***Vibrio* sp. associated with acute hepatopancreatic necrosis disease (AHPND) found in penaeid shrimp pond from east coast of peninsular Malaysia**

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Abstract

Aim: Acute hepatopancreatic necrosis disease (AHPND) is one of the fastest emerging disease in shrimp aquaculture industry. Based on a recent AHPND case reported in our local shrimp farm, this study aimed to isolate, identify and characterize the causal agent of AHPND isolated from a shrimp farm located in Malaysia.

Methodology : The isolates were first screened with AP4 primers, then identified based on phenotypic observations and phylogenetic analysis. Screening of selected virulence genes, antibiotic susceptibility test, and virulence study of the isolates using *Artemia* sp. as challenge model were carried out to further distinguish each isolates.

Results : Six positive AHPND isolates were reported in this study, where four of them were identified as *V. harveyi* and another two were *V. parahaemolyticus*. All the isolates appeared to be susceptible against tetracycline. *V. parahaemolyticus* (C2A, C4B) showed higher virulence than *V. harveyi* (C1B, C2B, C4A, D5) when tested in *Artemia* sp. challenge study.

Interpretation : This study reports that, in Malaysia, both *V. parahaemolyticus* and *V. harveyi* could be the causative pathogen of AHPND outbreak in local shrimp farm.

Key word : Acute hepatopancreatic necrosis disease, Aquaculture, Shrimp disease, *Vibrio harveyi*, *Vibrio parahaemolyticus*

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Introduction

Acute hepatopancreatic necrosis disease (AHPND) is an alarming threat to the shrimp aquaculture industry. AHPND impose a serious threat to the shrimp production, and globally it has been reported to cause a loss of USD 1 billion to the shrimp industries (Leaño and Mohan, 2012a). AHPND was first ever reported in Southern China in 2009 (Zhang et al., 2012). Shortly after that, the disease was reported in Vietnam in 2010 (Lightner et al., 2012). Subsequently, Malaysia in 2011 (Chu et al., 2016; Lightner et al., 2012), Thailand in 2012 (Leaño and Mohan, 2012b), Mexico in 2013 (Soto-Rodriguez et al., 2015), Philippines in 2015 (Dabu et al., 2015) and United States of America, USA in 2017 (Dhar et al., 2019). Malaysia has felt the impact as outbreak of AHPND in whiteleg shrimp farms in 2011, 2012 and 2013 has caused a significant reduction of shrimp production to 67,000 metric tonne, 55,000 metric tonne, and 50,000 metric tonne in 2011, 2012 and 2013, respectively (Annual Fisheries Statistics, 2005-2014). Based on the estimation of shrimp production losses from year 2011 to year 2014, the economic losses from AHPND episodes were estimated to reach USD 0.49 billion (Chu et al., 2016).

Vibrio parahaemolyticus has been reported the causative agent AHPND in shrimp (Joshi et al., 2014; Tran et al., 2013). However, not all strains of *V. parahaemolyticus* cause AHPND. Only those acquired pVA1 plasmid which encodes toxins are able to cause AHPND (Lee et al., 2015). The bacteria with this plasmid produces and releases toxins that is homologous to the Pir (Photorhabdus insect-related) toxin which consists of two subunits, PirA^{vp} and PirB^{vp} (Sirikharin et al., 2015). Though, predominantly *V. parahaemolyticus* has been reported as the main causal bacteria for the disease (Joshi et al., 2014; Tran et al., 2013), findings of the past ten years has shown that other *Vibrio* sp. can also carry the pVA1 plasmid which encodes the AHPND binary toxins (Lee et al., 2015). Studies have reported that the plasmid carrying the virulence plasmid has also been found in *V. harveyi* (Kondo et al., 2015), *V. owensii* (Liu et al., 2015) and *V. campbelli* (Dong et al., 2017). In a more recent findings, the AHPND virulent plasmid was found in *V. punensis* sp. nov. strain BA55 which belongs to the *Orientalis* clade, marking for the first time that a virulence plasmid found in species other than *Harveyi* clade (Restrepo et al., 2018).

Both environmental stress and the presence of causative agent, *V. parahaemolyticus*, are important factors for AHPND outbreaks in culture ponds (Akazawa and Eguchi, 2017). It is hypothesized that shrimps acquire infection by consuming raw sludge contaminated with the pathogen from the bottom of culture pond. Fluctuation in the pH of pond water causes stress and has weakened the immune system of white shrimp (Li and Chen, 2008). Other environmental influences such as temperature and salinity can also induce the occurrence AHPND (Moullac and Haffner, 2000).

This disease affects postlarvae and juvenile shrimp upon the first 20 to 30 days of stocking in grow-out pond (Lightner et al., 2012; Tran et al., 2013). Based on the observations, slow growth, loose shells and discoloration are the early clinical signs of this disease (Leaño and Mohan, 2012b). Pathologically, it degenerates hepatopancreas (sloughing of the hepatopancreas tubule epithelial cells), hemocytic infiltration, necrosis, and eventually death of the post larvae and juvenile shrimp (Tran et al., 2013). It has been reported that this disease has incurred great loss to farmers as it causes mass mortalities up to 100% of PLs during the first 20 to 30 days after stocking (Tran et al., 2013).

Based on a recent AHPND case reported in our local shrimp farm, in Terengganu, Malaysia, this study was carried out to isolate, identify and characterize the causal pathogen of AHPND from Malaysian shrimp farm. The bacterial virulence of the isolated strains were also determined using brine shrimp as the infection model to understand and compare the characteristics of AHPND causal *Vibrio* sp. strain found in Malaysia.

