MOLECULAR DETECTION OF PIRA/B GENE FROM *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM SHRIMPS IN KOTA BHARU WET MARKETS

By

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, University Malaysia Kelantan, in partial requirement of the course DVT 55204 - Research Project.

Acute Hepatopancreatic Necrotic Disease (AHPND) is a severe bacterial infection in shrimp aquaculture, caused by pathogenic strains of *Vibrio parahaemolyticus* harboring the plasmid-borne *pirA* and *pirB* toxin genes. While studies on AHPND detection, genetics and outbreaks have been conducted in Asia, data on the prevalence of these pathogenic strains in Malaysian retail markets are limited. This study aimed to detect and characterize *V. parahaemolyticus*, specifically screening for AHPND-causing strains, in white shrimp (*Penaeus vannamei*) from wet markets in Kota Bharu, Kelantan. Thirty fresh shrimp samples were collected randomly from three markets. Hepatopancreas tissue was processed for bacterial culture on Thiosulfate Citrate Bile Salt (TCBS) agar, followed by biochemical confirmation (oxidase, Triple Sugar Iron tests). Presumptive *Vibrio* spp. isolates were subjected to conventional PCR targeting the species-specific *pyrH* and *toxR* genes, with *toxR* amplicons sequenced for confirmation via NCBI BLAST analysis. Confirmed *V. parahaemolyticus* isolates were then screened for the *pirA* and *pirB* genes using multiplex PCR. Three isolates (from Pengkalan Chepa market) were confirmed as *V. parahaemolyticus*. However, none carried the *pirA* or *pirB* virulence genes. Therefore, while the detection of *V. parahaemolyticus* highlights a potential food safety consideration, the absence of the *pirA* and *pirB* genes suggests that the sampled shrimp from these markets did not harbor the specific virulent strain responsible for AHPND outbreaks in aquaculture.

Keywords: Vibrio parahaemolyticus, AHPND, white shrimp, Kelantan, pirA/B genes

ABSTRAK

Bagi kertas penyelidikan yang dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, bagi memenuhi sebahagian keperluan kursus DVT 55204 - Projek Penyelidikan.

Penyakit Nekrosis Hepatopankreas Akut (AHPND) ialah jangkitan bakteria serius dalam akuakultur udang, yang disebabkan oleh strain patogenik *Vibrio parahaemolyticus* yang membawa gen toksin *pirA* dan *pirB* yang terletak pada plasmid. Walaupun kajian mengenai pengesanan, genetik dan wabak AHPND telah dijalankan di Asia, data tentang kelaziman strain patogenik ini di pasaran basah Malaysia adalah terhad. Kajian ini bertujuan untuk mengesan dan mencirikan *V. parahaemolyticus*, khususnya menyaring strain penyebab AHPND, dalam udang putih (*Penaeus vannamei*) dari pasar basah di Kota Bharu, Kelantan. Tiga puluh sampel udang segar telah dikumpulkan secara rawak dari tiga pasar. Tisu hepatopankreas diproses untuk kultur bakteria di atas agar Thiosulfat Sitrat Garam Hempedu (TCBS), diikuti dengan pengesahan biokimia (ujian oksidase dan *Triple Sugar Iron*). Pemencilan *Vibrio* spp. presumptif kemudiannya dikenakan PCR konvensional yang mensasarkan gen spesies spesifik- *pyrH* dan *toxR*, dengan amplikon *toxR* dijujuk untuk pengesahan melalui analisis BLAST, NCBI. Pemencilan *V. parahaemolyticus* yang disahkan kemudiannya disaring untuk gen *pirA* dan *pirB* menggunakan PCR multipleks. Tiga isolat *V. parahaemolyticus* berjaya dipencilkan dari pasar Pengkalan Chepa. Justeru, pengesanan *V. parahaemolyticus* menunjukkan pertimbangan potensi keselamatan makanan, namun ketiadaan gen *pirA* dan *pirB* menunjukkan bahawa udang yang disampel dari pasar basah ini tidak mengandungi strain virulen khusus yang bertanggungjawab terhadap wabak AHPND dalam akuakultur.

