

Detection of *PirA/B* Toxin Genes for Acute Hepatopancreatic Necrosis Disease (AHPND) and *Vibrio parahaemolyticus* in *Penaeus vannamei* Culture from Major White Shrimp Producing Farms in Malaysia

Bakar Padilah^{1*}, Yahya Rohaiza-Asmini¹, Han-Ming Gan², Wan Ahmad Wan Rozana¹, Wan Muhammad Hazim Wan Sajiri¹ and Beng-Chu Kua¹

¹National Fish Health Research Division, Fisheries Research Institute (FRI), 11960 Batu Maung, Penang, Malaysia

²School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway 47500, Petaling Jaya, Selangor, Malaysia

ABSTRACT

The acute hepatopancreatic necrosis disease (AHPND) epidemic from 2010 to 2013 significantly affected white shrimp (*Penaeus vannamei*) production in Malaysia. This study aims to determine the status of AHPND in *P. vannamei* culture from detecting *PirA/B* toxin genes in hepatopancreas tissues and isolation of *Vibrio parahaemolyticus* for identification of pathogenic strain from major white shrimp producing farms in Malaysia. Bacteria from the hepatopancreas organ were cultured on tryptic soy agar and identified using API[®] 20 NE (bioMérieux, France) for *Vibrio* species. Confirmation of *PirA/B* toxin genes in hepatopancreas and *V. parahaemolyticus* isolates were determined by polymerase

chain reaction (PCR). Twenty-three *V. parahaemolyticus* isolates were identified from 7.7% of the analysed samples. Fourteen (14) (4.7%) were detected with *PirA/B* toxin genes from districts of Johor such as Batu Pahat (1) and Kota Tinggi (8), Alor Setar, Kedah (3), Tawau, Sabah (1), and Kuching, Sarawak (1). In contrast, the other nine isolates (3%) contained only the plasmid. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harbours

ARTICLE INFO

Article history:

Received: 6 September 2021

Accepted: 29 November 2021

Published: 24 January 2022

DOI: <https://doi.org/10.47836/pjtas.45.1.10>

E-mail addresses:

padilah@dof.gov.my (Bakar Padilah)

rohaizaasmini@dof.gov.my (Yahya Rohaiza-Asmini)

kuaben01@dof.gov.my (Han-Ming Gan)

gan.gseq@gmail.com (Wan Ahmad Wan Rozana)

wanrozana91@gmail.com (Wan Muhammad Hazim Wan Sajiri)

hazimsajiri@gmail.com (Beng-Chu Kua)

*Corresponding author

the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing *PirA/B* toxin genes. Hence, this study showed that six farms were negative from AHPND. In contrast, four farms were positive with *PirA/B* toxin genes in juveniles from Pekan, Pahang (26.7%), Kuching and Sarikei, Sarawak (10% respectively), and Alor Setar, Kedah (3.3%).

Keywords: AHPND, *P. vannamei*, *PirA/B* toxin genes, prevalence, *V. parahaemolyticus*

INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) or previously known as early mortality syndrome (EMS), is a bacterial disease caused by a unique strain of *Vibrio* spp., including *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio owensii*, *Vibrio campbelli*, *Vibrio punensis*, and other possible bacteria that contain ~70-kbp plasmid genes which encode homologous of the *Photorhabdus* insect-related toxins, *PirA/B* (Devadas et al., 2019; Lee et al., 2015; Yang et al., 2014). Nucleotide content [guanine-cytosine (GC)] of these two genes is only 38.2% and is substantially lower than the rest of the plasmid, which suggests that these genes are acquired (Feng et al., 2017; Han et al., 2015). In addition, a plasmid that contains *PirA/B* toxin genes was found

in the pathogenic AHPND strain of *V. parahaemolyticus* (VPAHPND) but was absent in non-pathogenic strain, suggesting *PirA/B* toxin genes as the causative agent for AHPND. These *PirA/B* toxin genes were also found in *Photorhabdus* spp., which are gram-negative, luminescent, rod-shaped bacteria members of the family Enterobacteriaceae. In nature, *Photorhabdus* spp. establish an obligate, symbiotic relationship with entomopathogenic nematode *Heterorhabditis* spp., which are parasites of insect larvae that have a wide geographic distribution. The first detection of AHPND was reported in China in 2009, Malaysia and Vietnam in 2011, Thailand in 2012, and the Philippines in 2015 (Dabu et al., 2015; Food and Agriculture Organization of the United Nations [FAO], 2013, 2016).

Malaysia produced significant quantities of *Penaeus monodon* in the early 2000s but then switched largely to *P. vannamei* until the AHPND epidemic hit the country between 2011 and 2013. Sentinel surveillance based on reports of mortality cases in white shrimp farms from Malaysia showed an increasing number of cases since 2011, with the first report on the east coast of Johor and subsequently in Pahang, Perak, and Penang (Kua et al., 2016). The prevalence of AHPND was 50% and 26% in 2011 and 2012, respectively. Confirmation of AHPND was based on observation of clinical signs and the characteristic pathology of acute and terminal stages of AHPND in hepatopancreas organs (Kua et al., 2016). Pale discolouration and atrophy of hepatopancreas accompanied by loose

shells, empty stomach or discontinuous midgut, and corkscrew swimming behaviour were reported. In addition, mixed bacteria of *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio cholerae*, *Aeromonas hydrophila*, *Enterobacter cloacae*, *Pseudomonas* spp., and *Photobacterium damsela* were isolated from the hepatopancreas.