Materials and Methods

The sampling was carried out in a shrimp farm, in Setiu, Terengganu located in the east coastal area of the peninsular Malaysia on 11th and 12th May, 2017. The farmers has reported the incidents of massive death of shrimp in a few newly stocked farm. Post larvae shrimp, *Penaeus vannamei*, at of approximately 5 g from ponds with mortalities were sampled. The shrimp was sampled based on the abnormal gross signs consistent to AHPND symptoms. Such signs inclusive of pale to whitish hepatopancreas with reduction in size.

In this study, hepatopancreas of the AHPND infected shrimp were aseptically separated, crushed and inoculated into flasks containing marine broth (Sigma, USA). The flasks were kept at 27°C for 18 to 24 hr to culture the pathogens. Individual colonies from the mix cultures were re-cultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar supplemented with 1.5% sodium chloride and kept at 27°C for 24 hr. The colony morphologies were observed, and then each colonies were cultured into tryptone soy broth (TSB) to confirm pure culture and were streaked again onto TCBS agar plates. All single isolates were stocked in tryptone soy broth (TSB) pre-added with 20% glycerol and stored at -80°C for other usages.

Genomic DNA extraction was done using GeneJET Genomic DNA Purification Kit, Thermo Scientific (USA). A single colony was inoculated into 10 ml marine broth, and kept for 18 hr at 27 °C. The cells were then spin-downed by centrifugation at 5000 g, for 10 min. The bacterial DNA extraction, GeneJET Genomic DNA Purification Kit was done as stated in the manual provided. Screening for AHPND positive strains were carried out using AP4 method. AP4 method is a two-step nested PCR

screening of AHPND isolates (Dangtip et al., 2015). The method targeted the tandem genes of *pirA^{vp}* and *pirB^{vp}* on pVA plasmid (Lee et al., 2015). The primers used in this method comprised of two pairs as stated in Table 1. PCR mixtures consisted of 5 PCR buffer, 2.0 µl MgCl₂ (100 mM), 1.0 µl dNTPs (10 mM each), 1.0 µl each forward and reverse primers (20 µM), 0.5 GoTaq Polymerase (5 U µl⁻¹) (Promega, USA), 5.0 µl of DNA template (50 ng µl⁻¹) and topped up with nuclease-free water (Thermo Fisher Scientific, USA) making up the final volume of 25 µl per reaction. PCR programmes were conducted using Eppendorf™ Mastercycler™ Nexus Thermal Cycler (Germany). The nested thermal PCR reactions consisted of two step, in the first step, PCR amplifications were carried out by the following programme:

denaturation 94 °C for 2 min then 30 cycles; each cycle, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, then the final elongation step at 72 °C for 2 min. For the second step of nested PCR, amplifications were done as follows: denaturation 94°C for 2 min then 25 cycles; each cycle, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, then the final elongation step at 72°C for 2 min. Further confirmation using duplex PCR was done to amplify *pirA* and *pirB* genes, which are unique to AHPND isolates. The PCR programme for duplex PCR carried out for *pirA* and *pirB* was as follows: denaturation 94°C for 3 min followed by 35 cycles; each cycle 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, then the final elongation step at 72°C for 7 min. Amplified PCR fragments were analysed by agarose gel electrophoresis (1.0%).

Table 1 : Summary of set of primers used in this study

Gene /Method	Primers	DNA Sequence (5' → 3')	Target product (bp)	Reference
AP4 Method	AP4-F1	ATGAGTAACAATATAAAACATGAAAC	1269	Dangtip et al. 2015
	AP4-R1	ACGATTTTCGACGTTCCCCAA		
	AP4-F2	TTGAGAATACGGGACGTGGG	230	
	AP4-R2	GTTAGTCATGTGAGCACCTTC		
<i>ftsZ</i>	F-ftsZ75F	GCTGTT GAACACATGGTACG	750	Sawabe et al. 2007
	R-ftsZ800R	GCACCAGCAAGATCGATATC		
<i>pyrH</i>	<i>pyrH</i> _F	GATCGTATGGCTCAAGAA	450	Chimetto et al. 2009
	<i>pyrH</i> _R	TAGGCATTTTGTGGTCACG		
<i>tdh</i>	<i>tdh</i> _F	GTAAGGTCTCTGACTTTTGGAC	269	Berj et al. 1999
	<i>tdh</i> _R	TGGAATAGAACCTTCATCTTCACC		
<i>trh</i>	<i>trh</i> _F	TTGGCTTCGATATTTTCAGTATCT	500	
	<i>trh</i> _R	CATAACAAACATATGCCCATTTCCG		
<i>tlh</i>	<i>tlh</i> _F	AAAGCGGATTATGCAGAAGCACTG	450	
	<i>tlh</i> _R	GCTACTTTCTAGCATTTTCTCTGC		
<i>pirA^{vp}</i>	VpPirA-284F	TGACTATTCTCAGGATTGGACTG	284	Han et. al 2015
	VpPirA-284R	CACGACTAGCGCCATTGTTA		
<i>pirB^{vp}</i>	VpPirB-392F	TGATGAAGTGATGGGTGCTC	392	
	VpPirB-392R	TGTAAGCGCCGTTAACTCA		

Table 2 : Phenotypic and biochemical characterization of AHPND positive isolates

Isolates code	Phenotypic observation								Identified species
	Gram staining	TCBS	Oxidase	Catalase	TSI	Lysinede carboxylase	ONPG	Hemolysin activity	
C1B	Negative, rod shaped	Yellow	+	+	A/A	+	+	γ	<i>V. harveyi</i>
C2A	Negative, rod shaped	Green bluish, pigmented	+	+	K/A	+	+	γ	<i>V. parahaemolyticus</i>
C2B	Negative, rod shaped	Yellow	+	+	A/A	+	+	γ	<i>V. harveyi</i>
C4A	Negative, rod shaped	Yellow	+	+	A/A	+	+	γ	<i>V. harveyi</i>
C4B	Negative, rod shaped	Green bluish, pigmented	+	+	K/A	+	+	γ	<i>V. parahaemolyticus</i>
D5	Negative, rod shaped	Yellow	+	+	A/A	+	+	γ	<i>V. harveyi</i>

Note : No gas production were observed for TSI analysis. α-hemolysis, α-hemolysin partially breaks down the red blood cells and leaves a greenish color; β-hemolysis, beta-hemolysin breaks down the red blood cells and hemoglobin completely, a clear zone around the bacterial growth; γ-hemolysis, does not produce hemolysins and does not break down the blood cells, no clearing will occur

Electrophoresis gel were viewed under the Alpha Imager (USA). 1kb Plus DNA Ladder (Thermofisher Scientific) were used as a guide to determine the amplicon size.