Kata kunci: Vibrio parahaemolyticus, AHPND, udang putih, Kelantan, gen pirA/pirB

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AHPND	-	Acute Hepatopancreas Necrotic Disease
EMS	-	Early Mortality Syndrome
FAO	-	Food and Agriculture Organization
Tdh	-	Thermostable direct hemolysin
Trh	-	TDH related hemolysin
Pir	-	*Photorhabdus* insect-related toxin
APW	-	Alkaline Peptone Water
TCBS	-	Thiosulfate Citrate Bile Salts Sucrose Agar
TSA	-	Tryptic Soy Agar
PCR	-	Polymerase Chain Reaction
DNA	-	Doeoxyribonucleic Acid
mRNA	-	Messenger Ribonucleic Acid

CHAPTER 1

1.0 INTRODUCTION

In Malaysia, shrimp production was started in the 1970s due to the increment in demand of shrimp supply (Natrah et al., 2025). *Penaeus vannamei*, the white shrimp, has a high demand locally and it occupies a large portion of Malaysia shrimp production (Harkell, 2018). In fact, it accounts for nearly 90% of farmed shrimps and dominates the local market, compared to the *Penaeus monodon* and the other species (Natrah et al., 2025). Pimentel et al. (2022) state that farming of *P. vannamei* is dispersed widely in different environments due to its significant adaptability and tolerance to broad range of temperature and salinity.

Shrimp production suffers significant losses due to Acute Hepatopancreatic Necrosis Disease (AHPND), formerly termed Early Mortality Syndrome (EMS), which is an infection caused by specific strains of *Vibrio parahaemolyticus* (Padilah et al., 2022). Based on the reports from Dabu et al. (2015) and Food and Agriculture Organization of United Nations (FAO). (2013, 2016), AHPND was first reported in Malaysia in 2011. Beginning in 2011, a significant increase in mortality rates among white shrimp was reported in Malaysian white shrimp farms (Ghee-Thean et al., 2016; Kua et al., 2016). The common clinical signs of AHPND include pale and atrophied hepatopancreas, soft shells, empty stomach, disrupted midgut and abnormal corkscrew swimming behavior in affected shrimps (Padilah et al., 2022).

Vibrio parahaemolyticus, a gram negative, straight or curved rod-shaped, halophilic, naturally occurring aquatic bacteria, that is commonly found in shallow coastal regions and aquatic creatures such as fish and shellfish (Jones, 2014). It is a significant pathogen in aquatic animals and humans. It enters the human body through ingestion of raw or undercooked seafoods and exposure to the open wound. Various illnesses can be caused by *V. parahaemolyticus* infections, for instance gastroenteritis, wound infections and sepsis (Rezny & Evans, 2023). Despite the non-pathogenic strain of *V. parahaemolyticus*, pathogenic AHPND strain contains the *pirA/B* toxin genes that produce the pirAB binary toxic protein which is the causative agent of the AHPND.

The objective of this study is to detect *pirA/B* genes from pathogenic strain of *V. parahaemolyticus* isolated from the shrimps obtained from Kota Bharu wet markets..

1.1 RESEARCH PROBLEM STATEMENT

V. parahaemolyticus is commonly found in shrimps but not all *V. parahaemolyticus* strains are pathogenic causing AHPND. Thus, it is important to detect the virulence factor which is the *pirA* and *pirB* gene. In Malaysia, shrimps, especially white shrimps (*Penaeus vannamei*) are highly consumed, yet there is no research on the *V. parahaemolyticus* isolation and *pirA/B* genes detection from shrimps in local wet markets in Kota Bharu, Kelantan. In this study, we will assess the detection of *pirA/B* genes in Kota Bharu shrimp samples in wet markets.

1.2 RESEARCH QUESTIONS

1) Do *pirA* and *pirB* genes present in *Vibrio parahaemolyticus* isolated from white shrimps in Kota Bharu wet markets?

1.3 RESEARCH HYPOTHESIS

1) *pirA* and *pirB* genes are present in *Vibrio parahaemolyticus* isolated from white shrimps in Kota Bharu wet markets.

1.4 RESEARCH OBJECTIVES

1) To detect the presence of *pirA* and *pirB* gene in *Vibrio parahaemolyticus* isolated from white shrimps in Kota Bharu wet markets.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 White Shrimps (*Penaeus vannamei*) Production

Globally, the Pacific white shrimp (*Penaeus vannamei*) and the black tiger shrimp (*Penaeus monodon*) are the primary shrimp species in shrimp production (Shinn, 2018). White shrimp is popular in shrimp farming because of its favourable traits such as a short cultivation time, fast growth, ability to tolerate high stocking densities, lower dietary protein needs with better utilization of plant proteins in feed, greater adaptability to low salinity and high resistance to viral diseases (Macusi et al., 2022; Liao & Chien, 2011)