About 67% of shrimp production in Malaysia comes from white shrimps (Harkell, 2018). AHPND had caused a significant drop in *P. vannamei* production from 87,000 metric tonnes in 2010 to 67,000 metric tonnes in 2011. A continuous drop in *P. vannamei* production was recorded in 2012 with 48,991.81 metric tonnes (RM61.59 million) to 45,473.74 metric tonnes (RM86.72 million) in 2013 (Department of Fisheries Malaysia [DOF], 2010, 2011, 2012, 2013). Statistical data in 2018 recorded *P. vannamei* production of 36,007.25 metric tonnes (RM79.8 million) (DOF, 2018). Despite increasing aquaculture areas and shrimp culture farms, diseases and mortalities have been identified as major obstacles to sustainable production. AHPND and hepatopancreatic microsporidians caused by *Enterocytozoon hepatopenaei* (EHP) have been reported as two emerging diseases from 2010 to 2015 that are usually occurred concurrently and have significantly affected shrimp production due to high mortalities (AHPND) and/or stunted growth (EHP) (FAO, 2017).

Giant tiger shrimp (*Penaeus monodon*), white leg shrimp (*Penaeus vannamei*), and oriental/Chinese white shrimp (*Penaeus*

chinensis) are known to be infected with AHPND that is characterised by mass mortalities between 40% and 100% in 20–30 days of post-stocking in grow-out ponds (Lightner et al., 2012). Management practices in farms, including pond management and maintenance of good water quality, are known to prevent or avoid stressing shrimp and making them more susceptible to disease. However, low compliance with standards in good biosecurity and good aquaculture practices at farm and hatchery facilities have been identified as major factors favouring the spread of disease from one farm to the other (FAO, 2016). Therefore, shrimp aquaculture needs to continuously develop a systematic approach that implements responsible and science-based farming practices. Hence, the objective of this study is to determine the status of AHPND in *P. vannamei* culture from detection of *PirA/B* toxin genes and bacteria *V. parahaemolyticus* from hepatopancreas organ in relation to several routine management practices of pond culture.

MATERIALS AND METHODS

Sampling Size and Locations

A cross-sectional study on the status of AHPND was carried out from major shrimp producing areas in Malaysia, involving ten shrimp farms from 10 districts in Kedah, Penang, Terengganu, Pahang, Johor, Sarawak, and Sabah that started from January to November in 2019. A minimum of 30 pieces of juvenile shrimps/day of culture (DOC) aged less than 30 or between

31–45 DOC were collected randomly from various pond cultures (maximum 5 to 6 culture ponds/30 pieces). A total of 300 pieces of juveniles were sampled and analysed individually. The sampling sites, states, districts, months of sampling, and the number of samples are shown in Table 1.

Table 1

A sampling of Penaeus vannamei at the day of culture (DOC) less than 30 days of age, or juveniles between 31–45 days old from 10 locations that show states, districts, months, and number of samples

States	District	Month (2019)	Number of samples
Kedah	Alor Setar	April	30
Penang	Bkt. Tambun	February	30
Terengganu	Setiu	March	30
Sarawak	Sarikei	May	30
	Kuching	May	30
Johor	Batu Pahat	August	30
	Kota Tinggi	August	30
Pahang	Pekan	October	30
Sabah	Kudat	November	30
	Tawau	November	30
TOTAL			300

Bacterial Culture, Isolation, and Identification

About 210 pieces of juvenile white shrimps aged less than 30 days old in pond culture (Kedah, Terengganu, Johor, Pahang, and Sabah) and 60 pieces of white shrimps (Sarawak) aged between 30–45 days old were tested to detect the presence of bacteria in the hepatopancreas organ. Bacteria were aseptically inoculated via direct streaking onto tryptic soy agar (TSA), which was incorporated with 1.5% sodium chloride (NaCl) and incubated at 30 °C for 18 to 24 hours, followed by sub-culture until pure isolate was obtained.

Vibrio parahaemolyticus bacterial was identified using Gram staining, oxidase test, sensitivity to vibrio static 0129-disc agent (2, 4-Diamino-6, 7-di-iso-propylpteridine phosphate) (150/10 µg) and observation of the colour colony on thiosulfate citrate bile salt (TCBS) agar (Austin et al., 1997). A biochemical test for confirmation of *Vibrio* species was carried out using API® 20 NE Kit (bioMérieux, France). The *V. parahaemolyticus* isolates identified with 98% to 99.9% identical to the reference strain in the analytical profile index (API) software database was further tested for the presence of *PirA/B* toxin genes using a PCR

method. An isolate of *V. alginolyticus* was used as rooting for phylogenetic analysis.

DNA Extraction of Hepatopancreas Tissues and Bacteria Cells

A total of 300 hepatopancreas tissues samples in 95% alcohol fixation were tested for *PirA/B* toxin genes using a PCR method. DNA extraction was carried out using the cetyl trimethyl ammonium bromide (CTAB) and dodecyle trimethyl ammonium bromide (DTAB) method (IQ2000™, GeneReach Biotechnology Corp., Taiwan). In contrast, bacteria *V. parahaemolyticus* was extracted using G-Spin™ Genomic Bacteria Extraction Kit (iNtRON Biotechnology, Korea). About 30 mg of hepatopancreas tissues fixed in 95% alcohol was placed into a 1.5 mL tube containing 600 µL DTAB solution. Tissues were ground with a sterile disposable grinder until they were completely dissolved into DTAB solution. After that, they were incubated at 75 °C for 5 minutes and cooled down to room temperature. The mixture was vortexed and spun down briefly and was then added with 700 µL of chloroform and centrifuged at 12,000 x g for 5 minutes. Next, the upper aqueous phase was transferred into a new 1.5 mL tube and added 100 µL of CTAB solution and 900 µL of deionised water. It was then vortexed briefly, incubated at 75 °C for 5 minutes and centrifuged at 12,000 x g for 5 minutes. Next, the pellet was re-suspended with 150 µL dissolving solution, incubated at 75 °C for 5 minutes and spun at 12,000 x g for 5 minutes. Finally, the supernatant was transferred into a new 1.5 mL tube

