Diagnostic approaches and contribution of next-generation sequencing technologies in genomic investigation of *Vibrio parahaemolyticus* that caused acute hepatopancreatic necrosis disease (AHPND)

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Abstract

A unique strain of *Vibrio parahaemolyticus* (designated as VP_{AHPND}) causes acute hepatopancreatic necrosis disease (AHPND), a deadly bacterial disease associated with mass mortality in cultured shrimps since 2009. AHPND is responsible for severe economic losses worldwide, causing multimillion-dollar loss annually. Because of the rapid and high mortality rates in shrimps, substantial research has been carried out to develop rapid detection techniques. Also, recent technological advances such as the next-generation sequencing (NGS) have made it possible to elucidate relevant information about a pathogen in a single assay. This review summarizes the current research pertaining to VP_{AHPND}, focusing on diagnosis and contribution of NGS technologies in the genomic studies of AHPND.

Keywords Acute hepatopancreatic necrosis disease · *Vibrio parahaemolyticus* · Diagnostics · Next-generation sequencing

Introduction

Shrimp aquaculture is the fastest-growing production sector in the fishery industry, and it plays a big role in worldwide economy, market demands and employment (FAO 2016). The

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increases in market demands has led the shrimp aquaculture to develop into an important export-oriented food industry especially in Southeast Asia, generating billions of US dollars in export income annually (FAO 2016; GOAL 2018). As the shrimp industry expands, more issues and challenges are faced by farmers due to intensification of shrimp farming. Among these issues and challenges, shrimp diseases remain the most critical challenge as a limiting factor for the expansion of shrimp production (GOAL 2018). The shrimp industry is challenged with a plethora of pathogens such as infection with infectious hypodermal and haematopoietic necrosis (IHHN), infectious myonecrosis (IMN), white tail disease (WTD), taura syndrome (TS), yellow head disease (YHD) and white spot disease (WSD), and it had caused a loss of approximately US\$ 15 billion in revenue over the past 15 years. In addition, lately a bacterial disease known as the acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus*, had superseded other diseases, causing high and rapid mortalities in all types of cultured shrimps and economical loss incurred more US\$ 1 billion, annually (Flegel and Timothy 2012; Tran et al. 2013).

AHPND, formerly known as EMS or AHPNS, is a shrimp disease that causes massive early mortality in shrimp ponds, reaching up to 100% within a few days after the infection (Lightner et al. 2012a, b; NACA 2012). The disease affects multiple shrimp species, especially the Asian tiger shrimp (*Penaeus monodon*) and white leg shrimp (*Litopenaeus vannamei*) via oral routes and cohabitation (Flegel and Timothy 2012; Leão and Mohan 2012; Lightner et al. 2012a, b). AHPND usually starts to develop approximately 8 days after the ponds are stocked, and severe mortalities could occur as early as the postlarvae stage on 12 to 40 days of culture. Clinical symptoms include pale atrophied hepatopancreas, soft shells, black spots or streaks that are visible in the hepatopancreas of the infected shrimps (NACA 2014). This disease was reported to have emerged in China (2009), followed by Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2014), Philippines (2015) and lastly in the Latin America (2016) (Dabu et al. 2017; Flegel and Timothy 2012; Gomez-Gil et al. 2014a; Leobert et al. 2015; Lightner et al. 2012a, b; Nunan et al. 2014; Restrepo et al. 2016; Tran et al. 2013) (Fig. 1).