2.2 Acute Hepatopancreatic Necrosis Disease (AHPND) in Shrimps

Santos et al. (2019) emphasize *V.parahaemolyticus* is responsible for motility in early stages of life of shrimps by causing acute hepatopancreatic necrosis disease (AHPND). It causes great loss in the growing shrimps within 30 to 35 days of postlarval stocking. Early life stages of shrimps are more susceptible to AHPND (Kumar et al., 2021). Grossly, affected shrimps will show clinical signs such as lethargy, slow growth, empty stomach and midgut as well as pale or white shrinked hepatopancreas and the shrimps will die at the bottom of the pond (Hong et al., 2015). However, gross signs are not reliable to diagnose AHPND. Histological examination is important for diagnosis. Histologically, it damages the hepatopancreas tubule epithelial cells and leads to disorganization and atrophy in hepatopancreas tissue (Soto-Rodriguez et al., 2014). At the initial stage to midstage of this disease, desquamation of the hepatopancreatic tubule epithelial cells will occur without the presence of any detectable causative pathogen (Tran et al., 2013). At the late stage, there are hemocytic aggregation and formation of melanized granulomas as well as a variety of bacteria in tubular lumen due to secondary bacteria infections (Hong et al., 2015).

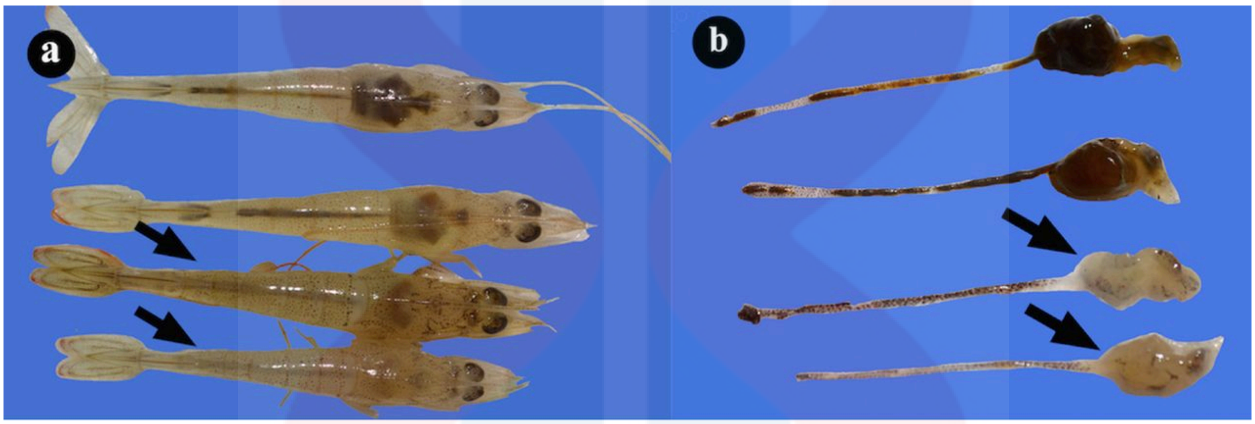

Figure 1. (a) Comparison between healthy shrimps and shrimps with AHPND (arrows). (b) the arrows show abnormal hepatopancreas (Soto-Rodriguez et al., 2014)

2.3 General Background on *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a motile Gram-negative bacteria with single polar flagellum that is resistance to a wide range of salinities (0.5 - 9.5%), pH (7.6 - 9.0) and temperature (7 - 43℃) (Chamberlain, 2013). It causes gastroenteritis in humans via consumption of raw seafood (Newton et al., 2012). Affected humans show signs of diarrhea, fever, headache, nausea, vomiting and abdominal pain (Su & Liu, 2007). Makino et al. (2003) discovered that *V. parahaemolyticus* contains several virulence factors such as adhesins, thermostable direct hemolysin (*tdh*), and TDH related hemolysin (*trh*). Bacteria may adhere to the surface of a host cell using adhesins, allowing them to secrete more toxins. The cytotoxicity and hemolysis capabilities of *tdh* and *trh* are the primary causes of damage to host cells (Broberg et al., 2010)..

2.4 *pirA/B* Toxic Genes in *Vibrio parahaemolyticus*

Kondo et al. (2014) reported that all pathogenic AHPND strains of *V. parahaemolyticus* carry a 70-kbp plasmid (pVA1) encoding genes corresponding to Photorhabdus insect-related (Pir) toxin genes. As the *pirAB* genes are harbored in conjugative plasmid, there is possibility for the genes to be transmitted to other *Vibrio* species horizontally. In fact, *Vibrio harveyi*, *V. owensii, V. campbellii*, and *V. punensis* are found to be possible AHPND-causing agents (Liu et al., 2018, Ahn et al., 2017, Soto-Rodriguez et al., 2022). *pirA* and *pirB* genes are responsible for the production of Pir toxins which act as binary proteins that affect the stomach, hepatopancreas and hemolymph of shrimps (Lai et al., 2015). *PirA* or *PirB* toxin alone only results in minimal histological change of hepatopancreas but combination of both toxins cause typical signs of AHPND which is sloughing of dead epithelial cells into hepatopancreas tubule (Lee et al., 2015). According to Sirikharin et al. (2015), a minimum concentration of 10 μg g−1 of PirAB toxins is able to reveal lesions of AHPND, while concentrations below 5 μg g−1 do not generate disease but can cause atrophy in tubular hepatopancreatic epithelium.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sample Collection and Preparation