containing 300 µL of 95% ethanol. This procedure was repeated twice, whereby the pellet was washed with 75% ethanol in the last procedure. The final pellet was dried and dissolved in deionised water or Tris ethylenediaminetetraacetic acid (TE) buffer. Dissolving DNA was stored at -20 °C until used for polymerase chain reaction (PCR).

Total genomic DNA from *V. parahaemolyticus* isolate was extracted using G-spin™ Kit (iNtRON Biotechnology, Korea). About 1 mL of bacteria cells was harvested from an overnight culture (18–24 hours) at 30 °C in tryptic soy broth incorporated with 1.5% NaCl (OD₆₀₀ 0.8–1.0) by centrifugation at 13,000 x g for 1 minute. The supernatant was removed, and cells were re-suspended by vortex and tapping. Bacteria cells pellet was extracted according to the manufacturer's instructions. The final collected DNA in a 1.5 mL tube was measured using a NanoDrop™ spectrophotometer (DS-11 Series, DeNovix, USA).

PCR Reaction Conditions

Nested PCR using AP4 primers was followed with some optimisation of annealing temperature using Maxime PreMix *i-Taq* (iNtRON Biotechnology, Korea) for detecting *PirA/B* toxin genes at 230 bp portion of a sequence, which includes 209 bp of the *ToxA* or *PirA* gene sequence plus 12 bp spacer sequence plus 9 bp of succeeding *ToxB* or *PirB* gene sequence (Dangtip et al., 2015). First-step PCR was performed using primer AP4-F1 with the sequence: 5'-ATGAGTAAACAATATAAAACAT

G A A A C - 3 ' a n d A P 4 - R 1 : 5 ' - A C G A T T T C G A C G T T C C C C A A - 3 ' , followed by nested PCR using AP4-F2 primer: 5'-TTGAGAATACGGGACGTGGG-3' a n d A P 4 - R 2 : 5 ' - G T T A G T C A T G T G A G C A C C T T C - 3 ' . In the first PCR reaction, 20 µL of the total volume reaction mixture was prepared, which consisted of DNA template 2 µL, AP4-F1 primer (0.4 µL 100 pmol/µL), AP-R1 primer (0.4 µL 100 pmol/µL), and deionised water (17.2 µL). Then, amplification was performed with the following parameters: initiation denaturation at 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 90 seconds, and a final extension at 72 °C for 2 minutes. For the second step (nested) PCR reaction, 1 µL of the final solution from the first-step PCR was diluted with deionised water at the ratio of 1 to 100, 2 µL of the diluted solution was used with nested primers AP4-F2 (0.4 µL 100 pmol/µL), AP4-R2 (0.4 µL 100 pmol/µL), and deionised water (17.2 µL) were used to make up for 20 µL of total volume. Then, amplification was performed with initiation denaturation at 94 °C for 2 minutes, followed by 25 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 30 seconds, and a final extension of 72 °C for 2 minutes. Following PCR, an aliquot of PCR products was analysed in a 1.5% gel containing green fluorescent dye nucleic acid staining solution (RedSafe™, iNtRON Biotechnology, Korea).

Sample Preparation for Sequencing and Phylogenomic Tree

Genomic deoxyribonucleic acid (gDNA) of five *Vibrio* spp., identified as IKK3, AAT22, PK3, Vp14, and SK6, were sent for sequencing via the iSeq100 Next Generation Sequencing System (GeneSEQ, Malaysia). Genome completeness analysis via BUSCO v4 was assessed for each assembled genome. In addition, protein-coding genes were briefly predicted from the assembly and were assessed for the presence of 1445 conserved genes found in *Vibrio* (*vibrio_odb10*). As expected from the high-quality assembly, each genome assembly exhibited genome completeness of >99.9%. The gDNA was fragmented using a Covaris ultra sonicator to approximately 350 bp. The fragmented DNA was end-repaired, adapter-ligated, and PCR-enriched using the NEBNext® Ultra™ II DNA Library Preparation Kit (New England Biolabs, USA) according to the manufacturer's instructions. The constructed libraries were normalised and sequenced on NovaSeq 6000 System (Illumina, USA) using a 2 x 150 bp read configuration (Simão et al., 2015).

Vibrio parahaemolyticus genome assemblies in National Center for Biotechnology Information (NCBI) that originated from Malaysia were used to infer a phylogenomic tree of five isolates (IKK3, AAT22, PK3, Vp14, and SK6) obtained from this study. The complete genome sequence of *V. parahaemolyticus* strain MVP1 was used as the reference genome. Each genome was subsequently aligned to

this reference genome to identify single nucleotide polymorphisms (SNPs) and generate a core SNP alignment. The Snippy v4.6.0 pipeline was used to perform these tasks. First, a maximum-likelihood tree was constructed using the FastTree2 setting, followed by visualisation and annotation in Figtree v1.4.1 (Price et al., 2010).

