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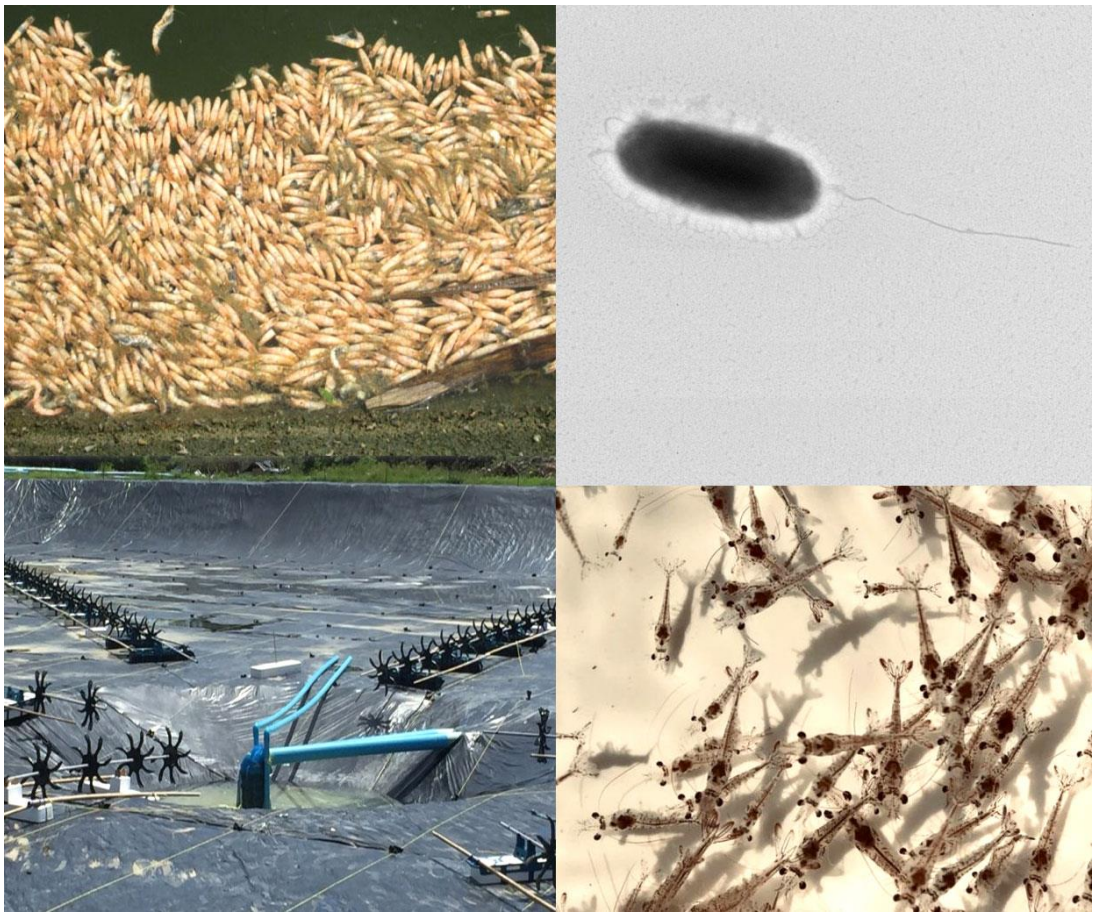
SHRIMP ACUTE HEPATOPANCREATIC NECROSIS DISEASE STRATEGY MANUAL



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Corrigendum

Updated on [3 December 2020]

The following corrections were made to the PDF of the report after it went to print.