Thirty fresh shrimps were purchased from 3 wet markets where ten shrimps were bought Siti Khadijah Market, Pengkalan Chepa Wet market and Pasar Tok Guru respectively. The shrimps were dissected under aseptic conditions using sterile scissors and forceps. All ten hepatopancreas tissues from each market were collected into a sterile incubation tube as the target sample for this study. The hepatopancreas tissues were homogenized in sterile saline solution. For enrichment, 1 ml of homogenized shrimp hepatopancreas tissue suspension were transferred into 9 ml Alkaline Peptone Water (APW) and were incubated at 28–30°C for 18–24 hours.

3.2 *Vibrio parahaemolyticus* Isolation and Confirmation

For bacteria isolation, the enriched cultures were streaked with a sterile loop on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS) (HiMedia, India) agar. The agar plates were incubated at 30°C for 24 hours. The green colonies on TCBS agar were selected and subcultured. The selected colonies were transferred onto Tryptic Soy Agar (TSA) with 2% NaCl to obtain pure cultures. The subculture plates were incubated at 30°C overnight.

Gram staining and biochemical tests such as oxidase test and Triple Sugar Iron (TSI) were performed to confirm *Vibrio* spp.

3.3 *Vibrio parahaemolyticus* Confirmation Using *pyrH* and *toxR* Gene Detection

To confirm the bacteria isolates were *Vibrio* sp, detection of *pyrH* gene was conducted using polymerase chain reaction (PCR) while detection of *toxR* gene using another PCR was also performed to confirm it is *V. parahaemolyticus*.

To extract DNA from the bacteria, one or two colony was transferred to 100□L of sterile water in 1.5 ml microfuge tube . The microfuge tube was vortexed to mix it well. Then, the cell suspension was incubated in a water bath at 60°C for 10 minutes. Then, the microfuge tube was placed vertically in ice for 5 minutes. The tube was centrifuged at 10,000 rpm for another 10 minutes and the supernatant was transferred to a new 1.5 ml centrifuge tube (Dashti et al., 2009).

The specific primers used in this study were selected based on the previous research. Table 1 shows specific primers that were used to detect the *pyrH* gene and *toxR* gene in *V. parahaemolyticus.* The reagents used and the volume for a single PCR reaction: PCR master mix (PCR buffer, Mg^{+}, Cl^{-}, DNTP, Taq polymerase) with the volume of 12.5ul, forward primer 1ul, reverse primer 1ul, nuclease free water 8.5ul and DNA template 2ul. The PCR reaction was done in T100 Thermocycler (Bio-Rad, USA) while the PCR conditions were summarised in Table 2 and 3.

Table 1. Primers used in PCR and their sequence

Primer	Sequence (5' to 3')	Amplicon size (bp)
pyrH-F (Nurliyana et al., 2019)	GATCGTATGGCTCAAGAAG	500
pyrH-R (Nurliyana et al., 2019)	TAGGC ATTTTGTGGTCACG	
toxR-F (Han et al., 2014)	AATCCATGGATTCCACGCGTTATTT	103
toxR-R (Han et al., 2014)	CACCAATCTGACGGAACTGAGATTC	

Table 2. PCR condition for *pyrH* gene (Nurliyana et al., 2019)

Initial denaturation	94 ℃	5 seconds	33 cycles
Denaturation	94 ℃	1 minute	
Annealing	55.3 ℃	2 minute	
Extension	72 ℃	1 minute 15 seconds	
Final extension	94 ℃	10 seconds	

Table 3. PCR condition for *toxR* gene modified from Han et al.(2014)

Initial denaturation	94 ℃	3 minutes	30 cycles
Denaturation	94 ℃	30 seconds	
Annealing	60 ℃	35 seconds	
Extension	72 ℃	30 seconds	
Final extension	72 ℃	5 minutes	

3.4 DNA Sequencing and BLAST Analysis

The PCR bands were excised using a sterile scalpel blade carefully from the agarose gel under a UV transilluminator. The excised gel slices were placed separately in Eppendoft tubes and the lids were sealed with paraffin tightly. The tubes were sent to Apical Sdn Bhd for DNA sequencing. The sequencing results were analysed using database Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information (NCBI).

