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Progress in research on acute hepatopancreatic necrosis disease (AHPND)

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Abstract Acute hepatopancreatic necrosis disease (AHPND) of shrimps is an important disease, first appeared in China in 2009. Since then, AHPND has caused serious drops in shrimp production (up to 20 % worldwide). Although AHPND [originally termed as acute hepatopancreatic necrosis syndrome (AHPNS)] first appeared in 2009, it was not until 2013 that a laboratory infection model was devised and the causative agent identified as certain strains of *Vibrio parahaemolyticus*. AHPND has caused mortality from 40 to 100 % which usually occurs early (within approximately 35 days) after stocking shrimp fry in shrimp ponds; therefore, it was initially referred to as early mortality syndrome (EMS). Confusingly, other pathogens and environmental factors also cause EMS and are often attributed to AHPND by shrimp farmers. Frequently, farmers do not send samples for confirmatory tests requiring detection of the unique histopathology at the acute stage of disease (massive sloughing of hepatopancreatic epithelial cells without any accompanying signs of a pathogen). The gross signs presumptive of AHPND (lethargy, slow growth, empty stomach and midgut, and a pale to white, atrophied hepatopancreas) are insufficient for confirmatory diagnosis. Recently, molecular detection of AHPND bacteria using PCR has been developed, which has sped up diagnosis and increased research on the causative agent, alternative detection methods, and possible therapies. We hope that this review of research progress on AHPND will serve as a useful introduction for researchers who are currently unfamiliar with AHPND, but have backgrounds in bacterial virulence, detection, and

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epidemiology, and may be encouraged to participate in the research effort to reduce AHPND's impact on shrimp cultivation.

Keywords Acute hepatopancreatic necrosis disease · AHPND · *Penaeus* (*Litopenaeus*) *vannamei* · *Vibrio parahaemolyticus*

Introduction

Asian shrimp cultivation is an important industry that generates billions of US dollars in export income annually (Flegel 2012). The main cultivated species are *Penaeus monodon* and *Penaeus* (*Litopenaeus*) *vannamei*, with the latter currently dominating the world market (FAO 2010). In China and southeast Asia, shrimp cultivation is a multi-billion dollar industry that contributed 3.9 million tons of production to the global shrimp market in 2010 (FAO 2010). However, widespread epidemic diseases caused by viruses, such as white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), Taura syndrome virus (TSV), and infectious hypodermal and hematopoietic virus (IHHNV), have caused serious losses for shrimps farmers, estimated at nearly US$15 billion over the last 15 years, with about 80 % of this loss occurring in Asia (Flegel 2012).

Covert mortality is a more recent, major disease that appeared in China before 2009. Its name is derived from the fact that most moribund shrimp die at the bottom of the pond rather than at the edges or in shallow water after swimming slowly at the surface (Huang 2012). The cause has been attributed to covert mortality nodavirus (CMNV)(Zhang et al. 2014). Affected shrimp exhibit hepatopancreatic atrophy and necrosis, empty stomachs and guts, soft shells, slow growth, and abdominal muscle whitening with necrosis (Zhang 2004; Huang 2012).

Since 2009, a novel disease of cultivated shrimp has emerged, termed acute hepatopancreatic necrosis disease (AHPND), which was initially called acute hepatopancreatic necrosis syndrome (AHPNS). It was reported from China in 2009 and then spread to Vietnam (2010), Malaysia (2011), Thailand (2012), and Mexico (2013) (Tran et al. 2013; Joshi et al. 2014; Nunan et al. 2014; Soto-Rodriguez et al. 2015). AHPND affects multiple shrimp species, including the *P. vannamei* and *P. monodon*. AHPND is characterized by severe atrophy of the shrimp hepatopancreas, accompanied by unique histopathology at the acute stage of disease (Tran et al. 2013). This consists of massive sloughing of hepatopancreatic epithelial cells in the absence of any accompanying pathogen within approximately the first 35 days after stocking shrimp ponds with fry (called post-larvae or PL). Gross signs of the disease include lethargy, slow growth, an empty stomach and midgut, and a pale to white atrophied hepatopancreas, with death at the bottom of the pond. In some aspects, these symptoms are similar to the gross signs of covert mortality disease. Unfortunately, farmers often lump these diseases together under the name early mortality syndrome (EMS), i.e., early, uncommon, and acute mortality in shrimp cultivation ponds. This has led to some confusion, because other pathogens and some environmental factors may also cause early mortality. Farmers frequently do not send samples for confirmatory tests to determine the precise cause of mortality (e.g., histological analysis to detect the characteristic histopathologies of AHPND, WSSV, YHV, CMNV, or traditional vibriosis); it is difficult to assess the proportional contribution of various pathogens to the EMS

phenomenon. As aquatic animals, shrimps are quite different to terrestrial animals, and the factors accounting for AHPND may be varied and complex. Both biological factors, such as toxic algae, bacteria, viruses, and parasites, and water chemical factors, such as nitrite and ammonia, could cause the same clinical signs. Secondary viral infections, such as YHV, could also lead to AHPND pathology. Here, we focus on AHPND, with the understanding that the gross signs presumptive of AHPND are not sufficient for confirmatory diagnosis.