RESULTS

Biochemical Confirmation of Bacteria Isolates

Twenty-three isolates of *Vibrio* spp. were subjected to a biochemical test to confirm the species using API® 20 NE (bioMérieux, France). Isolates Vp14, IKK3, AAT22, and PK3 were identified as *V. parahaemolyticus* with 90% to 99.1% identical to the reference strain in the API system. They were Gram-negative halophilic bacteria, which produced green colony growth on thiosulfate citrate bile salt sucrose (TCBS) agar, was sensitive to vibrio static 0129-disc agent (150/10 µg) and exhibited cytochrome oxidase with catalase activity. Enzymatic assays showed that they were nitrate (NO₃) reductase, tryptophanase, glucose fermentation, gelatinase, and β-galactosidase but produced a negative reaction to arginine dihydrolase, urease, and esculin hydrolase. Carbohydrate assimilation showed a positive reaction to D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-gluconate, and L-malate but a negative reaction to caprate, adipate, citrate, and phenylacetate. *Vibrio alginolyticus*

(SK6) produced similar results as *V. parahaemolyticus* isolates, except that it did not produce acid from glucose or decarboxylate β-galactosidase and did not break up arabinose.

Prevalence of AHPND from Detection of *PirA/B* Toxin Genes in Hepatopancreas of *Penaeus vannamei*

The highest prevalence of AHPND was found in juveniles/day of culture (DOC) of white shrimps from Pekan (8, 26.7%), Pahang. In contrast, the low prevalence was recorded in juvenile shrimps from Sarikei (3, 10%), Kuching (3, 10%), Sarawak, and Alor Setar (1, 3.3%), Kedah. The 7.3% of samples (22) were tested positive with *PirA/B* toxin genes with an overall mean prevalence of 5%. Results are shown in Table 2. AHPND prevalence was diagnosed by detecting *PirA/B* toxin genes from hepatopancreas tissues of white shrimps fixed in 95% alcohol and supported with or without the isolation of *V. parahaemolyticus* isolate that carries *PirA/B* toxin genes. AHPND was not detected in hepatopancreas tissues of juvenile white shrimps from Johor (60), Sabah (60), Terengganu (30), and Penang (30). However, culture isolates of *V. parahaemolyticus* obtained from white shrimp hepatopancreas at Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3%) showed that these strains are pathogenic which carry *PirA/B* toxin genes.

Table 2

Detection of AHPND with PirA/B toxin genes from the hepatopancreas of juvenile/DOC white shrimps

States	District	Sample number (n)	Positive sample for <i>PirA/B</i> toxin genes (Prevalence, %)
Kedah	Alor Setar	30	1(3.3)
Penang	Bukit Tambun	30	-
Terengganu	Setiu	30	-
Johor	Batu Pahat	30	-
	Kota Tinggi	30	-
Pahang	Pekan	30	8(26.7)
Sarawak	Kuching	30	3(10)
	Sarikei	30	3(10)
Sabah	Kudat	30	-
	Tawau	30	-
Total		300	15(50)
Mean (%)			5.0

Note. Mean (%): (Number of the sample with *PirA/B* toxin genes/Total samples) x 100%

Prevalence of *Vibrio parahaemolyticus* with *PirA/B* Toxin Genes

Twenty-three (23) *V. parahaemolyticus* isolates were obtained from 270 samples of juvenile white shrimps, whereby 14 isolates were detected with *PirA/B* toxin genes. AHPND isolates were found in samples from Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3.3%), Johor, Alor Setar, Kedah (3, 10%), and Kudat, Sabah (1, 3%). Although *V. parahaemolyticus* bacteria from culture media isolation were detected to have *PirA/B* toxin genes, direct PCR analysis of hepatopancreas tissues fixation (95% alcohol) of the similar samples failed to detect *PirA/B* toxin genes from these tissue samples. Virulence of AHPND-causing *V. parahaemolyticus* depends on the

amount of *PirA/B* toxin released and caused cellular damage to hepatopancreas when *V. parahaemolyticus* bacteria initially colonise in the shrimp stomach and eventually reach the hepatopancreas (Han et al., 2015). In this situation, *V. parahaemolyticus* was considered a relatively non-virulent bacterium until it released a potent toxin (*PirA/B*) in the host tissues/hepatopancreas organ or induced the clinical disease condition and mortality in white shrimps. PCR analysis of hepatopancreas tissue of white shrimp sample was negative from Johor (60) and Sabah (60). The detection of *PirA/B* toxin genes from *V. parahaemolyticus* isolates obtained through the propagation of bacteria cells in the laboratory was not considered a positive case

for AHPND in this surveillance study but rather for identifying the pathogenic strain. PCR analysis of the cultured cells showed that 13 *V. parahaemolyticus* AHPND strains were identified from Kota Tinggi (8) and Batu Pahat (1), Johor; Alor Setar (3), Kedah, and Kudat (1), Sabah. Another study has shown that infection of AHPND depends on sufficient bacteria cells count to secrete or release *PirA/B* toxins in the shrimp tissues rather than the number of copies of toxin genes (Tinwongger et al., 2016).

Genomic Sequence of *PirA/B* Toxin Genes and pVA1 Plasmid

Five isolates of *V. parahaemolyticus* from Sarawak (Vp14), Kedah (IKK3), Johor (PK3, AAT22), and *V. alginolyticus* from Sabah (SK6) were subjected to whole-

genomic sequences to determine the virulence of local strains obtained from this study. The whole-genome sequences were aligned to the pVA1 plasmid using blastN with an E-value of $1e^{-50}$ and subsequently visualised in Blast Ring Image Generator (Alikhan et al., 2011; Dong et al., 2019). Being consistent with the initial PCR screening result, strain Vp14 (Sarawak) was the only strain that harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, strain AAT22 (Johor) displayed significant sequence homology with at least 50% length of the plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segment containing the *PirA/B* genes (Figure 1).