3.5 PCR for *pirA* and *pirB* Gene

Multiplex-PCR was done to detect the toxic *pirA* and *pirB* genes. The primers used and PCR conditions in the study were listed in Table 4 and Table 5, respectively. The reagents used and the volume for a single PCR reaction: PCR master mix (PCR buffer, Mg^{+}, Cl^{-}, DNTP, Taq polymerase) with the volume of

12.5ul, forward primer 1ul, reverse primer 1ul, nuclease free water 8.5ul, and DNA template 2ul. The PCR reaction was done in T100 Thermocycler (Bio-Rad, USA)

Table 4. List of primers that were used in PCR for *pirA/B* toxic genes (Han et al., 2015)

Primer	Sequence (5' to 3')	Amplicon size (bp)
pirA-F	TGACTATTCTCACGATTGGACTG	284
pirA-R	CACGACTAGCGCCATTGTTA	
pirB-F	TGATGAAGTGATGGGTGCTC	392
pirB-R	TGTAAGCGCCGTTTAACTCA	

Table 5. PCR condition for *pirA/B* genes

Initial denaturation	94 ℃	3 minutes	35 cycles
Denaturation	94 ℃	30 seconds	
Annealing	60 ℃	30 seconds	
Extension	72 ℃	30 seconds	
Final extension	72 ℃	7 minutes	

3.6 Agarose Gel Electrophoresis

Amplified PCR products were visualized on 1.5% agarose gel and stained with 1□L Midori green. The electrophoresis set was run at 100V and 400 mA for 40 minutes. The electrophoresis gel was photographed using a gel documented system, UV Transilluminator (Bio-Rad, USA).

CHAPTER 4

4.0 RESULTS

The shrimp samples, labeled by their source market (A: Siti Khadijah, B: Pengkalan Chepa, C: Pasar Tok Guru), were cultured on TCBS agar. Sample A was notable for a complete absence of green colonies. In contrast, three yellowish green colonies were isolated from Sample B (labeled as B1, B2, B3) and one from Sample C (C1). All four colonies were subcultured onto both TSA and TCBS agars. On TCBS agar, the colonies were greenish, circular, convex, and measured about 1 mm (Figure 2). On TSA agar, they appeared as creamy white, circular, convex colonies resembling a ball-point (Figure 3).

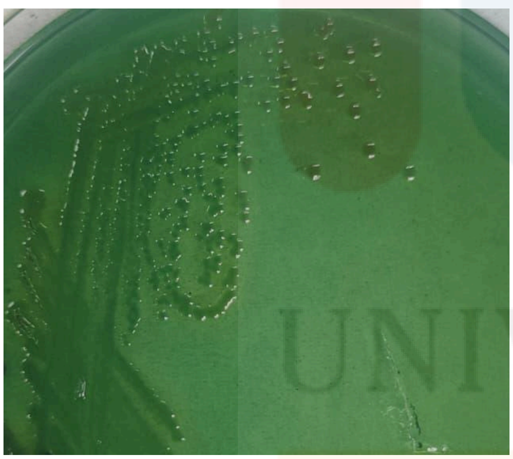

Figure 2. *Vibrio parahaemolyticus* colonies on TCBS agar

Figure 3. *Vibrio parahaemolyticus* Bacteria colonies on TSA agar

In Gram staining, the isolates were Gram-negative rods based on their eosinophilic, rod-shaped appearance. Results of oxidase and TSI tests were highlighted in Table 6. All the samples were positive in oxidase test with rapid colour change to dark purple or blue, indicating the bacteria are able to produce cytochrome oxidase. However, variable results for the TSI test . Samples B1 and B3 exhibited an alkaline (red) slant and an acid (yellow) butt, with no gas or hydrogen sulphide (H2S) production, identifying them as glucose fermenters. In contrast, Samples B2 and C1 showed an acid (yellow) slant, suggesting they are capable of fermenting lactose and/or sucrose.