The Thai Department of Fisheries reported that total shrimp production of Thailand in the first quarter of 2013 was 63,500 tons, while it was 94,400 tons during the same period in 2012, indicating a decrease of 30 % approximately, which was attributed to EMS outbreaks in the western coast of the gulf of Thailand (http://www.fisheries.go.th/ems/). Estimated total production loss in shrimps to below 1,000,000 tons in China and 600,000 in Vietnam was also attributed to EMS (FAO 2014).

Here, we present a brief overall review of research on AHPND. Most of the information in the review relates to clinical signs, histopathology, and potential methods of preventions. We hope that this review on AHPND will serve as a useful introduction for researchers with backgrounds in bacterial virulence, detection, epidemiology, and control who are currently unfamiliar with AHPND, but may be encouraged to join in the urgent research effort to reduce the negative impact of AHPND on shrimp cultivation.

AHPND: gross signs and histopathology

The signs of necrosis and mortality appeared on PL 30–35 days after stocking frequently. And the gross signs of AHPND include a pale and shrunken hepatopancreas, an empty stomach, and an empty gut (Fig. 1); however, these signs are only useful as a presumptive diagnosis, and a histological examination is required for confirmation. The key and unique histological feature at the early stage to midstage of AHPND is massive rounding and sloughing of hepatopancreatic tubule epithelial cells in the absence of any detectable causative pathogen (Tran et al. 2013). This is the critical feature for diagnosis, and it is recommended that a minimum of 10 specimens (but possibly more) can be taken and processed from any suspected pond to ensure that at least one specimen is at this stage of the disease. The reason for this is that the very early stages of AHPND (characterized by lack of mitotic activity in E cells, medial to distal dysfunction of B, F, and R cells, and prominent karyomegaly) and the late stages of infection (characterized by massive secondary bacterial infections) (Fig. 2) may be difficult to assess. For example, the late stage

Fig. 1 A pale and shrunken hepatopancreas, and an empty stomach, of a juvenile *P. vannamei*, indicative of AHPND (*source* D. V. Lightner)

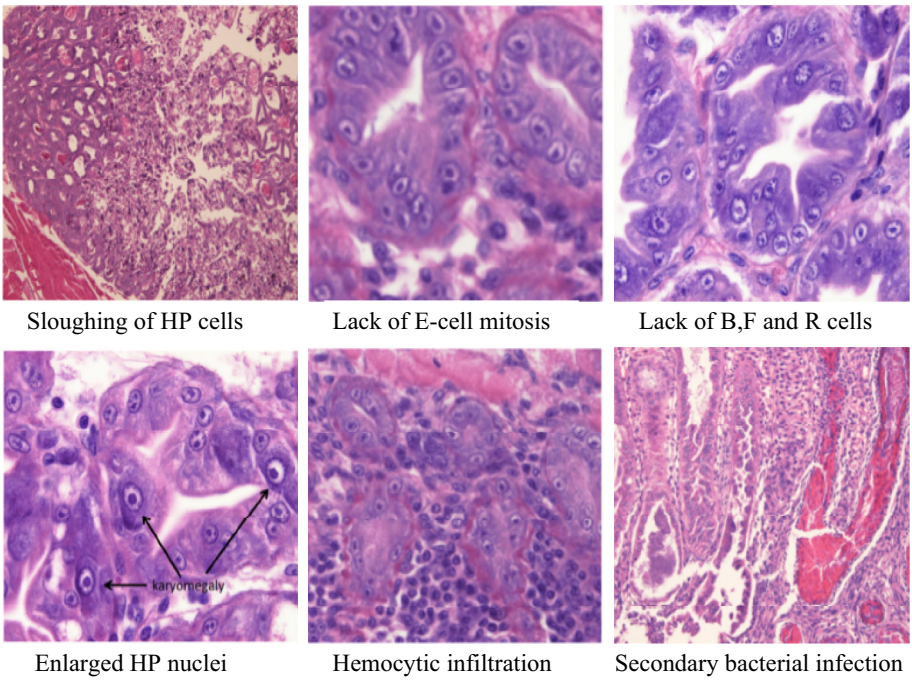

Fig. 2 Histopathology of *P. vannamei* hepatopancreases affected by AHPND in Thailand (*source* T. M. Flegel)

of infection is characterized by massive hemocytic aggregation and formation of melanized granulomas, accompanied by many colonies of a variety of bacteria in tubule lumens, which is indistinguishable from severe, traditional infections caused by non-AHPND isolates of various bacterial species. See section "Molecular detection methods" for molecular methods to confirm AHPND.