Fig. 1 Emergence of AHPND in different regions around the world. AHPND outbreaks have been reported in shrimp farms in China (2009), Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2014), Philippines (2015) and Latin America (2016)

The etiologic agent of the disease was identified as a unique bacterial strain of *V. parahaemolyticus* (NACA 2012). *V. parahaemolyticus* strain that causes AHPND in shrimps (designated as VP_{AHPND}) differs genetically from other *V. parahaemolyticus* strains as it carries an extrachromosomal plasmid (pVA) encoded homologues of the *Photobacterium* insect-related (Pir) PirA and PirB binary toxins, namely, as *pirA^{vp}* and *pirB^{vp}*, that are responsible for AHPND (Lee et al. 2015; Tran et al. 2013). However, AHPND-causing bacteria are not only limited to *V. parahaemolyticus* strains but are also associated with other species of *Vibrio* such as *V. harveyi*, *V. owensii* and *V. campbellii* (Dong et al. 2017; Kondo et al. 2015; Liu et al. 2015; Tran et al. 2013). These findings suggested the possibility of horizontal gene transfer events within the *Vibrio* genus, thus explaining the diversity of *Vibrio* spp. in causing AHPND (Castillo et al. 2018). This shows that the causative agent of AHPND is not restricted in a single species but can be found in multiple species of *Vibrio*, beyond the clade boundaries through the acquisition of the pathogenic plasmid (Restrepo et al. 2018). In this review, general information of AHPND and the causative agent, VP_{AHPND}, are discussed with an emphasis on the diagnosis and contribution of NGS technologies in the genomic studies of VP_{AHPND}.

***Vibrio parahaemolyticus*: isolation and preservation**

Vibrio parahaemolyticus is a Gram-negative, rod-shaped bacterium, belonging to the family *Vibrionaceae*, ubiquitous in marine, estuarine ecosystem as well as aquaculture farms (CDC 2018). It is polarly flagellated, halophilic and facultative anaerobic. This bacterium grows rapidly in high levels of bile salts and alkaline conditions. Therefore, media such as alkaline peptone water (APW), tryptic soy broth (TSB+) or Tryptic soy agar added with 1.5–2.5% of sodium chloride (NaCl), thiosulfate citrate bile sucrose agar (TCBS) and CHROMagar *Vibrio* are suitable to culture this bacterium (Nunan et al. 2014; Sirikharin et al. 2014; Soto-Rodriguez et al. 2015; Tran et al. 2013). *V. parahaemolyticus* grows ideally at temperatures between 28 and 35 °C; thus, inoculated broth or agar plates can be incubated at the temperature within this range for 18–24 h (Nunan et al. 2014; Soto-Rodriguez et al. 2015; Tran et al. 2013). To prepare the stock culture of *V. parahaemolyticus*, the culture must be preserved in TSB + (1–2% NaCl) with 50% glycerol and stored at –80 °C. As *V. parahaemolyticus* is sensitive to freezing (4 to –24 °C), preservation or storage of this bacterium under this range of temperature will lead to reduction in cell density for short-term storage (Han et al. 2015a; Nunan et al. 2014).

Diagnostic methods for the detection of VP_{AHPND}

Diagnosis of AHPND is a crucial aspect of proper control measure to minimize economic losses caused by VP_{AHPND}. However, in order to have accurate and reliable detection, several procedures are recommended prior to the detection of VP_{AHPND} from shrimp samples.

- i. A minimum of 10 shrimp specimens or more must be sampled from ponds that are suspected with AHPND infection to ensure that at least one specimen infected with this disease is detected.

- ii. Live shrimp, moribund shrimp and shrimp with gross clinical signs are preferred samples/specimens for examination.
- iii. The shrimp digestive system (hepatopancreas, stomach, midgut and hindgut) is the most suitable parts for the diagnosis of AHPND, and it must be excised under aseptic conditions.
- iv. Live shrimp or fresh shrimp specimens are preferred for examination as the causative agent; VP_{AHPND} strain is sensitive to cold temperature (4 to -24°C). Otherwise, the shrimp specimens can be kept in 100% ethanol or frozen at -80°C .
- v. Liquid medium should be used instead of solid medium to prepare and culture the VP_{AHPND} inoculum to induce the pathological signs of AHPND properly.