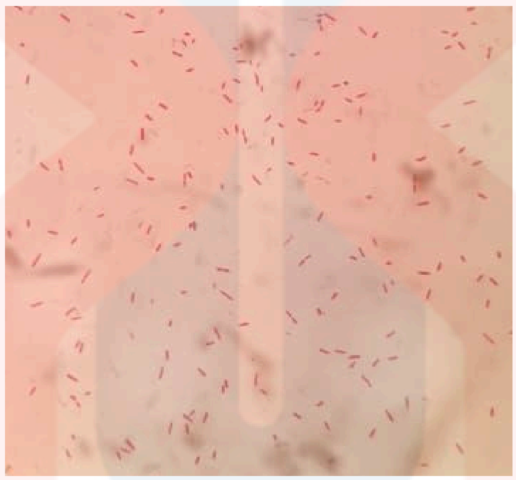

Figure 4. Gram negative bacilli in gram staining

Table 6. Results of TSI and Oxidase test

Sample	TSI	Oxidase Test
B1	K/A, Gas -ve, H_2S -ve	+ve
B2	A/A, Gas -ve, H_2S -ve	+ve
B3	K/A, Gas -ve. H_2S -ve	+ve
C1	A/A, Gas -ve, H_2S -ve	+ve

Indicators: +ve: positive result; -ve: negative result; K: alkaline; A: acidic

Only three isolates (B1, B2, B3) were confirmed as *Vibrio* spp.by PCR using *pyrH* gene as expected band size 500 bp was seen on agarose gel. To confirm the isolates as *Vibrio parahaemolyticus*, PCR which targets the *toxR* gene was conducted. BLAST analysis showed 99.79% - 100% of similarity for B1, B2, B3.

A multiplex-PCR was performed to detect the *pirA* and *pirB* genes to determine if they were the pathogenic type responsible for AHPND. The results were negative for all three samples, as neither the 284 bp nor the 392 bp amplification products were present. This indicates that these *V. parahaemolyticus* isolates lack the genes for the Pir toxin and are therefore not the AHPND-causing strain.

CHAPTER 5

5.0 DISCUSSION

In this present study, none of the isolates carried the *pirA/B* genes. This finding suggests that the shrimps from the sampled markets were not harboring the specific pathogenic strain that is responsible for AHPND. In addition, three isolates of *V. parahaemolyticus* were isolated only in Pengkalan Chepa wet market suggests a variable distribution of the bacteria in the wet markets of Kota Bharu, Kelantan. Such spatial variation in the prevalence of foodborne pathogens in retail environments is consistent with findings from other regional market surveys (Jun et al., 2018). While the absence of the AHPND strain is positive for local aquaculture, the presence of *V. parahaemolyticus* a known human pathogen (Nair et al., 2007) indicates that standard food safety and handling practices remain crucial for consumers.

The isolation process begins with culturing on the selective Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar. Instead of a typical, blue-green colony, the initial observation of yellowish-green colonies highlighted the variability in sucrose fermentation among *Vibrio* species. There is a limitation of relying solely on phenotypic characteristics for bacteria identification. This is because *V. parahaemolyticus* is not distinguishable from certain *Vibrio* species, including *V. vulnificus* and *V. mimicus,* by using TCBS agar (Su et al., 2005). Further biochemical and molecular confirmation are significant. Although TCBS was the best option to isolate vibrios, the other bacteria such as *Staphylococcus, Flavobacterium, Pseudoalteromonas, Streptococcus* and *Aeromonas* may grow on it as well. Environmental contamination may occur, affecting the results. The isolates were confirmed as Gram negative rods and were oxidase positive, consistent with the profile of most *Vibrio* sp (Haque et al., 2023). However, the variable results in Triple Salt Iron (TSI) test where B2 and C1 isolates exhibit an acidic slant and acidic butt (A/A), indicating fermentation of lactose and/ or sucrose is an atypical profile for classic *V. parahaemolyticus.* Generally, it produces an alkaline slant over acidic butt (K/A), indicating glucose fermentation. However, the accuracy of the TSI test is limited due to environmental factors such as temperature and humidity (Javed & Guo, 2021). Interestingly, most *V. parahaemolyticus* are unable to ferment sucrose as carbon source, but there are exceptions where *V. parahaemolyticus* with sucrose-positives were reported by Hammerl et al. (2021). This discrepancy further reinforces the necessity of molecular methods for accurate bacterial identification to species-level identification.

Definitive confirmation was achieved through a series of molecular assays. The initial screening using conventional PCR targeting the *pyrH* gene which is a conserved housekeeping gene in the Vibrio genus (Xiao et al., 2022). The *pyrH* gene has been shown to be a valuable tool for identifying and classification of various *Vibrio* species. It has higher specificity compared to other genes like *gyrB*, making it suitable for accurate identification of *Vibrio* species (Tall et al., 2013). For instance, in a study involving cultured groupers conducted by Amalina et al. (2019), the gene facilitated the differentiation and classification of diverse *Vibrio* strains into 11 groups, including *V. parahaemolyticus*. This suggests that C1 isolate was not the bacteria interested in this study. This result was conclusively validated by a second PCR targeting the species-specific *toxR* gene. The *toxR* gene is relatively specific for *V. parahaemolyticus*, making it a specific marker for this bacterium (Hou et al., 2024). It is involved in the virulence genes expression and regulation (Fabbro et al., 2010), which adds to its specificity for *V. parahaemolyticus* detection. This study shows that only isolates B1, B2 and B3 are confirmed to be *V. parahaemolyticus*. The concordance between the *pyrH* and *toxR* results, followed by successful BLASt analysis of the sequenced products has provided strong evidence for the presence of this bacterium in Market B.