Page	Location	Text in printed PDF	Text in corrected PDF
14	Para 2	A nested PCR with increased sensitivity (100 times than single-step PCR) and specificity has been developed for AHPND, this method can be used to detect low-levels of *Vp*AHPND and environmental samples (Dangtip *et al*., 2015).	A nested PCR (AP4 method) with increased sensitivity (100 times than single-step PCR) and specificity has been developed for AHPND, this method can be used to detect low-levels of Vp_{AHPND} and environmental samples (Dangtip *et al*., 2015)
23	Para 23	In **Bangladesh,** Vp_{AHPND} were isolated from *P. monodon* cultured in Satkhira (semi-intensive farms) and Cox's Bazar (hatcheries) in June 2017 (Eshik *et al*., 2018). The affected shrimp were detected by histology and PCR.	In **Bangladesh,** Vp_{AHPND} were isolated from *P. monodon* sampled from shrimp farms in districts of Satkhira and Bagerhat during June 2016 (Eshik *et al*., 2018). The bacterial isolates were determined to be Vp_{AHPND} by PCR using AP3 and AP4 methods (OIE, 2019b).
51	Reference 5	**Eshik, M.M.E., Punom, N.J., Begum, M.K., Sahai, T.K.M. & Rahman, M.S.** 2018. Molecular characterization of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* strains in cultured shrimp *Penaeus monodon* in south-west farming region of Bangladesh. *Dhaka Univ. J. Biol. Sci.*, 27: 57–68.	**Eshik, M.M.E., Punom, N.J., Begum, M.K., Khan, T., Saha, M. L. & Rahman, M.S.** 2018. Molecular characterization of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* strains in cultured shrimp *Penaeus monodon* in south-west farming region of Bangladesh. *Dhaka Univ. J. Biol. Sci.*, 27: 57–68.

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Top left: A mass mortality due to acute hepatopancreatic necrosis disease in *Penaeus vannamei* cultured in the People's Republic of China. Photo credit: ©Mr L. Hu, China and Dr D. Lightner, United States of America.
Top right: Electron micrograph of an acute hepatopancreatic necrosis disease-*Vibrio campbellii*. Photo credit: © Ms C. Li and Dr X. Dong, China.
Bottom left: The use of a central drain ("shrimp toilet") in shrimp pond to remove the bottom sludge. Photo credit: ©Dr P. Chiarahkhongman, Thailand.
Bottom right: Stocking shrimp pond with specific-pathogen-free *Penaeus vannamei*. Photo credit: ©Dr P. Chiarahkhongman, Thailand.

SHRIMP ACUTE HEPATOPANCREATIC NECROSIS DISEASE STRATEGY MANUAL

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PREPARATION OF THIS DOCUMENT

This manual provides guidance for policy makers, producers and other stakeholders for development of contingency plans in relation to outbreaks of acute hepatopancreatic necrosis disease (AHPND), a bacterial disease of farmed marine shrimp listed by the World Organisation for Animal Health (OIE) as notifiable.

The document is based, in large part, on information presented and discussed by participants at the Second Interregional Workshop of the Food and Agriculture Organization of the United Nations (FAO) Project TCP/INT/3502: Reducing and managing the risks of Acute Hepatopancreatic Necrosis Disease (AHPND) of cultured shrimp, one of a series of interregional workshops. These are: 1) TCP/VIE/3304: Technical workshop on early mortality syndrome or acute hepatopancreatic necrosis disease (AHPND) of cultured shrimp, which was hosted by the government of Viet Nam in 2013; 2) TCP/INT/3502: Reducing and managing the risk of AHPND of cultured shrimp, which was hosted by the government of Panama in 2015; 3) TCP/INT/3501 and 3502: Technical workshops on AHPND-this is the way forward, which was hosted by the Government of Thailand in 2016. Other publications emanating from these workshops can be found in *Asian Fisheries Science (2018) Vol. 31s: Acute hepatopancreatic necrosis disease (AHPND).*

The project contributes to the FAO Strategic Programme to increase resilience of livelihoods to threats and crises (SP5), particularly two outcomes, namely: Outcome 5.2 – Countries made use of regular information and early warning against potential, known and emerging threats; and Outcome 5.4 – Countries prepared for and managed effective responses to disasters and crises.