Before the introduction of PCR methods to detect VP_{AHPND}, the diagnosis of AHPND was confirmed through conventional culture-dependent and identification techniques. However, rapid, specific and reliable detection of infectious AHPND is important in preventing large disease outbreaks. In relation to AHPND detection, both culture-dependent and culture-independent methods are discussed below.

Culture-dependent techniques

Traditional diagnostic methods include interpretation of clinical and histological signs, culturing of pathogen in or on a selective medium and analysis of the morphological or biochemical characteristics of the presumptive pathogen. Different selective media such as TCBS or CHROMagar *Vibrio* have been developed for the detection of *V. parahaemolyticus* (Soto-Rodriguez et al. 2015). However, these media are not conclusive as other *Vibrio* spp. are also able to grow on these media (Bolinches et al. 1988). Biochemical properties of *V. parahaemolyticus*, such as nitrate reduction activity, oxidase and catalase activity and indole production, are used to confirm its identity (Buller 2004). However, identification based on metabolic fingerprinting is not always conclusive, requiring additional confirmation assays (Buller 2004). Furthermore, VP_{AHPND} strains are phenotypically like the common *V. parahaemolyticus* strains in the environment, so it is difficult to distinguish VP_{AHPND} from other *V. parahaemolyticus* strains that do not cause AHPND.

As a primary diagnostic approach, clinical signs of AHPND are used to examine infected shrimps. These early diagnostic clinical signs must be based on the OIE technical fact sheet and NACA disease card (NACA 2014; OIE 2013a). AHPND-infected shrimp generally show clinical symptoms which include pale atrophied hepatopancreas, soft shells, empty gut in early infection and black spots or streaks are visible in the hepatopancreas of the infected shrimp during the terminal phase (NACA 2014). These clinical signs are essential during the collection of shrimp samples for examination and can be further confirmed by histopathological observation and PCR.

Histopathological examination is an important classical method of detection and demonstration of transmission of pathogen to new hosts. To examine the samples, light microscope examination, transmission electron microscopy (TEM) and scanned electron microscopy (SEM) can be used for histological observation of the AHPND-infected shrimps (Bell and Lightner 1988; Lightner 1996). Generally, the AHPND-infected shrimp show sloughing of hepatopancreatic tubule epithelial cells and atrophy at the early stages. This is due to the medial to distal dysfunction of the R cells (resorptive), B cells (blister-like), F cells (fibrillar) and finally the E cells (embryonic). Subsequently, necrosis of hepatopancreas tubule epithelial

cells and massive haemolytic infiltration are observed at later stages (NACA 2014; OIE 2013b). At this stage, the HP are generally sloughed and provide as a substrate for secondary bacterial infection that causes complete destruction of the HP.

The methods discussed above permit pathogen detection but are time-consuming and labour-intensive and have limited accuracy and reliability. These conventional techniques are limiting and inadequate to manage large number of samples. These drawbacks have promoted the development of alternative, rapid and culture-independent detection and identification techniques. Nevertheless, these culture-dependent methods are still recommended to complement the culture-independent techniques.