However, none of the three confirmed isolates carried the *pirA* and *pirB* genes. The pirA/B genes are responsible for producing the PIr toxin, which is believed to be the direct cause of AHPND's pathology. In fact, PirAB toxin causes desquamation of the epithelial cells in hepatopancreatic tissue as the Pir A protein binds to the receptors on the shrimp target cells while PirB forms pores, leading to cell lysis (Lin et al., 2024). Hence, it can be concluded that *V. parahaemolyticus* strains present in the shrimps from Market B (Pengkalan Chepa wet market) are non-pathogenic strains. This finding is crucial for local aquaculture as it suggests a lower immediate threat of AHPND in the shrimp supply to the market but does not prove that there is complete absence of pathogenic strain in the environment as both non-pathogenic strain and AHPND strain can coexist in same environment.

The absence of AHPND-affected shrimps in this study could be contributed by several factors. The small sample size may cause missed detection of the AHPND strain in the wider area. It is possible that the pathogenic *V. parahaemolyticus* is present at a low frequency or is localized in certain farms or markets that are not included in this research. It is also possible that the source farms implement effective biosecurity measures and good husbandry management that prevent emergence of pathogenic strain as well as immunosuppression in shrimps. Also, the expression of virulence genes in *V. parahaemolyticus* AHPND strain is dependent on temperature and salinity which is supported by the experiment conducted by López-Cervantes et al. (2021). Maximum *pirA* expression was recorded at 3.5% sodium chloride (NaCl), while minimum expression was reached at 2.0% salinity. López-Cervantes et al. (2021) also suggested that NaCl concentration affects the expression of the pathogenic toxin gene and regulates virulence because sodium ion plays a role in signal transduction pathway in *pirA* transcription. Thus, proper salinity handling may be a factor to reduce impact of AHPND in the shrimp farms. On the other hand, temperature can influence the AHPND strain virulence by affecting the bacterial growth and toxin production (Prachumwat et al., 2018). Lower temperature (25℃ - 27℃) reduces the virulence of the bacteria compared to higher temperature (30℃ - 31℃) (Prachumwat et al., 2018). Not only increasing the host susceptibility due to stress, temperature fluctuation also influences the transcription of mRNA quantity which further results in increased bacterial virulent gene expression (Pragthong et al., 2020). Thus, this result should be interpreted as a brief summary indicating the absence of the pathogen within the specific sample set at specific time.

Despite the absence of AHPND-causing strain of *V. parahaemolyticus*, the finding of non-AHPND *V. parahaemolyticus* may possess the potential to affect public health. In fact, all AHPND-causing strains are tested to be lacking virulence factors associated with human pathogenic strain, indicating that the risk to human health by those strains is low (Karunasagar, 2018). Foodborne illness may occur in Kota Bharu consumers through the consumption of raw, undercooked or cross-contaminated shrimps. Wet markets with their ambient temperatures, high humidity and high potential of cross contamination via surfaces, wastewater and unhygienic handling practices can facilitate the survival and proliferation of *V. parahaemolyticus*. It is important to raise public awareness on it. Furthermore, the presence of non-AHPND strain of *V. parahaemolyticus* should not be overlooked as it represents a potential to obtain the virulence genes through *pirA/B* plasmid horizontal gene transfer (Liu et al., 2018, Ahn et al., 2017, Soto-Rodriguez et al., 2022). The *pirAB* plasmid, specifically the pVA1-type plasmid, can be transferred through conjugation (Dong et al., 2019). The plasmid DNA is passed from between two cells via a mating pilus (Beltran et al., 2023). A study done by Dong et al. (2019). have shown that the pVA1-type plasmid can be transferred from AHPND-causing *V. parahaemolyticus* to non-pathogenic strains Vibrios, resulting in the recipient strain acquiring the ability to produce the PirAB toxins and cause AHPND. Thus, if the non-pathogenic strain is exposed to the pathogenic strain, there is a possibility for horizontal gene transfer to occur and result in AHPND outbreak.