To screen the positive isolates of *Vibrio* having AHPND virulence, phylogenetic analysis were carried out using *ftsZ* and *pyrH* gene. Identification of positive AHPND isolates was performed by using primers as stated in Table 1. PCR reactions were inclusive of 5.0 µl PCR buffer, 2.0 µl MgCl₂ (100 mM), 1.0 µl dNTPs (10 mM each), 1.0 µl each forward and reverse primers (20 µM), 0.5 GoTaq (Promega, USA) Polymerase (5 U µl⁻¹), 5.0 µl of DNA template (50 ng µl⁻¹) and topped up with nuclease-free water (Thermo Fisher Scientific, USA) to make up the final volume of 25 µl. For amplification of partial *ftsZ* gene, the PCR programme was started with denaturation at 94°C for 3 min followed by 30 cycles; each cycle; 94°C for 1 min, 53.8°C for 1 min and 72°C for 1 min, then the final elongation step was carried out at 72°C for 5 min. Meanwhile, for amplification of partial *pyrH* gene, temperature-cycling programme was carried out, denaturation at 94°C for 3 min followed by 30 cycles; each cycle, 94°C for 1 min, 50.9°C for 1 min and 72°C for 1 min, then the final elongation step at 72°C for 5 min. All PCR fragments were analysed for sequencing by MEGA version 7.0.

Gram staining was carried out with Gram staining set kit (Merck, USA). Growth and colonial pigmentation were observed on TCBS (Oxoid, USA). Oxidase test was performed using oxidase strips (Oxoid, USA). Catalase test by catalase reagent (Sigma-Aldrich). Triple sugar iron agar (TSI) (Oxoid, USA) test was performed to characterize sugar fermenters and hydrogen sulphide production. Besides that, lysine decarboxylase test (Oxoid, USA) was carried out by culturing the isolate in decarboxylase broth. Ortho-Nitrophenyl-β-galactoside (ONPG) test was also performed to identify β-galactosidase activity of the

isolates. All analysis were carried out according to manual supplied by the manufacturers. Observations were based on colour changes occurred before and after the test carried out. All the AHPND positive isolates were then characterize to their susceptibility to different types of antibiotics (Table 3) using disk diffusion method as stated in the Clinical and Laboratory Standards Institutes (CLSI, 2015) on Mueller-Hinton agar (MHA). Antibiotics discs (Oxoid, UK) were put onto the agar and kept at 27°C for 18 to 24 hr. The zone of inhibitions were observed and the diameters were recorded. The acceptable susceptible zone of inhibition values for antibiotics (Table 3) used were based on (CLSI, 2015). All AHPND positive isolates were subjected to hemolytic activity screening. A single colony culture from each isolate were inoculated onto blood agar and kept for 24 hr at 27°C. The type of hemolysin was determined by pigmentation production and hemolysis area on the plate agar.

All positive AHPND isolates were assessed for virulence gene analysis. Multiplex PCR were used to amplify *thermostable direct hemolysin (tdh)*, *TDH-related hemolysin (trh)* and *thermolabile haemolysin (tlh)*. PCR mixtures composed of 5.0 µl of PCR buffer, 2.0 µl of MgCl₂ (100 mM), 1.0 µl of dNTPs (10 mM each), 1.0 µl of each forward and reverse primers (20 µM), 0.5 µl GoTaq Polymerase (Promega, USA) (5 U µl⁻¹), 5.0 µl of template (50 ng µl⁻¹) and topped up with nuclease-free water (Thermo Fisher Scientific, USA) to make up the final volume of 25 µl. Meanwhile, the programme for multiplex PCR for *tdh*, *trh* and *tlh* amplification were as follows: denaturation at 94 °C for 3 min followed by 30 cycles; each cycle, 94°C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, then the final elongation step at 72 °C for 5 min. Amplified DNA fragments were analysed by agarose gel electrophoresis (1.0%) and ethidium bromide staining. Electrophoresis gel were viewed under the Alpha Imager (USA).

Table 3 : Acceptable susceptible zone of inhibition values for antibiotics used based on CLSI (2015)

Antibiotic	Concentration	Zone diameter interpretive criteria (nearest whole, mm)		
		Susceptible	Intermediate	Resistance
Ampicilin (AMP)	10 µg	≥ 17	14 - 16	≤ 13
Bacitracin (B)	10 units	≥ 13	-	-
Cefepime (FEP)	30 µg	≥ 25	19-24	≤ 18
Ceftriaxone (CRO)	30 µg	≥ 23	20 - 22	≤ 19
Chloramphenicol (C)	30 µg	≥ 18	13-17	≤ 12
Ciprofloxacin (CIP)	5 µg	≥ 21	16-20	≤ 15
Cephalothin (KF)	30 µg	≥ 18	15-27	≤ 14
Erythromycin (E)	15 µg	≥ 23	14 - 22	≤ 11
Gentamicin (CN)	10 µg	≥ 15	13 - 14	≤ 12
Kanamycin (K)	30 µg	≥ 18	14 - 17	≤ 13
Streptomycin (S)	25 µg	≥ 15	12 - 14	≤ 11
Tetracycline (TE)	30 µg	≥ 15	12 - 14	≤ 11
Vancomycin (VA)	30 µg	≥ 17	15 - 16	≤ 14

*CLSI: Clinical and Laboratory Standards Institute, 2015.