Culture-independent techniques

Before the introduction of PCR methods for AHPND-causing bacterial strains detection, the diagnosis of AHPND was confirmed through the gross clinical signs and histopathology of shrimp bodies (NACA 2014). However, specific and rapid detection methods are needed for the early detection of AHPND-causing bacterial strains (Santos et al. 2020). These nucleic acid-based techniques have the advantage of being both highly specific and sensitive. Up to date, several PCR-based methods have been developed for the detection of VP_{AHPND}. The first PCR-based method utilized two sets of primers (AP1 and AP2) (Flegel and Lo 2014). These primers served as an interim detection tool for AHPND as it only targets the plasmid sequences and not the toxin genes that are responsible for AHPND (Flegel and Lo 2014). Next, another improved PCR primer (TUMSAT-Vp3) which is also based on plasmid sequences was developed (Tinwongger et al. 2014). Later, Flegel and Lo introduced another set of new and improved AP3 primer based on the gene sequence of a putative protein that was present only in AHPND-causing strains (Flegel and Lo 2014). The utility of the AP3 primer has been shown to give higher sensitivity and specificity compared with the previous AP1 and AP2 primers (Sirikharin et al. 2014). In addition, an enhanced nested two-step PCR method (AP4) was developed as an alternative to AP3-PCR (Sritunyalucksana et al. 2015). This approach was able to detect low levels of AHPND-causing bacteria, and the sensitivity was 100 times higher than AP3-PCR method (Sritunyalucksana et al. 2015). Commercial diagnostic test kits such as the IQ Plus TM AHPND/EMS KIT (POCKIT) (Zorriehzahra and Banaederakhshan 2015) and AHPND/EMS Toxin 1 Detection and Prevention System PCR kit are available for the detection of AHPND (Leobert et al. 2015). In addition, real-time PCR method such as the VP_{AHPND}-specific TaqMan real-time PCR (Han et al. 2015b), isothermal loop-mediated amplification (LAMP) method (Arunrut et al. 2016), duplex PCR and multiplex PCR methods were introduced and have high sensitivity in the detection of VP_{AHPND}.

Several studies have shown that VP_{AHPND} mutants with *pirA*^{vp} and *pirB*^{vp} genes knock out could still induce AHPND in shrimps (Phiwsaiya et al. 2017; Restrepo et al. 2016). This has raised concern as PCR methods based on AP1, AP2 and TUMSAT-Vp3 for the VP_{AHPND} detection only target the plasmid sequences, and this could lead to false positive results. Thus, the AP3-PCR and duplex PCR methods that were designed to detect both the *pirA*^{vp} and *pirB*^{vp} genes, respectively, are recommended for specific detection of VP_{AHPND} (Devadas et al. 2019).

Besides PCR methods, several new detection approaches such as monoclonal antibodies assays and biosensor are introduced to detect VP_{AHPND}. Antibody-based assays present a simple, rapid and low-cost detection system with high sensitivity and specificity for detection of diseases. A monoclonal antibody (MAb) specific to ToxA and ToxB that are present in

VP_{AHPND} has been developed (Wangman et al. 2017). This MAb can be used in the dot-ELISA approach for complex samples for the detection of VP_{AHPND}. Biosensing technology has also been applied in the detection of VP_{AHPND} (Rizan et al. 2018). A biosensor is an analytical device which converts a biological response into an electrical signal. It acts to detect a biological analyte such as nucleic acids, antibodies, enzymes, proteins or whole cells and transmits the signals to a transducer which then convert the biological signal into an electrical signal (Karunakaran et al. 2015). Research in the development of biosensors is becoming more extensive because it provides accurate, simple, fast, cost-effective, sensitive and specific detection of diseases. So far, there is one study which demonstrated the use of a biosensor for the identification and detection of VP_{AHPND} strains (Rizan et al. 2018). Generally, this approach involved DNA extraction from VP_{AHPND} strains, and the extracted DNA is then used to create a DNA-metal (semiconductor-metal) device. The identification of the bacteria is determined by studying the effect of electric current conductivity across the biosensor (Rizan et al. 2018). Thus, the advances in biotechnology, nanotechnology, and information technology are crucial in the development of rapid diagnostic technologies to prevent the spread of AHPND.