Excluding other possible causes of early mortality

It is very important when studying suspected farm outbreaks of AHPND and laboratory experiments on AHPND that AHPND pathology is confirmed in any moribund shrimp examined. This should involve confirmation by a combination of histological and molecular methods. This section describes other possible causes of early mortality, which may be subdivided into biological and non-biological causes. EMS encompasses causes ranging from environment factors to pathogens, such as WSSV and YHV (NACA 2012),

Viruses

Shrimps affected by covert mortality disease exhibit gross signs similar to AHPND (Zhang 2004; Huang 2012); therefore, histological or molecular examination for the presence of CMNV (Zhang et al. 2014) may be necessary if tests on shrimps showing such gross signs of disease do not give positive results for AHPND by histological or molecular analysis. In

China, some shrimp samples showing gross signs, including a pale and partially atrophied hepatopancreas, an empty stomach and gut, and slightly whitish abdomen muscles, were found to be infected with a new genotype of yellow head virus (YHV) (Liu et al. 2014). In Thailand, examination of shrimps submitted by farmers from suspected outbreaks of AHPND turned out to be outbreaks caused by YHV or WSSV after histological and/or molecular analysis (T.W. Flegel, personal communication).

Bacteria

Vibriosis caused by non-AHPND bacteria, such as *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio penaeidida*, and other *Vibrio* species, have long been known as shrimp pathogens associated with disease outbreaks in hatcheries and cultivation ponds (Lightner 1996; Longyant et al. 2008). These are often cases of opportunistic infections that result from environmental stress caused by the weather, water quality, or pond mismanagement (e.g., overfeeding or overstocking). On the other hand, this may also occur when specific isolates possess particular virulence factors (Goarant et al. 2000; Austin and Zhang 2006; Intaraprasong et al. 2009). In the event of very severe infections of the shrimp hepatopancreas by *Vibrio* species, the histological picture may be difficult to distinguish from the late stage of AHPND, which is characterized by severe secondary bacterial infections. To distinguish between the two, it is necessary to examine several shrimp specimens to determine the earlier stages of disease, one characterized by sloughing in the absence of bacteria followed by heavy bacterial infection (AHPND), and the other by initially light bacterial infections progressing to severe infections (vibriosis). A good example of experimental vibriosis was *P. vannamei* challenged with 10^9 cell/ml *V. harveyi*, which resulted in mortality of 61.9 % significantly and histopathological lesions, dominated by necrosis and sloughing of hepatopancreatic epithelial cells, deterioration of acinar structure of tubules and hemocyte infiltration in sinusoidal spaces of the hepatopancreas (Peyghan et al. 2009).

Algae

Algal blooms can produce hypoxia or anoxia, which results in shrimp mortality at any time during cultivation. This is usually unlikely during the early stages of cultivation because of the small size of the post-larvae; however, it is possible if there is a high organic load in the water or if there are very dense algal blooms, which can result in very low oxygen levels overnight. For example, a massive bloom of the dinoflagellate *Alexandrium tamarense* in Taiwan caused massive mortality of *P. monodon* in 1989 (Su et al. 1993). Some flagellates, such as *Euglena* spp., have also been reported to cause problems when ammonia and mucus (abnormal secretion in the gills of fishes) were expelled into the water and eventually killed *Penaeus orientalis* or made it vulnerable to diseases (Alonso-Rodriguez and Paez-Osuna 2003).

Parasites

Tissue squashes of infected hepatopancreases and histological examination of infected shrimps in Central America from 2004 to 2010 revealed that the massive mortality might have been caused by an endoparasite. The determined DNA sequence was novel, and phylogenetic analysis placed the *P. vannamei* parasite within the phylum Haplosporidia as

a sister taxon to a clade that included *Bonamia* and *Minchinia* species (Nunan et al. 2007). A similar pathogen caused high mortality of juvenile *P. vannamei* (<1 month old) in Indonesia, which showed 96 % sequence identity to the haplosporidian from Central America (Utari et al. 2012). *Enterocytozoon hepatopenaei* causes white feces syndrome and has no causal relationship with AHPND; it seems likely that the increased prevalence of *E. hepatopenaei* and AHPND bacteria has resulted in the contamination of brood stock or post-larvae (Sriurairatana et al. 2014).

Environmental factors

Some cases of early mortality were related to environment factors, such as nitrite, ammonia, salinity, and pH. The acute toxicity of nitrite in *P. vannamei* juveniles was demonstrated to be related to salinity: When exposed to higher salinity, the LC50 values of nitrite on *P. vannamei* were higher (Lin and Chen 2003). The toxicity of ammonia in *P. vannamei* is similar to that of nitrite (Lin and Chen 2001). The early mortality observed under high concentrations of nitrite and ammonia shows similar clinical signs to covert mortality (Zhang 2004).

Both pathogens and environmental factors cause clinical signs including atrophic hepatopancreas, empty stomach and gut, soft shell, and slow growth, which are similar to AHPND to some degree; however, when compared with D.V. Lightner's definition described above, it is clear that none of them are the cause of AHPND.