Virulence study of all the AHPND positive isolates was carried out using brine shrimp, *Artemia* sp. (Mermaid, China). Before the challenge, 50 µl of stock bacterial culture were transferred into 5 ml Marine broth were kept overnight at 28 °C under constant agitation (180 rpm). All the equipments used in this study were autoclaved at 120 °C for 20 min prior to use and all culturing activities were carried out under a laminar flow hood with axenic conditions. Hatching of brine shrimp larvae were performed as per Defoirdt et al. (2005). A 200 mg of *Artemia* sp. cysts was soaked in 18 ml distilled water for 1 hr with strong filtered aeration through 0.22 µm filter to avoid bacterial contamination. After hydration, 660 µl of sodium hydroxide (30% w/v) and 10 ml of sodium hypochlorite (17.3% w/v) were added to initiate the decapsulation process. The reaction of decapsulation were stopped after 120 sec by adding 14 ml of autoclaved sodium thiosulphate pentahydrate (1% w/v) into the mixture. Decapsulated cysts were washed with 1 l of filtered autoclaved artificial sea water and the cysts were collected over a 100 µm sterile sieve. Decapsulated cysts were transferred to sterile 50 ml falcon tubes pre-added with 30 ml of marine broth. The tubes with decapsulated cysts were placed on a rotor (4 rpm), constantly exposing to incandescent light (2000 lux) at 28 °C for 24 hr for hatching. The hatched *Artemia* sp. nauplii was used for challenge trials. For the challenge activity, each isolate suspension was inoculated into each 50 ml Falcon tubes pre-added with 20 nauplii in 20 ml seawater. The challenge trial was carried out at three different bacterial concentration, 1×10^4 cells ml⁻¹, 1×10^5 cells ml⁻¹ and 1×10^6 cells ml⁻¹ in triplicates. The falcon tubes were kept at 28 °C and monitoring was carried out every 12 hr to check survival of *Artemia* nauplii. Survival percentage was calculated to determine the most virulence isolate. Statistical significance of data between groups was evaluated by Analysis of variance (ANOVA), while multiple comparisons were made by Tukey tests. P-value <0.05 was considered significant.

Results and Discussion

Generally, the symptoms showed by AHPND infected shrimp were consistent as those previously reported (Tran et al., 2013). Lethargy, notably smaller size, discolouration of shrimp and hepatopancreas turning whitish have been observed (Leaño and Mohan, 2012b; Soto-Rodriguez et al., 2015; Tran et al., 2013). Thirty three isolates were isolated from the hepatopancreas of diseased shrimp. Fifteen isolates that showed growth on TCBS were further screened with AP4 primers to identify the presence of AHPND virulence genes. Based on all the isolates screened with AP4 method, six isolates showed positive results and the isolates were coded as C1B, C2A, C2B, C4A, C4B and D5.

AP4 method is widely used to detect AHPND cases (Dabu et al., 2015; Dhar et al., 2019). This method has been carefully designed and gives 100% confirmation for AHPND-causing isolates (Sritunyaluksana et al., 2015). As compared to the

predecessor method used, this method has been devised to achieve minimum sensitivity as low as 1 fg of DNA only. This enables direct detection of AHPND-causing isolates on tissue samples and environmental samples (water, sludge and sediment) that may have low to very low level of AHPND bacteria (Dangtip et al., 2015). The designation of the method were based on the toxins that contributed uniquely to the virulence of this disease which consists of two subunits, PirA^{vp} and PirB^{vp} (Lee et al., 2015; Han et al., 2015; Sirikharin et al., 2015). The causative pathogen of AHPND, *V. parahaemolyticus* (Lee et al., 2015) carries a pVA1 plasmid containing both virulent toxin genes, *pirA* and *pirB*. Though, *V. parahaemolyticus* was the first *Vibrio* species found to carry the plasmid (Sirikharin et al., 2015), other *Vibrio* species have been also reported to carry the virulent plasmid (Dong et al., 2017; Kondo et al., 2015; Liu et al., 2015; Restrepo et al., 2018) and recent findings evidently show the occurrence of horizontal transfer of pVA1 plasmid in between *Vibrio* species (Dong et al., 2019). However, it is still debatable to pin-point which *Vibrio* species is the original carrier of plasmid as Wangman et al. (2018) had discovered both PirA and PirB toxins in an archived *V. campbelli*, isolated seven years before the first case of AHPND occurred in 2009 in China (Zhang et al., 2012).

Phylogenetic analyses were carried out using neighbour-joining method to confirm their taxonomic position in the genus *Vibrio*. Based on the phylogenetic analysis using *ftsZ* gene, C2A and C4B were clustered with *V. parahaemolyticus* and C1B, C2B, C4A and D5 were clustered with *V. harveyi* (Fig. 1). Phylogenetic analysis using *pyrH* gene also showed C2A and C4B clustered with *V. parahaemolyticus* and C1B, C2B, C4A, and D5 clustered with *V. harveyi* (Fig. 2). Both analysis were carried out using neighbour-joining (Kimura 2-parameter model) of concatenated phylogenetic tree. Both *ftsZ* and *pyrH* were commonly used in multilocus sequence analysis (MLSA) (Al-Saari et al., 2015; Hoffman et al., 2012; Sawabe et al., 2013; Steinum et al., 2016; Tanaka et al., 2018; Thompson et al., 2007). Both gene codes for housekeeping genes, *ftsZ* a cell division protein and *pyrH*, uridylyl transferase and were also among the genes that gave the highest resolution in distinctively differentiating *V. harveyi* and *V. campbelli* (Thompson et al., 2007). Both genes were used along with other genes to confirm a novel *Vibrio* species, *V. caribbeanicus* sp. nov (Hoffman et al., 2012), *Vibrio tritonis* sp. nov (Sawabe et al., 2013) and to construct the phylogeny of *Rumoiensis* clade species (Al-Saari et al., 2015), a relatively new clade of *Vibrionaceae* (Sawabe et al., 2013). According to Steinum et al. (2016), multilocus sequence analysis similarities for all eight genes used (including *ftsZ* and *pyrH*), were between 97.5% to 99.0% and this percentage is well above the 95% threshold to distinguish between *Vibrio* species as proposed by Sawabe et al. (2007).