Contribution of next-generation sequencing technologies in the genomic studies of VP_{AHPND}

Infectious shrimp disease is one of the critical issues faced in the management of shrimp aquaculture. Traditionally, studies on pathogens detection depend on conventional microbiological tests that are laborious and time-consuming (Fournier et al. 2014). In the early 2000s, the emergence of next-generation sequencing (NGS) technologies has revolutionized the field of OMICS, permitting new directions to address pertinent research areas that could not be considered previously. The NGS technologies (Illumina/Solexa, ABI/SOLiD, 454/Roche and Helicos) have provided unprecedented opportunity for high-throughput functional genomic research (Moorthie et al. 2011). These technologies have been made available on an increasing range of platforms designed to suit different applications and capacity requirements from large Genome Centres and clinical laboratories (Moorthie et al. 2011). With the large data collection of bacterial genomes, NGS allows one to consider all the relevant information such as species identification, strain typing, virulence determination, molecular epidemiology, mechanism of pathogenesis and antibiotics resistance about a pathogen in one single test (Fournier et al. 2014). In spite of the promising outcomes obtained using this technology, NGS was underutilized in the study of shrimp diseases at the beginning as it requires high costs (Nkili-Meyong et al. 2016). Fortunately, the cost of sequencing has decreased significantly, and studies on shrimp diseases that involved NGS have increased significantly over the past few years. Since the emergence of AHPND, numerous studies have applied NGS to sequence the causative agent to obtain detailed information that are relevant for the mitigation of this disease. Here, we summarize the contribution of NGS towards the studies of VP_{AHPND} (Table 1). Further details on the progress of studies in VP_{AHPND} using NGS are described.

Whole genome sequencing (WGS) and targeted amplicon sequencing are the two common approaches that are used in NGS studies of bacterial pathogens (Lefterova et al. 2015). WGS is the analysis of the entire genomic DNA sequence of a bacterium and relies on non-targeted library preparation (Lefterova et al. 2015). The use of WGS is to determine the genomic content and functional potential of organisms. On the other hand, targeted amplicon

Table 1 NGS studies conducted with AHPND-causing *Vibrio* spp. strains

Strain	Accession no.	Findings	Reference
TUMSAT_DE1_S1	BAVF01000000	Identified unique sequences of a conjugative plasmid that were conserved in VP _{AHPND} strains	Kondo et al. (2014)
TUMSAT_DE2_S2	BAVG01000000		
TUMSAT_D06_S3	BAVH01000000		
M0605	JALL00000000		Gomez-Gil et al. (2014a, 2014b)
FIM-S1708+	JPLV00000000		Gomez-Jimenez et al. (2014)
NCKU_TV_3HP	JPKS00000000	Identified one large extrachromosomal plasmid (~69 kbp) carrying two toxin genes that homologue to the insecticidal <i>Photobacterium</i> insect-related binary toxin (PirAB)	Yang et al. (2014)
NCKU_TV_5HP	JPKT00000000		
NCKU_TV_CHN	JPKU00000000		
13-028/A3 and NCKU_TV_3HP	JOKE00000000	Completed sequence of the plasmids (pVPA3-1/pVA1) that present only in VP _{AHPND} strains Suggested Pir toxin-like genes (pirAB ^{VP}) could be the causative factor for AHPND The plasmid might be self-transmissible	Han et al. (2015a, 2015b); Lee et al. (2015)
Ba94C2	PRJNA335761	SWAT-3 type transposase was found flanked with pirAB ^{VP} toxin	
KC13.17.5 (<i>V. harveyi</i>)	BBXN01000000	<i>Vibrio</i> species (non- <i>V. parahaemolyticus</i>) that cause AHPND in shrimp	
SH-14 (<i>V. owensii</i>)	LCKM00000000		Kondo et al. (2015)
Vc 3S01 (<i>V. campbellii</i>)	CP020076-CP020081		Liu et al. (2015)
LA16-V1(<i>V. campbellii</i>)	CP021145-CP021150		Dong et al. (2017)
BA55 (<i>V. punensis</i>)	PRJNA412371		Ahn et al. (2017)
1335	MYFF00000000	The VP _{AHPND} strains harbour an antibacterial type VI secretion system (T6SS)	Fu et al. (2017); Li et al. (2017)
12297B	MYFG00000000		
D4	MYFH00000000		
PB1937	CP022243-CP022246		
1930	SRX3093854	The VP _{AHPND} strains were genomically diverse	Fu et al. (2017)
HZ-7	SRX3093852		
MVP1	MQMQ01000000	Identical sequences of seven housekeeping genes with MVP2 and MVP6, classifying as the same sequence type	Foo et al. (2017)
MVP2	MSBY01000000		
MVP6	MSCA01000000		
KS17.S5-1	PJJY00000000	The Malaysian VP _{AHPND} strains harbour multiple antibiotic resistance genes	Devadas et al. (2018a, 2018b)
ST17.P5-S1			
M1-1	PDDQ00000000	A Vietnamese VP _{AHPND} strain causes a mild form of AHPND	Kumar et al. (2018)
XN87	KU145395-KU145400	A mutant strain that killed shrimps but did not produce Pir ^{VP} toxins or AHPND lesions	Phiwsaiya et al. (2017)
3HP	WIAZ00000000	Comparative genomics of VP _{AHPND} strains from Thailand, Malaysia and Vietnam have revealed several genes such as F-pilus assembly, TA system, cholera toxin and vibrio core oligo-saccharides biosynthesis that could contribute to the variation in the virulence of VP _{AHPND} strains	Yu et al. (2020)
SEB2	WHOI00000000		
SEA5	VTWX00000000		
SAB6	VTWY00000000		
SDA4	WIBC00000000		