The AHPND causative agent and bioassay development

AHPND was shown to be the main cause of EMS in Vietnam and Thailand (Tran et al. 2013; Joshi et al. 2014). In 2013, a group from the University of Arizona identified that the causative agents of EMS/AHPND were specific virulent strains of *V. parahaemolyticus*. They used immersion challenge tests for infectivity, which induced 100 % mortality with typical AHPND pathology, which satisfied with Koch's postulates (Tran et al. 2013). In 2012, samples collected from 92 AHPNS-affected ponds in Vietnam mostly contained vibrio isolates, with the majority being *V. parahaemolyticus* (Oanh et al. 2013).

Vibrio parahaemolyticus is a Gram-negative (G^-), halophilic, mesophilic, and rod-shaped species that is found in estuarine and marine environments worldwide (Drake et al. 2007). As an important human pathogen, *V. parahaemolyticus* could cause gastroenteritis when consumed raw or partially cooked food. *V. parahaemolyticus* tolerates a high range of salinities (0.5–9.5 %), pH (7.6–9.0), and temperatures (7–43 °C), attaches to marine plankton readily, and spreads by ocean currents (Chamberlain 2013).

The research group in Mexico found moribund shrimp with AHPND from farms in northwestern Mexico and identified the presence of *V. parahaemolyticus* using the *tlh* gene. In immersion challenges and farmed and challenged shrimps presented the same clinical and pathological symptoms: lethargy, empty gut, pale and aqueous hepatopancreas, and expanded chromatophores. Using histological analysis and bacterial density counting, three stages of AHPND were identified (initial, acute, and terminal) in the affected shrimps. Pathognomonic lesions indicating severe desquamation of tubular epithelial cells in the hepatopancreas were observed. Some of the less virulent strains do not induce 100 % mortality, and mortality rates also increase more slowly than they do for the more virulent strains. The virulence of *V. parahaemolyticus* strains was dose dependent, where the

threshold infective density was 10^4 cfu/ml; below that density, no mortality was observed. Field and experimental results showed that the *V. parahaemolyticus* strain that causes AHPND acts as a primary pathogen for shrimps in Mexico compared with the *V. parahaemolyticus* strains reported to date (Soto-Rodriguez et al. 2015). The possibility of diversity in isolates of *V. parahemolyticus* may cause early mortality without AHPND symptoms (Joshi et al. 2014).

Bacteriophages, commonly referred as phages, are natural pathogens of bacteria and thus may be adapted to fight bacterial infections. Lytic phages capable of lysing pathogens can be a safe and effective alternative to control bacterial infections in aquatic animals; however, lysogenic phages have a serious problem of enhancing the virulence armory of bacterial pathogens that infect aquatic animals, thus threatening aquaculture (Rao and Lalitha 2015). A change in the phenotype of *V. harveyi* isolates from non-virulent to virulent caused by two bacteriophages belonging to *Myoviridae* and *Siphoviridae* caused luminous bacteriosis in shrimp hatcheries and farms (Flegel et al. 2005). Phage may be involved in AHPND-related *V. parahaemolyticus* (Zhang et al. 2012; Tran et al. 2013).

EMS appears to be a septicemic *Vibriosis* involving at least two *Vibrio* species infected by a bacteriophage (FAO 2013). In 2012, samples collected from 92 AHPNS-affected ponds in the Mekong Delta found a number of Vibrio isolates, with the majority being *V. parahaemolyticus*. Three isolates were found to carry phages. Experimental challenge of white shrimp showed that a *V. parahaemolyticus* strain that carried a phage was capable of causing AHPNS pathology in non-infected shrimps (Oanh et al. 2013).

Bacillus thuringiensis is a Gram-positive bacterium that has important roles in agriculture and forests, with entomopathogenic properties. Many *B. thuringiensis* strains contain a δ-endotoxin that acts against many insects of different orders (Lepidoptera, Coleoptera, and Diptera) and other invertebrates. *B. thuringiensis* was isolated from the moribund shrimps' hepatopancreas and caused hepatopancreas necrosis and mortality among the shrimps (He et al. 2014). Moreover, a plasmid found in some AHPND *V. parahaemolyticus* strains showed some similarity to that found in *B. thuringiensis* (Lo et al. 2014).

Based on 16S RNA sequences, the isolate from East Malaysia shrimps was closely associated with *Vibrio sinaloensis* (85 % homology) and could also produce AHPND histopathology (LinThong et al. 2014). The AHPND-related strains have significant genomic variations; the concentrations of *Delftia*, *Rhodococcus*, and *Leifsonia* in AHPND ponds were higher than in normal ponds. The PCR results demonstrated their existence in shrimps; however, using the method of immersion could lead to a high level of mortality without significant pathological characteristics in the hepatopancreas (Flegel 2014).

To determine the lemology and the route of transmission, subsequent research could use oral methods, such as injection of healthy specific pathogen-free (SPF) shrimps with AHPND-related pathogens in a tank and compare their hepatopancreases with those of non-injected shrimps. Further study of the causative agent of AHPND might identify other *Vibrio*, in addition to *V. parahaemolyticus*, which are responsible for AHPND epidemics.