All the isolates were negative and rod shaped (Table 2). This indicated that all the six isolates belonged to Gram-negative bacteria. The growth and colonial pigmentation of six isolates

Fig. 1 : Neighbor-joining phylogeny (Kimura 2-parameter model) of concatenated partial *ftsZ* gene sequences from *Vibrio* sp. strains. Bootstrap values represent 1,000 replications. Reference sequences were acquired from the NCBI GenBank.

Fig. 2 : Neighbor-joining phylogeny (Kimura 2-parameter model) of concatenated partial *pyrH* gene sequences from *Vibrio* sp. strains. Bootstrap values represent 1,000 replications. Reference sequences were acquired from the NCBI GenBank.

Table 4 : Antibiotic resistance profile and MAR index of AHPND positive isolates to thirteen antibiotics

Isolate code	Species	AMP	B	FEP	CRO	C	CIP	KF	E	CN	K	S	TE	VA	MAR Index
C1B	<i>V. harveyi</i>	R	R	R	I	S	S	S	R	S	S	S	S	R	0.4
C2A	<i>V. parahaemolyticus</i>	S	R	S	S	S	I	I	I	S	S	S	S	R	0.2
C2B	<i>V. harveyi</i>	S	S	R	R	S	I	S	I	S	S	R	S	S	0.2
C4A	<i>V. harveyi</i>	S	S	R	R	S	S	I	I	R	R	R	S	S	0.4
C4B	<i>V. parahaemolyticus</i>	R	R	S	S	S	I	S	I	S	S	S	S	R	0.2
D5	<i>V. harveyi</i>	S	R	S	R	R	I	S	R	S	S	S	S	I	0.3
Total isolates tested		6	6	6	6	6	6	6	6	6	6	6	6	6	
Total resistance isolates		2	4	3	3	1	0	0	2	1	1	2	0	3	
Percentage of resistance isolates (%)		33.3 (2)	66.7 (4)	50.0 (3)	50.0 (3)	16.7 (1)	0.0 (0)	0.0 (0)	33.3 (2)	16.7 (1)	16.7 (1)	33.3 (2)	0.0 (0)	50.0 (3)	
Percentage of intermediate resistance isolates (%)		0.0 (0)	0.0 (0)	0.0 (0)	16.7 (1)	0.0 (0)	66.7 (4)	33.3 (2)	66.7 (4)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	16.7 (1)	
Percentage of susceptible isolates (%)		66.7 (4)	33.3 (2)	50.0 (3)	50.0 (3)	83.3 (5)	33.3 (2)	66.7 (4)	0.0 (0)	83.3 (5)	83.3 (5)	66.7 (4)	100.0 (6)	33.3 (2)	

Note: Symbol "R": resistant, "I": intermediate "S": susceptible, AMP: ampicillin, B: bacitracin, FEP: cefepime, CRO: ceftriaxone, C: chloramphenicol, CIP: ciprofloxacin, KF: cephalothin, E: erythromycin, CN: gentamicin, K: kanamycin, S: streptomycin, TE: tetracycline, VA: vancomycin

Table 5 : Presence of virulence genes in AHPND positive isolates

Isolates	Virulence index				
	<i>pirA</i>	<i>pirB</i>	<i>tdh</i>	<i>trh</i>	<i>tliH</i>
C1B	+	+	-	-	+
C2A	+	+	-	-	+
C2B	+	+	-	-	+
C4A	+	+	-	-	+
C4B	+	+	-	-	+
D5	+	+	-	-	+

observed on TCBS agar revealed that, four out of six isolates showed growth and formed yellow colonies and another two isolates showed growth and formed green-bluish pigmented colonies. The oxidase and catalase test of all isolates were positive (Table 2). Triple Sugar Iron Test was used to detect bacterial ability to ferment lactose, sucrose and glucose and their ability to produce H₂S (Farmer and Hickman-Brenner, 1992). Based on the TSI results, two isolates produced alkaline-over-acid reaction (K/A) and another four produced acid-over-acid reaction (A/A). No gas production observed during TSI analysis. All the isolates were positive for the lysine decarboxylase test and ONPG test. Based on the phenotypic characterization, two of the positive isolates were *V. parahaemolyticus* and another four were *V. harveyi*. Table 2 showed the results of haemolysin analysis of all the isolates. All the isolates showed γ -hemolysis, as no breakdown of blood cells occurred (no clearing occur).