sequencing is to selectively sequence the genomic regions of interest by using target-specific primers in PCR-mediated amplification, and this approach is frequently performed to examine well-characterized genomic regions such as known virulence genes (Lefterova et al. 2015). Usually, multiple sequenced bacterial genomes are compared to elucidate the pathogenesis of bacteria. Through the application of NGS, one of the initial discoveries was from the comparative genomics of three draft genomes of VP_{AHPND} strains originated from Thailand (TUMSAT_DE1_S1, TUMSAT_DE2_S2 and TUMSAT_D06_S3) with the non-VP_{AHPND} strains, in which unique sequences that encode for type IV pilus and conjugal transfer proteins were identified only in the genomes of VP_{AHPND} strains (Kondo et al. 2014). The findings were in concordance with the analysis of the genomes of the VP_{AHPND} strains from Mexico (Gomez-Jimenez et al. 2014; Gomez-Gil et al. 2014b). Another study on the comparison of two draft genome sequences from Thailand (NCKU_TV_3HP and NCKU_TV_5HP) as well as one draft genome sequence from China (NCKU_TV_CHN) found one large extrachromosomal plasmid (~69 kbp), pVA carrying two toxin genes which are homologue to the insecticidal *Photorhabdus* insect-related binary toxin PirAB present in all VP_{AHPND} strains but not in the non-VP_{AHPND} strain (Yang et al. 2014). The recognition of the pVA plasmid in the genome of VP_{AHPND} strains suggested that the plasmid is responsible for AHPND in shrimp.

Following the identification of the pVA plasmid via WGS, two subsequent studies increased the sequencing depth of the plasmid sequence by using targeted amplicon sequencing to fill in the gaps. The complete sequences of the plasmid, namely, pVPA3-1 and pVA1, have been described in two studies for the Vietnamese strain 13-028/A3 and Thai strain 3HP (Lee et al. 2015; Han et al. 2015a). Both of these plasmid sequences have a similar genome feature such as genome sizes (69–70 kbp), G + C contents (~45.9%) and similar number of predicted open reading frames (ORFs: 90–92) (Han et al. 2015a; Lee et al. 2015). The open reading frames consist of mobilization protein, replication enzymes, transposases, virulence-associated proteins and proteins similar to *Photorhabdus* insect-related (Pir) toxins. Both studies suggested that these Pir toxin-like proteins could be the determinant for AHPND. Based on previous findings, various primer sequences were developed for PCR typing of VP_{AHPND} strains by targeting the plasmid sequences or the toxin genes that are responsible for AHPND in shrimp (Flegel and Lo 2014; Tinwongger et al. 2014; Sritunyalucksana et al. 2015). Hence, NGS has helped researchers to gain greater insights in discovering novel biomarkers for VP_{AHPND}.