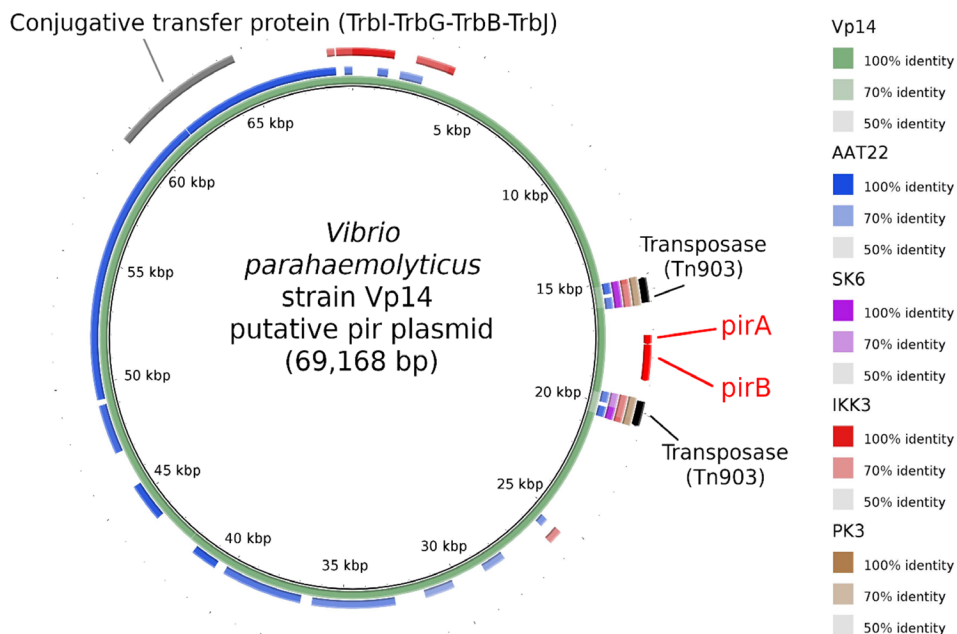


Figure 1. Circular visualization of the pVA1 plasmid and selected genes. Coloured rings indicate a genomic region with significant homology to the strain of interest (Vp14, AAT22, SK6, IKK3, and PK3)

Phylogenomic Tree of *Vibrio parahaemolyticus* Associated with Genome Assemblies in NCBI from Aquaculture in Malaysia

Four *V. parahaemolyticus* isolates, which were identified as Vp14 (Sarawak), AAT22 and PK3 (Johor), IKK 3 (Kedah), and SK6 (Sabah), were identified as *V. alginolyticus* that were subjected to genomic and phylogenetic analysis. The assembled genomes were compared to *Vibrio* spp. type strains to confirm their taxonomic assignment. Strains AAT22, IKK3, PK3, and Vp14, showed a pairwise average nucleotide identity of more than 98% to *V. parahaemolyticus*. In contrast, strain SK6 showed an average nucleotide identity of 98.5% to *V. alginolyticus* and less than 90% to other tested *Vibrio* species. Strain SK6 (Sabah) expectedly displayed a long branch length and was thus chosen as the outgroup for rooting, given its taxonomic assignment as *V. alginolyticus*. Strain Vp14 harbours the *PirA/B* toxin genes formed a monophyletic cluster with high SH-like support of a few MVP strains isolated from a shrimp pond water sample located in Negeri Sembilan in 2016. In contrast, strains IKK3 (Kedah), AAT22 and PK3 (Johor) only shared a relative distance ancestor with some of the publicly available strains, as evidenced by their relatively longer branch length (Figure 2). It suggested that they could be novel genomic lineages of *V. parahaemolyticus*, which was not reported previously in Malaysia.

DISCUSSION

This study showed that grow-out or juvenile white shrimps aged less than 30 days of culture in pond and post-larvae appeared most susceptible to AHPND infection with an overall mean prevalence of 5% and 4.7%, respectively. AHPND often occurs within 20–30 days of post stocking in grow-out ponds and causes mass mortalities in post-larvae shrimps (De Schryver et al., 2014). Therefore, most farms practise routine screening for major shrimp diseases that are known in aquacultures, such as white spot disease that is caused by white spot syndrome virus (WSD/WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), AHPND, and EHP as parts of prevention programmes in their farms whereby the infected stocks will be destroyed.

This study identified 23 *V. parahaemolyticus* isolates from 7.7% of total samples. Fourteen (14) strains of *V. parahaemolyticus* were found to have *PirA/B* toxin genes, whereas nine isolates were detected with plasmid. Gross observation during sampling showed only mild changes in hepatopancreas organs with pale coloured, atrophied, empty midgut, and soft shell in several samples, suggesting that subclinical infection may go unnoticed. However, AHPND infection caused by *V. parahaemolyticus* that produce *PirA/B* toxin genes will usually cause severe pathological changes to hepatopancreas organ, which are shown via degeneration and massive sloughing of hepatopancreas cells, followed