Thirteen antibiotics were used to screen the antibiotic susceptibility of isolates (Table 3). Based on the results (Table 4), 66.6% (n=6) of all the isolates showed resistancy towards bacitracin. This was the highest resistancy observed among all

the tested antibiotics. 50.0% (n=6) resistancy against cefepime, ceftriaxone and vancomycin. Only 33.3% (n=6) of the isolates were resistance against ampicillin, erythromycin and streptomycin. 16.7 % (n=6) of the isolates recorded against chloramphenicol, gentamicin and kanamycin. No resistancy was observed against ciprofloxacin, cephalothin and tetracycline. Multiple antibiotic resistance (MAR) were calculated for all the antibiotics. MAR index more than 0.2 indicates that the isolates were isolated from a high risk source of exposure where antibiotics are often used (Krumperman, 1983). Three isolates (50.0%) had MAR index greater than 0.2. The MAR index calculated indicated that there is a potential that the isolates come from a high risk of source where antibiotics were used (Table 4). Further tests are required for larger number of isolates to confirm this. The isolates showed more than 50 percent resistancy against bacitracin, cefepime, ceftriaxone and vancomycin. It is not uncommon that in fish industry, particularly in developing countries like Malaysia, a lot of antibiotics have been used to avoid infection (Cabello *et al.*, 2006). Though the virulency of AHPND isolates has been linked to tetracycline resistancy before (Dong *et al.*, 2017; Han *et al.*, 2015), all the isolates in this study

Fig. 3 : Survival (%) of *Artemia* nauplii after 60 hours of challenged with isolate A) C1B B) C2A C) C2B D) C4A E) C4B and F) D5 at three different bacterial concentrations; 1×10^4 cells ml⁻¹, 1×10^6 cells ml⁻¹ and 1×10^8 cells ml⁻¹. Control represents non-challenge group. Significant difference between challenged and control groups were evaluated at $P < .05$ (*), $P < .01$ (**) and $P < .001$ (***) using one-way ANOVA analysis.

were found susceptible against tetracycline. This is in agreement with the AHPND isolates from Vietnam that did not carry *tetB* and

susceptible against tetracycline (Han *et al.*, 2015). This could shed some lights to the origin of this local isolates.

Fig. 4 : Comparison of survival (%) of *Artemia* sp. nauplii at 1×10^8 cells ml^{-1} for 60 hr of challenged with isolate C1B, C2A, C2B, C4A, C4B and D5. Control represents non-challenge group.

Table 5 shows the results of virulence gene screening of all the AHPND positive isolates. The isolates were screened for *thermostable direct hemolysin (tdh)*, *TDH-related hemolysin (trh)* and *thermolabile haemolysin (tlh)*. All the isolates were *tdh* and *trh* negative, and *tlh* positive. All the AHPND isolates showed negative results upon screening with thermostable direct hemolysin (*tdh*), TDH-related hemolysin (*trh*) (Miyamoto *et al.*, 1969; Honda and Lida, 1993). This is true as these isolates were isolated from an environmental source. Observation on the hemolysin plate also showed no breakdown of blood cells occurred (no clearing occur) indicating γ -hemolysis. Both these genes are actually vital for the virulence of pathogen as these genes encode the pore-forming proteins involved in the invasiveness of pathogen. All isolates were *thermolabile haemolysin gene (tlh)* (Taniguchi *et al.*, 1986) positive and has been reported earlier that this gene is widespread in *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *V. fischeri*, *V. mimicus*, *V. natriegens*, *V. proteolyticus*, *V. anguillarum* and *V. vulnificus* (Wang *et al.*, 2007).

As reported by Kreger and Lockwood (1981), highly virulent strains of bacteria can produce higher amount of toxins as compared to less virulent strains. The virulence study of isolates using *Artemia* sp. nauplii was conducted. *Artemia* sp. nauplii was challenged with all six positive AHPND isolates. Three different bacterial concentrations 1×10^4 cells ml^{-1} , 1×10^6 cells ml^{-1} and 1×10^8 cells ml^{-1} were selected in this study. Fig. 3 shows the survival percentage of *Artemia* sp. challenged with all six AHPND isolates at three different bacterial concentrations. At 1×10^4 cells ml^{-1} , after 48 hrs of challenge, isolate C2A and C4B (*V. parahaemolyticus*), showed less than 40% survival, as compared to other isolates (Fig. 3). Meanwhile, at 1×10^6 cells ml^{-1} , after 48 hrs of challenge, both isolates also showed less than 30% survival as compared to other isolates (Fig. 3). All the isolates were also compared and

analysed at the bacterial concentration 1×10^8 cells ml^{-1} . Fig. 4 shows that *Artemia* sp. challenged with C2A and C4B (*V. parahaemolyticus*) showed 100% mortality after incubation for only 12 hr. Meanwhile, *Artemia* sp. challenged with C1B, C2B, C4A and D5 (*V. harveyi*) started showing more than 100% mortality only after 48 hrs. In this study, AHPND positive isolates of *V. parahaemolyticus* showed more virulence than *V. harveyi*. In this study, *V. parahaemolyticus* was found as the most virulent isolate. However, Dong *et al.* (2017) demonstrated that *P. vannamei* infected with both AHPND positive *V. parahaemolyticus* and AHPND positive *V. campbelli* presented similar mortality and suggested that the pathogenicity encoded of AHPND is independent within closely related *Vibrio* species (*Harveyi* clade).

In conclusion, this study showed that, in Malaysia, both *V. parahaemolyticus* and *V. harveyi* could both be the causative pathogen of AHPND outbreak in local shrimp farm. Although after ten years of first outbreak in this country, AHPND cases still occur. Looking further into the genome construction of each locally isolated species and analyzing the pathogenic plasmids would give us more insights on how to manage and control the disease.