Metagenomics for virulence of AHPND bacteria

Hemolysins from many *Vibrio* spp. are recognized as virulence factors (Zhang and Austin 2005). The type III secretion system (T3SS), which is a complex structure that enables G-bacteria to secrete and inject bacterial effector proteins into the cytoplasm of eukaryotic

host cells, has been observed in many pathogenic bacteria (Hueck 1998). The *V. parahaemolyticus* have two sets of genes (T3SS1 and T3SS2). T3SS2 is only found in human pathogenic *V. parahaemolyticus* isolates which possessed the thermostable direct hemolysin (*tdh*) or tdh-related hemolysin (*trh*) gene (Park et al. 2004; Okada et al. 2009). The type VI secretion system (T6SS) is a common export pathway in G-bacteria that can translocate effector protein into many target cell types. T6SS is a complex bacteriophage-like structure, which is correlated to the ability of organisms to induce host diseases (Yu et al. 2012; Salomon et al. 2013).

A variety of *V. parahaemolyticus* isolates could be obtained from a single Thai shrimp farm exhibiting early mortality, including high lethal isolates that caused 100 % mortality and were accompanied by typical AHPND pathology when immersion challenge with 10^6 cfu/ml in 96 h post-infection; and a less lethal isolate that caused 73.3 % mortality and did not cause AHPND pathology. However, the isolates from the Thai shrimp farm gave negative PCR results for two human pathogen markers *tdh* and *trh* (Joshi et al. 2014), which revealed that these strains could not lead to zoonosis.

A team from Taiwan sequenced four strains of *V. parahaemolyticus*, three of which caused serious AHPND. The sequence analysis of the toxic strains revealed not only genes related to cholera toxin and the type IV pilus/type IV secretion system, but also a unique, previously unreported, large extrachromosomal plasmid that encodes a homolog to the insecticidal *Photorhabdus* insect-related binary toxin PirAB (Yang et al. 2014). Another Thai team sequenced three AHPND and three non-AHPND strains of *V. parahaemolyticus* and found that all isolates lacked the pathogenicity island related to human infection. A unique sequence encoding a type IV pilus/type IV secretion system was found in the three AHPND strains (Kondo et al. 2014). A research group in Mexico isolated one strain of *V. parahaemolyticus* M0605 from the stomach of *P. vannamei*. M0605 has two chromosomes, both of which were identified as having pathogenicity (Gomez-Gil et al. 2014).

Isolates from India were negative for virulence genes associated with human pathogenic strains of *V. parahaemolyticus* and were also PCR negative for genomic regions considered specific for AHPND strains (Karunasagar et al. 2014). The isolates from southern Thailand possessed a unique O antigen, but small variations in the K antigens were detected, and similar DNA profiles were detected from different farms. It is hypothesized that the causative agent of AHPND might have originated from one clone from which slightly different serotypes subsequently developed (Kongrueng et al. 2014b).

Proteomics for virulence of AHPND bacteria

Lightner's team found that AHPND is caused by a unique strain of *V. parahaemolyticus* that is infected by a phage, which causes it to release a potent toxin (Lightner 2013). Toxin genes of AHPND *V. parahaemolyticus* are located on a plasmid, including toxin A and toxin B. Toxin B was detected only on AHPND strains, which suggested that the crucial toxic factors of AHPND *V. parahaemolyticus* are homologs of insecticidal toxins (Tinwongger et al. 2014). When sequenced, most AHPND-specific contigs on purified plasmids from AHPND strains matched to pVA1, which contains an operon that encodes homologs of *Photorhabdus* insect-related (Pir) binary toxin, PirA and PirB, which are critical in inducing AHPND (Lee et al. 2014; Yang et al. 2014) The AHPND *V. parahaemolyticus* strains possess a unique plasmid called pVPA3-1, containing insecticidal related genes PirA and PirB (Lightner 2014). These two potent toxin genes are used for PCR diagnosis;

the non-virulent strains carrying the plasmid completely lack the toxin gene, but possess an insertion sequence that might have transposes activity and be involved in deletion and/or insertion of toxin genes (Nochiri et al. 2014). However, when the entire genomes of eight *V. parahaemolyticus* strains were sequenced, the zonular occludens toxin (ZOT) protein gene was always accompanied by the presence of E-family virulence factors. The ZOT proteins are related to those from a *Shewanella* species, which indicated that this virulence factor may have been phage-transfected from this species into *V. parahaemolyticus* (Ung et al. 2014). Three insecticidal toxin complex (tc)-like genes were identified in *V. parahaemolyticus*: a tcd A-like gene (7710 bp), predicted to encode a 284-kDa protein; a tcd B-like gene (4272 bp), predicted to encode a 284-kDa protein; and a tcC-like gene (2916 bp), predicted to encode a 107-kDa protein. A genomic island was also identified that contains 21 transposase genes, suggesting that it was acquired through horizontal transfer from other organisms (Tang and Lightner 2014).