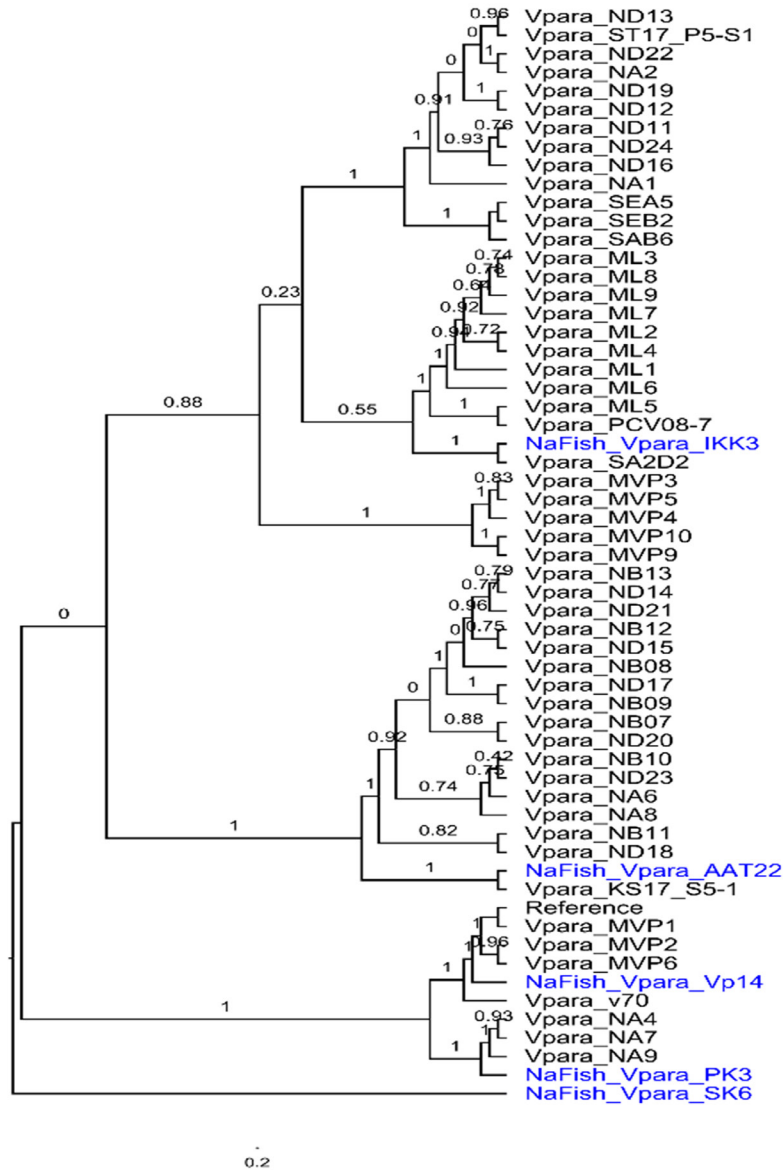


Figure 2. Maximum likelihood tree constructed based on the core SNP alignment of publicly available Malaysian *Vibrio parahaemolyticus* genomes and NaFish *Vibrio* genomes (blue labels). Strain SK6 was rooted as the outgroup, given its taxonomic classification as *Vibrio alginolyticus*. The branch lengths indicate the number of substitutions per site, while the SH-like bootstrap supports values that were indicated by the colour of the nodes.

by high mortalities in disease outbreaks (Lightner et al., 2012; Nunan et al., 2014). However, typical gross pathological changes associated with clinical disease of AHPND was not observed on-site, and farmers have not reported high mortalities. Nevertheless, the occurrence of *V. parahaemolyticus* bacteria with *PirA/B* toxin genes in the hepatopancreas of shrimp will increase their risk to AHPND under stressful conditions. Routine management practices applied in farms such as regular health screening of stocks, strict biosecurity measures, hygienic practices at the entrance and within farms culture area, and pond management and its water quality are among many factors that impact the management of disease-free culture.

The pVA1 plasmid is the source of the AHPND-causing toxin, whereas *PirA/B* genes, irrespective of other plasmidic factors of pVA1, are sufficient to produce symptoms associated with AHPND (Lee et al., 2015). Culturable cells of *V. parahaemolyticus* local strains were recovered from -20 °C storage. Genomic sequencing of four local strains of *V. parahaemolyticus* carrying *PirA/B* toxin genes showed that only one strain (Vp14) harboured the *PirA/B* complete genes, which displayed a full sequence of pVA1 virulent plasmid. The risk of AHPND outbreak can be reduced by controlling the *Vibrio* spp. activities, in particular, *V. parahaemolyticus* cells count. The bacterial concentration ranging ($5 \times 10^4 - 5 \times 10^5$ cfu/ml) from AHPND strain has been proven to be able to cause significant mortalities to *P. vannamei* from 60% to 100% within 3 to 6

days of post infections from the immersion challenge test (Tinwongger et al., 2016). Hence, the virulence of AHPND depends on sufficient bacterial count to release or secrete toxin rather than the number of copies of toxin genes (Tinwongger et al., 2016). Other studies showed that phytoplankton/green water and nutrient enrichment affect the microbial community in the ecosystem, especially bacterial load and interaction with the shrimp immune responses (De Schryver et al., 2014). Probiotic and/or algae-rich green waters are known to be able to create microbially matured water systems, whereby environments that are primarily colonised by slow-growing harmless bacteria may best guarantee the prevention of AHPND outbreaks (De Schryver et al., 2014). Probiotics and molasses help to increase the diversity of heterotrophic bacteria, including *V. parahaemolyticus* thus, effectively inhibiting pathogens (Bhatnagar & Pooja, 2013; Hu et al., 2016). Molasses are known to improve water quality, and it is suggested to be used during the nursery and grow-out phase of *P. vannamei* under limited water discharge or close system (Tzachi et al., 2007).