Next, the Pir toxin-like proteins were found to be encoded by *pirA*-like (336 bp) and *pirB*-like (1317 bp) genes and were flanked with inverted repeats of transposase, suggesting that this virulent plasmid is acquired through horizontal gene transfer and may be self-transmissible (Han et al. 2015a, b; Kumar et al. 2018; Lee et al. 2015; Restrepo et al. 2016). The self-transmissible characteristic of the plasmid pVA has led researchers to speculate that the plasmid pVA could transfer the toxin genes not only among *V. parahaemolyticus* strains but also to other members of the *Vibrio* species. Indeed, pVA plasmid that harbours *pir^{vp}* toxin genes has high similarity to other pVA plasmid present in other *Vibrio* species including *V. harveyi*, *V. owensii*, *V. campbellii*, *V. sinaloensis* and *V. punensis* (Thong et al. 2014). The existence of this self-transmissible plasmid harbouring toxin genes could possibly increase the complexity of the causative agents of AHPND, thereby providing a caveat for future research in considering other *Vibrio* species.

Although the *pir^{vp}* toxin genes are the primary causes of AHPND, there might be other virulence components or antibiotic resistant genes that play important roles during shrimp infection. Previous studies reported that the VP_{AHPND} strains possess a T6SS protein secretion

apparatus that is known to play a role in inter-bacterial competition (Yang et al. 2018; Yu et al. 2020). The T6SS possesses antibacterial activities mediated by delivery of toxic effectors into neighbouring cells and therefore the acquisition T6SS might confer a fitness advantage to VP_{AHPND} strains over other competing bacteria and facilitates shrimp infection (Yang et al. 2018). In addition, multiple antibiotic resistance genes encoding resistance to tetracycline, sulfanamide, bleomycin, chloramphenicol and vancomycin were reported from the draft genomes of Malaysian VP_{AHPND} strains (Devadas et al. 2018a, b; Foo et al. 2017). The NGS technologies have provided us relevant information on virulence factors and the antibiotic resistance genes of the VP_{AHPND} strains, and these could reveal a potential target for disease control. Besides, the application of NGS can be implemented to improve our knowledge about the genetic diversity of VP_{AHPND} strains. By using the single-nucleotide polymorphism (SNP) based on WGS data, the genetic diversity of bacteria could be accurately presented especially if established typing schemes are not available. Moreover, SNP has higher resolution than other existing sequence-based typing schemes (Schürch et al. 2018; Yu et al. 2020). In the study of the genetic diversity of VP_{AHPND} strains in Asia and Mexico using the SNP-based method, the VP_{AHPND} strains are genetically distinct in different localities, and there are no epidemiological links between the Asian and Mexico outbreaks (Fu et al. 2017). The findings were further supported by another study that involved 28 global VP_{AHPND} strains from different countries (Yu et al. 2020). An understanding of the genetic diversity of VP_{AHPND} strains could hasten the intervention strategies, providing information such as the global spread of pathogens and rational development of diagnostics and therapeutics and vaccines to mitigate the risk of AHPND since such data are currently lacking.