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References

- Akazawa, N. and M. Eguchi: Pond sludge and increased pH cause early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in cultured white shrimp. *BjoMSA*, **1**, 92–96 (2017).
- Al-Saari, N., F. Gao, A.K.M.A. Rohul, K. Sato, K. Sato and S. Mino: Advanced microbial taxonomy combined with genome-based approaches reveals that *V. astriarenae* sp. Nov., an agarolytic marine bacterium, forms a new clade in *Vibrionaceae*. *PLoS ONE*, **10**, 1–17 (2015).
- Annual Fisheries Statistics. 2005–2014. Department of Fisheries Malaysia.
- Bej, A.K., D.P. Patterson, C.W. Brasher, M.C.L. Vickery, D.D. Jones and C.A. Kaysner: Detection of total and hemolysin-producing *V. parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J. Microbiol. Methods*, **36**, 215–225 (1999).
- Cabello, F.C.: Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environ. Microbiol.*, **8**, 1137–1144 (2006).
- Chu, K.B., I. Ahmad, A.S. Zahrah, J. Irene, J. Norazila, N.Y. Nik Haiha, Y. Fadziilah, M. Mohammed, B.S. Rokhaiya, M. Omar and N.P. Teoh: Current status of acute hepatopancreatic disease (AHPND) of farmed shrimp in Malaysia. In: Addressing acute hepatopancreatic necrosis disease (AHPND) and other transboundary disease for improved aquatic animal health in Southeast Asia (Eds.: R.V. Jr. Pakingking, E.G.T. de Jesus-Ayson and B.O. Acosta) Tigbauan,

- Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center, p. 55 (2016).
- Chimetto, L.A., M. Brocchi, M. Gondo, C.C. Thompson, B. Gomez-Gil, and F.L. Thompson: Genomic diversity of vibrios associated with the Brazilian coral *Mussismilia hispida* and its sympatric zoanthids (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*). *J. Appl. Microbiol.*, **106**, 1818-1826 (2009).
- CLSI: Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI. Wayne, Pennsylvania, USA (2015).
- Dabu, I.M., J.J. Lim, P.M.T. Arabit, S.J.A.B. Orense, J.A. Tabardillo, V.L. Corre and M.B.B. Maningas: The first record of acute hepatopancreatic necrosis disease in the Philippines. *Aquacult. Res.*, **48**, 792-799 (2015).
- Dangtip, S., R. Sirikharin, P. Sanguanrut, S. Thitamadee, K. Sritunyalucksana, S. Taengchaiyaphum, R. Mavichak, P. Proespraiwong and T. Flegel: AP4 method for two-tube nested PCR detection of AHPND isolates *V. parahaemolyticus*. *Aquacult. Rep.*, **2**, 158-162 (2015).
- Defoirdt, T., P. Bossier, P. Sorgeloos and W. Verstraete: The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *V. anguillarum* and *V. harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. *Environ. Microbiol.*, **7**, 1239-1247 (2005).
- Dhar, A.K., P. Piamsomboon, L.F.A. Caro, S. Kanrar, R. Jr. Adami and Y.S. Juan: First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. *Dis. Aquat. Organ.*, **132**, 241-247 (2019).
- Dong, X., D. Bi, H. Wang, P. Zou, G. Xie, X. Wan, Q. Yang, Y. Zhu, M. Chen, C. Guo, Z. Liu, W. Wang and J. Huang: pirAB⁺-bearing *V. parahaemolyticus* and *V. campbellii* pathogens isolated from the same AHPND-affected pond possess high similar pathogenic plasmids. *Front. Microbiol.*, **8**, 1-9 (2017).
- Dong, X., J. Chen, J. Song, H. Wang, W. Wang, Y. Ren, C. Guo, X. Wang, K. F. J. Tang, and J. Huang: Evidence of the horizontal transfer of pVA1-type plasmid from AHPND causing *V. campbellii* to non-AHPND *V. owensii*. *Aquaculture*, **503**, 396-402 (2019).
- Farmer, J.J. and F.W. Hickman-Brenner: The General *Vibrio* and *Photobacterium*, in *The Prokaryotes*. Vol. 3, Springer-Verlag, NY (1992).
- Han, J.E., K.F.J. Tang, L.H. Tran and D.V. Lightner: *Photobacterium* insect-related (Pir) toxin-like genes in a plasmid of *V. parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Organ.*, **113**, 33-40 (2015).
- Hoffman, M., S.R. Monday, M.W. Allard, E.A. Strain, P. Whittaker, M. Naum, P.J. McCarthy, J.V. Lopez, M. Fischer and E.W. Brown: *V. caribbeanicus* sp. nov., isolated from the marine sponge *Scleritoderma cyanea*. *Int. J. Syst. Evol. Microbiol.*, **62**, 1736-1743 (2012).
- Honda, T., and T. Iida: The pathogenicity of *V. parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. *Clin. Microbiol. Rev.*, **4**, 106-113 (1993).
- Joshi, J., J. Srisala, V.H. Truong, I.T. Chen, B. Nuangsaeng, O. Suthienkul, C.F. Lo, T.W. Flegel, K. Sritunyalucksana and S. Thitamadee: Variation in *V. parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, **428**, 297-302 (2014).
- Kondo, H., P.T. Van, L.T. Dang and I. Hirono: Draft genome sequence of non-*V. parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. *Genome Announc.*, **3**, 1-2 (2015).
- Kreger, A.S. and D. Lockwood: Detection of extracellular toxin(s) produced by *V. vulnificus*. *Infect. Immun.*, **33**, 583-590 (1981).
- Krumperman, P.H.: Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.*, **46**, 165-170 (1983).
- Le Moullac, G. and P. Haffner: Environmental factors affecting immune response in Crustacea. *Aquaculture*, **191**, 121-131 (2000).
- Leaño, E.M. and C.V. Mohan: Early mortality syndrome threatens Asia's shrimp farms. *GAA*, **8**, 38-39 (2012a).
- Leaño, E.M. and C.V. Mohan: Emerging threat in the Asian Shrimp Industry: Early Mortality Syndrome (EMS)/Acute Hepatopancreatic Necrosis Syndrome (AHPNS). Network of Aquaculture Centres in Asia-Pacific. Asian Fisheries Society (2012b).
- Lee, C.T., I.T. Chen, Y.T. Yang, T.P. Ko, Y.T. Huang, J.Y. Huang, M.F. Huang, S.J. Lin, C.Y. Chen, S.S. Lin, D.V. Lightner, H.C. Wang, A.H.J. Wang, H.C. Wang, L.I. Hor and C.F. Lo: *V. parahaemolyticus*: An opportunistic marine pathogen becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proc. Natl. Acad. Sci.*, **112**, 10798-10803 (2015).
- Li, C.C. and J.C. Chen: The immune response of white shrimp *L. vannamei* and its susceptibility to *V. alginolyticus* under low and high pH stress. *Fish Shellfish Immunol.*, **25**, 701-709 (2008).
- Lightner, D.V., R.M. Redman, C.R. Pantoja, B.L. Noble and L. Tran: Early mortality syndrome affects shrimp in Asia. *GAA*, **15**, 40-41 (2012).
- Liu, L., J. Xiao, X. Xia, Z. Pan, S. Yan and Y. Wang: Draft genome sequence of *V. owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. *Genome Announc.*, **3**, 1 (2015).
- Miyamoto, Y., T. Kato, S. Obara, S. Akiyama, K. Takaziwa and S. Yamai: In vitro characteristics of *V. parahaemolyticus*: Its close correlation with human pathogenicity. *J. Bacteriol.*, **100**, 1147-1149 (1969).
- Nunan, L., D. Lightner, C. Pantoja and S. Gomez-Jimenez: Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Dis. Aquat. Organ.*, **111**, 81-86 (2014).
- Restrepo, L., B. Bayot, S. Arciniegas, L. Bajana, I. Betancourt, F. Panchana and A.R. Munoz: PirVP genes causing AHPND identified in a new *Vibrio* species (*V. punensis*) within the commensal *Orientalis* clade. *Sci. Rep.*, **8**, 13080 (2018).
- Saravanan, V., H. Sanath-Kumar and I. Karunasagar: Putative virulence genes of *V. cholerae* from seafoods and the coastal environment of Southwest India. *Int. J. Food Microbiol.*, **119**, 329-333 (2007).
- Sawabe, T., K. Kita-Tsukamoto and F.L. Thompson: Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J. Bacteriol.*, **189**, 7932-7936 (2007).
- Sawabe, T., Y. Ogura, Y. Matsumura, G. Feng, A.K.M.R. Amin, S. Mino, S. Nakagawa, T. Sawabe, R. Kumar, Y. Fukui, M. Satomi, R. Matsushima, F. L. Thompson, B. Gomez-Gil, R. Christen, F. Maruyama, K. Kurokawa and T. Hayashi: Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *V. trinitinus* sp. nov. *Front. Microbiol.*, **4**, 1-14 (2013).
- Sirikharin, R., S. Taengchaiyaphum, P. Sanguanrut, T.D. Chi, R. Mavinchak and P. Proespraiwong: Characterization and PCR detection of binary, Pir-like toxins from *V. parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. *PLoS ONE*, **10**, 1-16 (2015).

