and characterised from the local shrimp farm in Malaysia.
   $H\alpha$: AHPND causing *Vibrio* spp. could be isolated and characterised from the local shrimp farm in Malaysia.

2. Objective 2:
   H_0 : QS signals are not detected in the AHPND causing *Vibrio* spp. In addition, QS does not control biofilm formation in AHPND causing *Vibrio* spp.
   $H\alpha$: QS signals are detected in the AHPND causing *Vibrio* spp. In addition, QS does control the biofilm formation in AHPND causing *Vibrio* spp.

3. Objective 3:
   H_0: QS signals in the AHPND causing *Vibrio* spp. does not regulate the AHPND virulence genes.
   $H\alpha$:QS signals in the AHPND causing *Vibrio* spp. does regulate the AHPND virulence genes.

4. Objective 4:
   H_0: Horizontal gene transfer (HGT) does not take place from an AHPND positive bacterium to a non-*Vibrio* and non-AHPND bacterium.
   $H\alpha$: Horizontal gene transfer (HGT) does take place from an AHPND positive bacterium to a non-*Vibrio* and non-AHPND bacterium.

1.4 Objectives

Therefore, this dissertation seeks to gain fundamental information on the type of bacterial species that causing AHPND in Malaysian shrimp farms, the mechanisms of pathogenic AHPND positive bacteria by identifying the virulence genes, studying the *in vitro* and *in vivo* QS expression, and investigating the role of horizontal gene transfer (HGT) from AHPND positive bacterium to a non-AHPND bacterium. Thus, the objectives of this research are as follows:

1) To identify and characterise the local *Vibrio* spp. causing AHPND and their toxin productions.
2) To screen for QS signalling molecules in *Vibrio* spp. causing AHPND.
3) To determine the effect of QS on the virulence genes regulation in *Vibrio* spp. causing AHPND when challenged with whiteleg shrimp, *Penaeus vannamei* and
4) To investigate the horizontal gene transfer (HGT) mechanism from AHPND positive bacterium to a non-AHPND bacterium.

The objectives in this study could be outlined as described below:

1. **Objective 1**: This objective determined the presence of AHPND causing *Vibrio* spp. in local shrimp farms. The isolated AHPND causing *Vibrio* spp. were then characterised based on molecular and biochemical techniques. The virulence of AHPND causing *Vibrio* spp. were determined by brine shrimp and whiteleg challenged tests. The virulence of AHPND causing *Vibrio* spp. were evaluated based on mortality rate, clinical signs and histopathological analysis. Data from the objective 1 had been published in Aquaculture Journal, 511: 734227, 2019. https://doi.org/10.1016/j.aquaculture.2019.734227.
2. **Objective 2**: This objective aims at determining the presence of quorum sensing (QS) signals in all the local AHPND causing *Vibrio* spp. in *in vitro* experiment using specific biosensors. The presence of QS master regulatory gene *luxR* and S-ribosylhomocysteinase gene *luxS* were screened using molecular method. The relationship of biofilm in regards to QS was examined using quantitative and qualitative methods.
3. **Objective 3**: *In vivo* expression of AHPND virulence factor (*pirA* and *pirB*) and virulence gene regulator (*luxR* and *toxR*) in the AHPND causing *Vibrio* spp. during the infection was investigated using realtime PCR method. The superoxide dismustase (SOD) and protein content analysis were performed to access the conditions of the whiteleg shrimp during the infection of AHPND causing *Vibrio* spp.. Data from objective 2 and 3 had been submitted to *Journal of Applied Microbiology.*
4. **Objective 4**: The horizontal gene transfer of *pirA* and *pirB* genes from an AHPND positive *V. parahaemolyticus* to non-AHPND and non-*Vibrio* bacterium was studied in this objective. Then, the transconjugants were challenged with whiteleg shrimp to observe the mortality rate, clinical signs and pathology of the challenged shrimp to access the virulence of transconjugants. Data of the objective 4 had been submitted to *Journal of Environmental Microbiology.*

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BIODATA OF STUDENT

CURRICULUM VITAE
Sarmila Muthukrishnan
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Sarmila Muthukrishnan was a Lab Manager in Biovalence since Feb 2014-Feb 2017. She was leading the Shrimp Disease Centre, under the purview of Blue Archipelago. She has experience and vast knowledge in shrimp disease screening (DNA viruses, RNA viruses and bacterial infection), water quality analysis, isolation and identification of the potential probiotics, *in vivo* and in vitro analysis of the potential probiotic, toxicity tests of the pathogen, primer designs for the targeted genes and bioinformatics analysis. She has successfully mentored and managed over 20 interns from various universities. Her key areas of expertise include R&D in aquaculture wastewater treatment and shrimp disease screening. She also has in depth knowledge in scientific reporting, laboratory management and R&D related work.

Prior to Biovalence, she was a Researcher at University of Malaya (UM). She has worked in various aspects of bacterial studies (*Actinomycetes*, *Bacillus*, *Vibrios*, nitrifying bacteria and denitrifying bacteria), fungus and best-fit-design for wastewater treatment bio filter. She has also well exposed to techniques such as mouse bioassays and toxin extraction using chemicals during her bachelor's. She has published scientific papers in the field of aquaculture wastewater treatment.

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