CONCLUSION

The health status of AHPND from grow-out/ juvenile white shrimps aged less than 30 days of culture was determined through a cross-sectional study using a random sampling method. AHPND with the detection of *PirA/B* toxin genes and identification of *V. parahaemolyticus* with *PirA/B* toxin genes were determined from 10 major shrimp

producing areas in Malaysia. AHPND with *PirA/B* toxin genes detection from the hepatopancreas of white shrimps was found in Kuching and Sarikei, Sarawak at 10% prevalence, respectively, followed by 3.3% in Alor Setar, Kedah, and 26.7% from Pekan, Pahang. *PirA/B* toxin genes of AHPND were not detected in white shrimps from farms in Penang, Johor, Terengganu, and Sabah. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing the *PirA/B* toxin genes.

Many factors are known to influence the clinical manifestation of AHPND, such as the presence of *PirA/B* toxin genes and *V. parahaemolyticus* bacteria that carry *PirA/B* toxin genes in hepatopancreas tissues, as well as water quality and bacterial cell counts in pond culture. Health screening through regular observation of shrimp behaviour and health checks for important diseases in shrimp culture provides the best solution for early detection and management of health problems. The risk of spreading the disease to other farms can be prevented through removal and safe disposal of sick or dead shrimps, as well as emergency harvesting if necessary, or destruction

of infected stocks as appropriate. Strict biosecurity measures and disinfection have been strictly implemented in almost all farms that the researchers visited or surveyed. This practice is believed to contribute significantly to controlling and preventing AHPND.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the R&D Fund (220501-039), the Department of Fisheries Malaysia (DOF), and the Ministry of Agriculture Malaysia (MOA). The authors want to thank the Director of Fisheries Research Institute (FRI), Batu Maung, Dr Zainuddin Jamari, for providing financial and moral support. The authors would also like to express their sincere gratitude to all farmers, company managers, officers, and staff from various States of Fisheries Department (Pejabat Perikanan Negeri), Biosecurity and Aquaculture Division from Kedah, Terengganu, Johor, Pahang, Sarawak and Sabah for their active participation and continuous support throughout the process of conducting this study.

REFERENCES

- Alikhan, N. F., Petty, N. K., Ben-Zakour, N. L., & Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics*, *12*(1), 402. <https://doi.org/10.1186/1471-2164-12-402>
- Austin, B., Austin, D. A., Blanch, A. R., Cerdà, M., Grimont, F., Grimont, P. A. D., Jofre, J., Koblavi, S., Larsen, J. L., Pedersen, K., Tiainen, T., Verdonck, L., & Swings, J. (1997). A comparison of methods for the typing of

- fish-pathogenic *Vibrio* spp. *Systematic and Applied Microbiology*, 20(1), 89-101. [https://doi.org/10.1016/S0723-2020\(97\)80053-7](https://doi.org/10.1016/S0723-2020(97)80053-7)
- Bhatnagar, A., & Pooja, D. (2013). Water quality guidelines for the management of pond fish culture. *International Journal Environmental Science*, 3(6), 5-30. <https://doi.org/10.6088/ijes.2013030600019>
- Dabu, I. M., Lim, J. J., Arabit, P. M. T., Orense, S. J. A. B., Tarbadillo, J. A., & Corre, V. L. (2015). The first record of acute hepatopancreatic necrosis disease in Philippines. *Aquaculture Research*, 48(3), 792-799. <https://doi.org/10.1111/are.12923>
- Dangtip, S., Sirikharin, P., Sanguanrut, S., Thitamadee, K., Sritunyalucksana, S., Taengchaiyaphum, R., Mavichak, P., Proespraiwong, P., & Flegel, T. W. (2015). AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. *Aquaculture Representative*, 2, 158-162. <https://doi.org/10.1016/j.aqrep.2015.10.002>
- De Schryver, P., Defoirdt, T., & Sorgeloos, P. (2014). Early mortality syndrome outbreak: A microbial management issue in shrimp farming? *PLOS Path*, 10(4), e1003919. <https://doi.org/10.1371/journal.ppat.1003919>
- Department of Fisheries Malaysia. (2010). Perangkaan Perikanan Tahunan 2010 [Annual fisheries statistics 2010]. DOF. <https://www.dof.gov.my/en/resources/fisheries-statistics-i/>
- Department of Fisheries Malaysia. (2011). Perangkaan Perikanan Tahunan 2011 [Annual fisheries statistics 2011]. DOF. <https://www.dof.gov.my/en/resources/fisheries-statistics-i/>
- Department of Fisheries Malaysia. (2012). Perangkaan Perikanan Tahunan 2012 [Annual fisheries statistics 2012]. DOF. <https://www.dof.gov.my/en/resources/fisheries-statistics-i/>
- Department of Fisheries Malaysia. (2013). *Perangkaan perikanan tahunan 2013* [Annual fisheries statistics 2013]. DOF. <https://www.dof.gov.my/en/resources/fisheries-statistics-i/>
- Department of Fisheries Malaysia. (2018). *Perangkaan tahunan perikanan 2018 jilid 1* [Annual fisheries statistics 2018 volume 1]. DOF. <https://www.dof.gov.my/en/resources/i-extension-en/annual-statistics/>
- Devadas, S., Banerjee, S., Yusoff, S. F. M., Bhasu, S., & Shariff, M. (2019). Review: Experimental methodologies and diagnostic procedures for acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, 499, 389-400. <https://doi.org/10.1016/j.aquaculture.2018.06.042>
- Dong, X., Song, J., Chen, J., Bi, D., Wang, W., Ren, Y., Wang, H., Wang, G., Tang, K. F. J., & Wang, X. (2019). *Conjugative transfer of the pVA1-type plasmid carrying the PirAB^{vp} genes results in the formation of new AHPND-causing Vibrio*. *Frontiers in Cellular and Infection Microbiology*, 195(9), 1-11. <https://doi.org/10.3389/fcimb.2019.00195>
- Feng, B., Liu, H., Wang, M., Sun, X. H., Pan, Y., & Zhao, Y. (2017). Diversity analysis of acute hepatopancreatic necrosis disease-positive *Vibrio parahaemolyticus* strains. *Aquaculture and Fisheries*, 2(6), 278-285. <https://doi.org/10.1016/j.aaf.2017.10.001>
- Food and Agriculture Organization of the United Nations. (2013). *FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp*. FAO. <https://www.fao.org/3/i3422e/i3422e00.htm>
- Food and Agriculture Organization of the United Nations. (2016). *Second international technical seminar/workshop on acute hepatopancreatic necrosis disease (AHPND) there is a way forward!*. FAO. <https://www.fao.org/documents/card/en/c/28b6bd62-5433-4fad-b5a1-8ac61eb671b1/>