- Soto-Rodriguez, S.A., B. Gomez-Gil, R. Lazano-Olvera, M. Betancourt-Lazano and M.S. Morales-Convarrubias: Field and experimental evidence of *V. parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultures shrimp (*L. vannamei*) in northwestern Mexico. *Appl. Environ. Microbiol.*, **81**, 1689-1699 (2015).
- Sritunyalucksana, K., S. Dangtip, P. Sanguanrut, R. Sirikharin, S. Taengchaiyaphum, S. Thitamadee, R. Mavichak, P. Proespraiwong and T.W. Flegel: A two-tube, nested PCR detection method for AHPND bacteria. Network of Aquaculture Centres in Asia-Pacific (NACA) (2015).
- Steinum, T.M., S. Karatas, N.T. Martinussen, P.M. Meirelles, F.L. Thompson and D.J. Colquhoun: Multi-locus sequence analysis of the close relatives *V. anguillarum* and *V. ordalii*. *Appl. Environ. Microbiol.*, **82**, 5496–5504 (2016).
- Tanaka, M., S. Mino, Y. Ogura, T. Hayashi and T. Sawabe: Availability of nanopore sequences in the genome taxonomy for *Vibrionaceae* systematics: *Rumoiensis* clade species as a test case. *Peer. J.*, **6**, 1-12 (2018).
- Taniguchi, H., H. Hirano, S. Kubomura, K. Higashi and Y. Mizuguchi: Comparison of the nucleotide sequences of the genes for the thermolabile hemolysin from *V. parahaemolyticus*. *Microb. Path.*, **1**, 425-432 (1986).
- Thompson, F.L., B. Gomez-Gil, A.T. Vasconcelos and T. Sawabe: Multilocus sequence analysis reveals that *V. harveyi* and *V. campbelli* are distinct species. *Appl. Environ. Microbiol.*, **73**, 4279-4285 (2007).
- Tran, L., L. Nunan, R.M. Redman, L.L. Mohney, C.R. Pantoja, K. Fitzsimmons and D.V. Lightner: Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Organ.*, **105**, 45-55 (2013).
- Wang, X.H., X.H. Zhang, Y.B. Zhong, B.G. Sun and J.X. Chen: Genes encoding the *V. harveyi* haemolysin (*vhh*)/thermolabile haemolysin (*tlh*) are widespread in vibrios. *Acta Microbiol. Sin.*, **47**, 874-881 (2007).
- Wangman, P., S. Longyant, S. Taengchaiyaphum, S. Senapin, P. Sithigorngul and P. Chaivisuthangkura: Pir A & B toxins discovered in archived shrimp pathogenic *V. campbellii* isolated long before EMS/AHPND outbreaks. *Aquaculture*, **497**, 494–502 (2018).
- Zhang, B.C., F. Liu, H.H. Bian, J. Liu, L.Q. Pan and J. Huang: Isolation, identification, and pathogenicity analysis of a *V. parahaemolyticus* strain from *L. vannamei*. *Prog. Fish. Sci.*, **3**, 56–62 (2012).