In extremely dense populations, the colonies coordinate the release of a potent toxin through a process known as quorum sensing (Hardman et al., Lightner et al. 2013). Studies suggest that this toxin may be released when a *V. parahaemolyticus* population reaches 10^6 cfu/ml. A group from Taiwan has demonstrated that Pir proteins are crucial to the infectivity of *V. parahaemolyticus* (Lo et al. 2014).

Molecular detection methods

Lightner's team found that EMS/AHPND is caused by a unique type *V. parahaemolyticus*, which is infected by a phage (Lightner 2013). Current research indicates that virulence is governed by a large episomal plasmid (Flegel 2014; Lo et al. 2014).

PCR method to detect AHPND

Current research indicated that virulence is variable among isolates and is governed by a large episomal plasmid. Tentative PCR detection methods (AP1, 2, and 3) for AHPND strains were published in 2014 (Flegel and Lo 2014). However, the false-positive PCR results of AP2 were thought to have arisen because of mutation of plasmids that lack the toxin gene. The AP3 primer set showed the best sensitivity and specificity. Thus, a more sensitive AP3 detection method based on AHPND toxin detection, and with 100 % sensitivity and specificity, was released (Flegel 2014).

The method of PCR-based AHPND detection method using two AHPND-specific contig sequences that are both located on an AHPND-related plasmid was also developed (Chen et al. 2014). The primers used to detect AHPND isolates are shown in Table 1.

Loop-meditated isothermal amplification (LAMP) method to detect AHPND

The coding gene of virulence protein VP19 is 336 bp, and a fluorescence-based loop-meditated isothermal amplification (FRt-LAMP) method, with a sensitivity of 2.6×10^1–2.6×10^2 copies per reaction tube, and an on-site detection kit, has been developed. Moreover, quantitative real-time PCR (qPCR) could be used in the laboratory method to detect AHPND samples (Wang et al. 2014). A special set of six primers was designed using the sequence of a plasmid fragment from AHPND bacteria, with DNA templates extracted from isolates of AHPND bacterial in the LAMP reaction, and was proposed as a new

Table 1 Primers used to detect AHPND isolates

Name	Oligonucleotide sequence (5′–3′)	References
AP1 F	CCTTGGGTGTGCTTAGAGGATG	Flegel and Lo (2014)
AP1 R	GCAAACTATCGCGCAGAACACC	Flegel and Lo (2014)
AP2 F	TCACCCGAATGCTCGCTTGTGG	Flegel and Lo (2014)
AP2 R	CGTCGCTACTGTCTAGCTGAAG	Flegel and Lo (2014)
AP3 F	ATGAGTAACAATATAAAACATGAAAC	Sirikharin et al. (2014)
AP3 R	GTGGTAATAGATTGTACAGAA	Sirikharin et al. (2014)
TUMSAT-Vp3 F	GTGTTGCATAATTTTGTGCA	Tinwongger et al. (2014)
TUMSAT-Vp3 R	TTGTACAGAAACCACGACT	Tinwongger et al. (2014)

method that is 100 times more sensitive (100 CFU) than the 1-step PCR detection method (10^4 CFU) for the same target sequence, using amplicon detection by electrophoresis or spectrophotometry. This new LAMP-AuNP assay for detecting AHPND-related bacteria significantly reduced the time, ease, and cost of molecular detection (Suebsing et al. 2014). Two sets of primers (LAMP-A2 and LAMP-A3) were developed and validated for use in a LAMP assay to identify *V. parahaemolyticus* causing AHPND specifically. LAMP-A2 and LAMP-A3 detected all 33 AHPND-related *V. parahaemolyticus* isolates, but not the non-AHPND *V. parahaemolyticus* isolates and 19 other closely related bacterial species. In pure culture and in spiked shrimp experiments, the LAMP assay was superior to PCR to detect AHPND (Kongrueng et al. 2014a).

Pond-side strip tests for immunodetection of AHPND toxin

The World Animal Health Organization recommends polymerase chain reaction (PCR)-based methods to detect shrimp viruses. In all cases, immunological-based assays are virus specific, with optimum sensitivity (10^5 cfu/ml). Further development of immunochromatographic strip tests that can be used by shrimp farmers to monitor certain shrimp virus infections is discussed.

Virulence mechanisms inducing AHPND

Some studies of AHPND *V. parahaemolyticus* highlighted certain similarities with *B. thuringiensis* infection. In this way, research into *B. thuringiensis* pathogenesis could give some clues to AHPND.