- Food and Agriculture Organization of the United Nations. (2017). *FishStatJ – Software for fishery and aquaculture statistical time series*. FAO.
- Han, J. E., Tang, Kathy F. J., Tran, Loc H., & Lightner, D. V. (2015). Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Disease Aquatic Organisms*, *113*, 33-40. <https://doi.org/10.3354/dao02830>
- Harkell, L. (2018). *Half of shrimp farmers in key Malaysia region switch to black tiger: Seafood business news from beneath the surface*. <https://www.undercurrentnews.com/2018/08/27/half-of-malaysian-shrimp-farmers-in-key-region-switch-to-black-tiger/>
- Hu, X. J., Cao, Y. C., Wen, G. L., Zhang, X. Y., Xu, Y., Xu, W., & Li, Z. J. (2016). Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. *Aquaculture Research*, *48*(6), 2691-2705. <https://doi.org/10.1111/are.13101>
- Kua, B. C., Ahmad, I. A. R., Siti-Zahrah, A., Irene, J., Norazila, J., Nik-Haiha, N. Y., Fadzilah, Y., Mohammed, M., Siti-Rokhaiya, B., Omar, M., & Teoh, T. P. (2016). Current status of acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia. In *Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia* (pp. 55-59). Southeast Asian Fisheries Development Center.
- Lee, C. T., Chen, I. T., Yang, Y. T., Ko, T. P., Huang, Y. T., Huang, J. Y., Huang, M. F., Lin, S. J., Chen, C. Y., Lin, S. S., Lightner, D. V., Wang, H. C., Wang, A. H., Hor, L. I., & Lo, C. F. (2015). The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proceedings of the National Academy of Sciences*, *112*(34), 10798-10803. <https://doi.org/10.1073/pnas.1503129112>
- Lightner, D. V., Redman, R. M., Pantoja, C. R., Noble, B. I., & Tran, Loc H. (2012). Early mortality syndrome affects shrimp in Asia. *Global Aquaculture Advocate*, *15*(1), 40.
- Nunan, L., Lightner, D. V., Pantoja, C., & Gomez-Jimenez, S. (2014). Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Disease Aquatic Organisms*, *111*, 81-86. <https://doi.org/10.3354/dao02776>
- Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS One*, *5*(3), e9490. <https://doi.org/10.1371/journal.pone.0009490>
- Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210-3212. <https://doi.org/10.1093/bioinformatics/btv351>
- Tinwongger, S., Nochiri, Y., Thawonsuwan, J., Nozaki, R., Kondo, H., Awasthi, S. P., Hinenoya, A., Yamasaki, S., & Hirono, I. (2016). Virulence of acute hepatopancreatic necrosis disease *PirAB*-like relies on secreted proteins not on gene copy number. *Journal Applied Microbiology*, *121*(6), 1755-1765. <https://doi.org/10.1111/jam.13256>
- Tzachi, M. S., Susmitas, P., Mike, S., Abduk-Mehdi, A., Josh, M. B., Rodrigo, V. A., Zarrein, A., Margasanto, H., Ami, H., & David, L. B. (2007). Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. *Aquacultural Engineering*, *36*(2), 184-191. <https://doi.org/10.1016/j.aquaeng.2006.10.004>

Bakar Padilah, Yahya Rohaiza-Asmini, Han-Ming Gan, Wan Ahmad Wan Rozana,
Wan Muhammad Hazim Wan Sajiri and Beng-Chu Kua

Yang, Y. T., Chen, I. T., Lee, C. T., Chen, C. Y.,
Lin, S. S., Hor, L. I., Tseng, T. C., Huang Y. T.,
Sritunyalucksana, K., Thitamadee, S., Wang, H.
C., & Lo, C. F. (2014). Draft genome sequences
of four strains of *Vibrio parahaemolyticus* three
of which cause early mortality syndrome/acute
hepatopancreatic necrosis disease in shrimp in
China and Thailand. *Genome Announcement*,
2(5), e00816-14. [https://doi.org/10.1128/
genomeA.00816-14](https://doi.org/10.1128/genomeA.00816-14)