In addition, NGS technologies can be used to identify the genetic variability of bacterial strains through comparison of genomic sequences from different sources. Since different rates of mortality were observed in AHPND-infected shrimps, researchers have suggested that the genetic difference among the VP_{AHPND} strains could be one of the reasons that contributed to this phenomenon (Kumar et al. 2018). In a challenged study, Kumar et al. showed that a Vietnamese VP_{AHPND} strain, M1-1, had a lower mortality rate than the Thai VP_{AHPND} strains 3HP and 5HP (Kumar et al. 2018). Sequence analysis of M1-1 showed that this particular strain M1-1 also harboured the pVA1 plasmid and expressed Pir toxin just like the 3HP and 5HP strains. Comparative genomic studies revealed that while these strains shared most of the genes, several additional genes were identified in the genome of M1-1 alone, which suggested there was a lot of genetic variation between these strains of different virulence status (Kumar et al. 2018). The additional genes that were present only in M1-1 may provide important clues to the pathogenicity of M1-1 which was less virulent than 3HP and 5HP strains. Phiwsaiya et al. isolated a natural *V. parahaemolyticus* mutant strain (XN87) that produced no Pir^{vp} toxin but caused mortality in shrimp without AHPND lesions (Phiwsaiya et al. 2017). Sequence analysis of this XN87 strain showed the presence of pVA plasmid and the *pirAB*^{vp} toxin genes; however, the *PirA*^{vp} gene was interrupted by out-of-frame insertion of a transposon gene fragment, suggesting that the VP_{AHPND} strains carrying mutant pVA plasmid can also cause mortality in shrimps without showing the AHPND lesions (Phiwsaiya et al. 2017).

In addition, comparative genomics analysis of VP_{AHPND} strains with varying degree of virulence from Thailand, Vietnam and Malaysia revealed a cluster of F-pilus assembly genes which are only present in the most virulent Thai VP_{AHPND} strain 3HP. Among the Malaysian VP_{AHPND} strains (SEB2, SEA5 and SAB6), no remarkable genetic differences were observed although these strains exhibited different levels of virulence. Also, several gene clusters such as TA system, cholera toxin and *Vibrio* core oligosaccharides biosynthesis were present in the

Thai and Malaysian strains but absent in the genome of Vietnamese VP_{AHPND} strain SDA4 which has the lowest virulence among the studied strains. This suggests that the identified genes might contribute to the variation in virulence among the VP_{AHPND} strain (Yu et al. 2020). Genetic variation is one of the problems that is often faced in the development of vaccine, and NGS could provide new critical insights into vaccine development strategies. In short, the integration of NGS into studies of shrimp diseases is exceptionally important and plays a vital role for the development of effective approach and solution to mitigate the disease.

Conclusion

Since the emergence of AHPND in 2009, the shrimp aquaculture industry has faced massive economic losses, especially in Southeast Asian countries. Many research progress and efforts taken to mitigate AHPND were observed in the last 10 years. Following the identification of the causative agent, VP_{AHPND} and its unique histopathological manifestation in 2013, research on AHPND has progressed rapidly, especially on the development of diagnostic methods as well as the implementation of control and preventive measures. Genomic studies contributed largely to a deeper understanding of the genomes of VP_{AHPND} strains, providing significant genetic information which allows the design of targeted disease interventions, particularly, in the development of PCR-based methodologies for pathogen study as well as detection of AHPND-causing bacteria in shrimps. We have highlighted the importance of genomics towards the study of aquatic diseases, emphasizing the purpose to integrate genomics in aquaculture for future research. Clearly, rapid diagnostic technologies are crucial to prevent the spread of AHPND. PCR seems as one of the promising detection methods for rapid and sensitive detection of AHPND-causing bacteria, and so far, PCR method using the AP3 and duplex primers is the most recommended approach for the detection of VP_{AHPND}. Advances in biotechnology, nanotechnology and information technology allowed the evolution of rapid diagnostic technologies. There is a delay in developing an effective solution for controlling AHPND mainly due to the lack of understanding on the interactions between microbes and their effects on shrimps. National programmes should be routinely held to exchange information between farmers, researchers and responsible parties. This could serve as a preventive measure to prepare for similar future emergency.

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Cindy Shuan Ju Teh: Review and project administration
Kien Pong Yap: Review
Kwai Lin Thong: Idea, supervision and funding acquisition

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with animals performed by any of the authors.

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