The δ-endotoxin of *B. thuringiensis* shares the same three-dimensional structure with domain II and domain III of PirA and PirB. The endotoxin exerts its pathological role by forming lytic pores in the cell membrane of insect midgut epithelial cells. Domain II and domain III are involved in the process of activated toxin binding to the receptor on the membrane of tubular epithelial cells. There are three types of receptors on the membrane of tubular epithelial cells: a cadherin-like protein (CAD), a glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase (ALP), and a GPI-anchored aminopeptidase (APN). The mechanism of crystal protein insertion includes three steps: receptor binding, pre-pore

formation, and membrane insertion. The activities at each step of this mechanism could be modulated against a particular insect, which affects the overall specificity of a toxin. Many researchers have demonstrated that APN is an important toxin receptor, and some reconstitution studies have linked toxin binding to APN with functional pore formation in artificial membranes. After ingestion by insect, crystals are digested in the gut; isolated toxin could be separated from the tubular epithelial cells. These protoxins bind to CAD, leading to proteolytic activation and protease secretion. The monomeric protoxins then form an oligomeric structure, which binds to APN and ALP. Following binding, at least part of domain I inserts into the membrane as part of an oligomer, to form an aqueous pore with other toxin molecules. Domain I is similar to other pore-forming or membrane translocation domains of bacterial toxins. By analogy to those, membrane entry might start by insertion of a hydrophobic two-helix hairpin. In an umbrella-like model, based on mutational and biophysical studies, α-helices four and five of several toxin molecules in an oligomer comprise the pore, with the rest of the protein spreading over the membrane surface. Finally, the protoxin is transplanted on the membrane and forms pores. The leak through the plasma membrane results in extracellular cations entering the cell freely, leading to cell lysis. Gut microflora can then enter the hemocoel of the larva and propagate (de Maagd et al. 2001; Bravo et al. 2007).

The 1 μg crude protein in the 60 % ammonium sulfate precipitate from AHPND-related *V.parahaemolyticus* broth culture caused AHPND pathology by reverse gavage; however, the combination of the two independently expressed Pir-like toxins A and B requires 10 μg each to cause the same pathology. This indicated that another protein or proteins have a large influence on the virulence of these binary toxins, and this should be a focus of further research.

Other factors affecting AHPND virulence

Exposure to high concentrations of nitrite and ammonia can inhibit the immune response of *P. vannamei* and increase its susceptibility to AHPND-related *V. parahaemolyticus*, eventually leading to increased mortality (Ge et al. 2014a, b). When shrimps were exposed to a combination of pesticides and *V. parahaemolyticus*, mortality increased. However, exposure to pesticides without *V. parahaemolyticus* did not show typical EMS/AHPND pathology (Oanh 2014). The likely reason for this association might be the contamination of the pathogen in the shrimp production line, especially at the hatchery or nursery stages via live feed of brooder stock or larvae. In addition, inappropriate management practices in nursery farms might increase the number and virulence of the pathogen (Boonyawiwat and Kasornchandra 2014).

The authors believe that AHPND may occur when the amount of cultivated shrimps surpasses the environmental tolerance. The direct cause of EMS/AHPND is infection of vibrio, and the fundamental reason is related to breeding.

Research on prevention and therapy

Until now, research on AHPND has mainly focused on pathology and etiology. In most cases, diseases caused by bacterial pathogens could be prevented by appropriate management of shrimp cultivation, including adding probiotics or phages, appropriate water quality, stocking density, aeration, feed quality, feed quantity, and seed quality.

Bacteriophage infection to control and prevent AHPND

Phage therapy is an alternative method to control bacterial pathogens in shrimp cultivation. Under the evaluated conditions, phage therapy was effective to prevent *V. parahaemolyticus* in brine shrimps; however, in advanced infections, their ability to control *Vibriosis* was limited (Martínez-Díaz and Hipólito-Morales 2013). *P. vannamei* larvae infected with *V. parahaemolyticus* were treated with different doses of selected phages, and selected lytic phages (A3S and Vpms1) were effective to reduce mortality (Lomelí-Ortega and Martínez-Díaz 2014).

Ecological approaches to control and prevent AHPND

Recent outbreaks of EMS/AHPND suggest that modern intensive shrimp cultivation needs to be critically reviewed. Current pond culture practices result in microbial communities that very often include a large fraction of opportunistic pathogens (De Schryver et al. 2014), such as AHPND-related *V. parahaemolyticus*. It is necessary to not only focus on the causative agent of the disease, but also to study the microbial community as a whole system. Total disinfection of the pond bottom and water to kill possible vectors of EMS/AHPND may contribute to reducing the epidemic spread of EMS/AHPND disease rather than controlling it, and microbial management strategies and ecological theories may be vital to minimize the risk of EMS/AHPND outbreaks (De Schryver et al. 2014; Sorgeloos et al. 2014).

Microbial management practices (including growing animals in microbially mature ecosystems and applying biocontrol strategies that are compatible with these systems) are key factors to deal with these problems (De Schryver et al. 2014). AHPND will serve as a game changer; better management practices should be adopted as an important national strategy by tropical countries, to ensure reliable and sustainable aquaculture production, with minimal negative impact on the environment. To deal with this disease, multidisciplinary input from scientists in many fields (e.g., aquaculture engineering, biochemical engineering, materials science, aquaculture biology, microbial ecology, aquatic animal health and nutrition) will be required (Flegel 2014). Efficient implementation of measures to control horizontal transmission, including appropriate pond preparation, sufficient water surface dedicated for water treatment and reservoirs, implementing farm biosecurity, eradicating accumulated pathogens by ploughing and drying pond bottoms, fish–shrimp or rice–shrimp crop rotation, polyculture, and aging water in reservoirs using tilapia, and protecting shrimp gut health using probiotics, can all be performed at the farm (Tran et al. 2014). The shrimps from the slightly infected ponds could survive in laboratory conditions for 1 month when water parameters were controlled (Phuoc and Hao 2014).