It is important to note the two limitations of the current study. First, this study was conducted using a limited number of samples from only selected wet markets in the city of the state and does not cover all the wet markets in Kelantan despite the focus of this study being the detection of pathogenic *pirA/B* gene in *V. parahaemolyticus*, where the disease has never been documented before in Kelantan. Thus, a thorough study of AHPND should be conducted involving a larger number of shrimp farms and wet markets in Kelantan to further evaluate the disease in shrimps. Besides that, the bacteria culture plays a significant role in this research. The success of obtaining pure bacterial cultures through streaking is heavily dependent on the researcher's technique. It requires careful handling to prevent contamination or mixed colonies as these are unfavourable errors that will impact the accuracy of the result. Future research should involve larger scale samples from both wet markets and shrimp farms across Kelantan.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, three isolates of *V. parahaemolyticus* were successfully isolated from the shrimp samples from Pengkalan Chepa wet market in Kelantan. The isolates were confirmed through *toxR* gene sequencing. However, none of the *pirA* and *pirB* genes were detected, indicating absence of AHPND strain in the sampled white shrimps in the present study. Therefore, this research suggests that the shrimps from the analysed wet markets in Kota Bharu pose a low immediate threat of AHPND transmission and shrimp as carriers of the disease. Nonetheless, the presence of *V. parahaemolyticus* suggests the importance of further surveillance and research in both aquaculture farms and wet markets. Future studies with a larger sample size and wider geographical scope within Kelantan, including the shrimp farms, are recommended to assess the risk of AHPND comprehensively in the white shrimp production.

7.0 APPENDIX

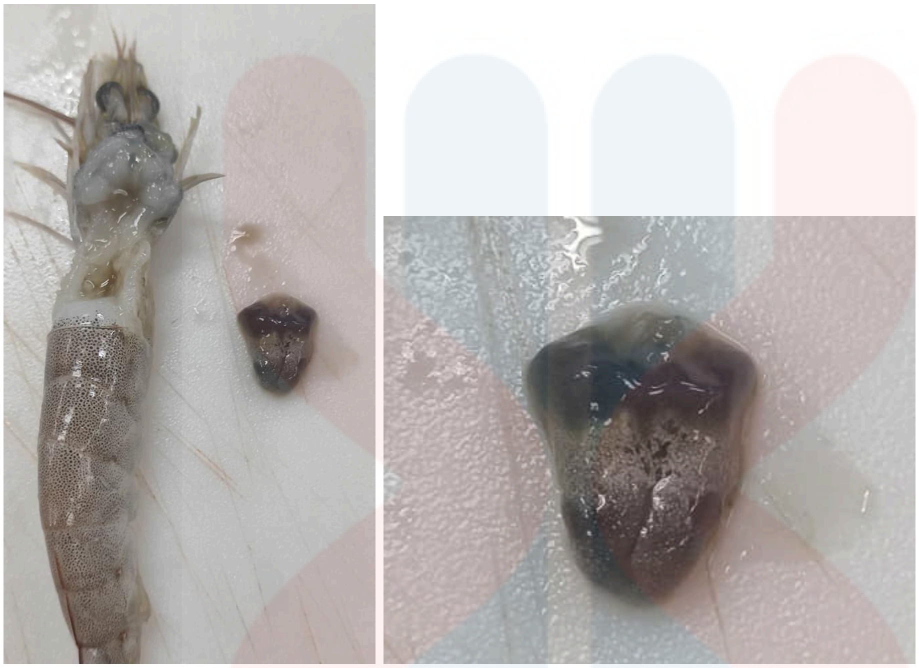

Figure 5 and 6: Dissected Hepatopancreas from Shrimp

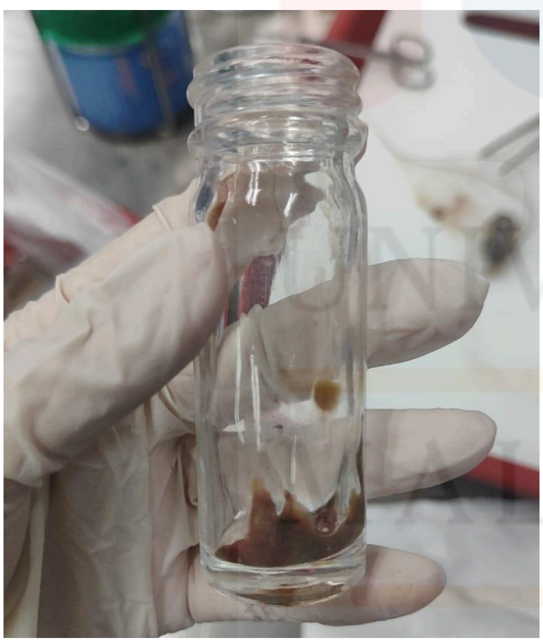

Figure 7 : Homogenized Hepatopancreas

8.0 REFFERENCE

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