From multivariate analysis, the pond level factors that are significantly associated with increased risk of EMS/AHPND were the stocking of PLs from some hatcheries and increasing total feed within 1-month period per 100,000 PLs after stocking; thus, increasing the frequency of adding water into the pond could reduce the risk of EMS/AHPND (Kasornchandra et al. 2014).

Breeding and genetics strategies for shrimp farming

Until the WSSV pandemic, the penaeid shrimp farming industry in Asia and the Americas remained largely dependent on wild shrimps to stock their farms, and biosecurity was not

part of the shrimp farming industry's vocabulary (Lightner 2011). Domesticated stocks made it possible to better manage the health status of the farmed stocks, and with *P. vannamei* SPF, domesticated lines were readily available (Lightner 2005). However, AHPND is a newly emerging bacterial disease and is becoming more difficult to deal with, and control measures used to date will not be effective to prevent AHPND because the causative agent is a free-living organism that can persist in marine water and sediment for a long time, even in the absence of carriers. Breeding and genetics might have a slight effect on this problem; however, good management practice, such as post-larvae quality assessment, good preparation of the pond before stocking, natural feed enrichment in the pond before stocking and in the early period after stocking, promotion of heterotrophic microbials to control the optimum water quality and produce a biofloc, and control of the level of *V. parahaemolyticus* in water and in the shrimp gut environment with probiotics and other natural products, were the valuable measures to prevent the AHPND (Boonyawiwat and Kasornchandra 2014).

The pathogens causing AHPND need to be included in the detection of SPF stocks. The best way to deal with the AHPND is to use SPF stocks in completely closed culture systems, which could provide the best biosecurity.

Feed adjustment for shrimp farming

When organic acid was used to supplement the diet of *P. vannamei*, hepatopancreatic protective properties against *V. harveyi* were observed (Romano et al. 2015).

When shrimps received extract of betel leaves (*Piper betle*) and lemongrass (*Cymbopogon citratus*) in their basal diet, the betel extract showed a good potential to treat bacterial diseases and cold minimize the occurrence of AHPND (Kua et al. 2014). Other studies showed that *Ricinus communis*, *Phyllanthus niruri*, *Leucus aspera*, *Manihot esculenta*, and seaweeds (*Ulva lactuca* and *Sargassum wightii*) could improve resistance to *V. parahaemolyticus* in *Penaeus indicus* juveniles (Immanuel et al. 2004). Lo et al. (2014) have produced a vaccine by injecting chicks with PirA and PirB. When added to the basal diet, the vaccine could improve the immunity of *P. vannamei* to AHPND (Lo et al. 2014).

Immune stimulation of biofloc-grown shrimps is an very important method of disease control and could explain the lower prevalence of AHPND observed in farms that apply biofloc technology (NACA 2012).

Biocontrol and Biosecurity for shrimp farming

The occurrence of EMS/AHPND outbreaks in nearby farms could increase the risk of the disease in shrimp farms (Kasornchandra et al. 2014); thus, management, including quarantine, is necessary. Report of AHPND outbreaks in Mexico, which is a long way from Asia, indicated that biosecurity should be an important consideration for local government.

Summary

EMS/AHPND has recently become the most serious disease which the tropical shrimp aquaculture facing. AHPND has been ascribed to specific *V. parahaemolyticus* strains. However, other Vibrio also cause AHPND histopathology. Generally, AHPND/EMS is determined according to the histopathology of the hepatopancreas and the time of stocking

for 20–30 days. Thus, the detection of a single AHPND-related pathogen may be inaccurate. *Vibriosis* only occurs in a susceptible organism when the bacteria reach a certain concentration; therefore, the concentration of Vibrio could be used to assess the risk of AHPND.

Hopefully, further research on *V. parahaemolyticus* will focus on virulence factors. PirA and PirB have been detected as the virulence factors and could be used to develop antibodies that could be added into a basal diet, thereby resisting AHPND by enhancing shrimps' immunity. They could also be developed for rapid detection methods, such as pond-side strip tests. The mechanisms of AHPND-related bacterium infection of shrimps, including how receptors on the hepatopancreas cell membrane meditate the pathogens entry into cells, and the pathogenesis of AHPND-related pathogens should be further researched. The virulence factors isolated with *Vibriosis* share similar characters with *B. thuringiensis*, which suggests that further research on *B. thuringiensis* pathogenesis in insects may provide additional clues to AHPND by *V. parahaemolyticus*. Moreover, further metagenomic and proteomic research into the virulence of AHPND bacteria will lead to a full understanding of the molecular pathogenesis of AHPND.

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