ABSTRACT

The contents of this *Shrimp acute hepatopancreatic necrosis disease strategy manual* provides information and guidance relevant to the development of policies to respond to outbreaks of acute hepatopancreatic necrosis disease (AHPND) in farmed marine shrimp. The etiologic agents for AHPND are virulent strains of bacteria belonging to the genus *Vibrio parahaemolyticus* and related species, which harbor specific toxin genes. While these bacterial species are part of the normal microflora of the marine environment, they may cause substantial mortalities in whiteleg shrimp (*Penaeus vannamei*) and giant tiger prawn (*Penaeus monodon*) cultured in countries in Asia and the Americas. These strains of these *Vibrio* bacteria secrete a PirAB^{vp} binary toxin resulting in sloughing of tubule epithelial cells and dysfunctions of the hepatopancreas in the acute form; mortality can reach 100 percent in affected ponds. Chronic presentation of this disease involves secondary bacterial infection of hepatopancreas and running mortality over the culture cycle. Acute or chronic presentation would greatly depend on the culture conditions. This disease can be considered a toxicosis rather than an infection. Economic losses due to this disease have amounted to over USD 7 billion annually. Further outbreaks of AHPND, particularly in areas that are currently free of the disease, would be expected to experience similar devastating effects on local shrimp producers and the surrounding communities; and thus, there is an urgent need to develop a contingency plan to control and eradicate this disease. This manual includes information on: 1) the nature of AHPND: a brief review of current knowledge in disease etiology, susceptible species and global distribution; 2) diagnosis of disease: a description of gross clinical signs and laboratory methods; 3) prevention and treatment: farm management, the use and development of antibiotics, bacteriophages, probiotics, disease-tolerant shrimp, shrimp immunity and vaccination; 4) epidemiology: AHPND's geographic distribution, genotype, persistence in the environment, reservoir hosts, modes of transmission, risk factors, and economic impacts; 5) principles of control and eradication: methods for containment, mitigation and eradication of AHPND, and trade and industry considerations; and 6) policy development and implementation: AHPND-specific objectives, options and strategies for eradication and control, education, capacity building, funding, and compensation.

CONTENTS

TABLES

FIGURES

ACKNOWLEDGEMENTS

This publication was supported by the Food and Agriculture Organization of the United Nations (FAO) Project TCP/INT/3502: Reducing and managing the risks of Acute Hepatopancreatic Necrosis Disease (AHPND) of cultured shrimp.

The preparation of this manual was initiated during the project workshops held in Viet Nam, Panama, and Thailand. The Governments of Viet Nam, Panama and Thailand are gratefully acknowledged for graciously hosting the workshops. We thank the contributions of all participants to this effort, and Dr Pathrarpol Piamsomboon, Chulalongkorn University, Thailand for providing a questionnaire form to survey farm's risk factors.

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ABBREVIATIONS AND ACRONYMS

AHPND	Acute hepatopancreatic necrosis disease
AHPNS	Acute hepatopancreatic necrosis syndrome
AMR	Antimicrobial resistance
APW	Alkaline peptone water
ASEAN	Association of Southeast Asian Nations
B cells	Blastozellen cells
CCRF	Code of Conduct for Responsible Fisheries
CFU	Colony forming unit
Chr	Chromosome
DAFF	Department of Agriculture, Forestry and Fisheries (Australia)
E cells	Embryozellen cells
EHP	*Enterocytozoon hepatopenaei*
ELISA	Enzyme-linked immunosorbent assay
EMS	Early mortality syndrome
FAO	Food and Agriculture Organization of the United Nations
F cells	Fibrillenzellen cells
H&E	Haematoxylin and eosin
HP	Hepatopancreas
LAMP	Loop-mediated isothermal amplification
MAb	Monoclonal antibody
MIC	Minimum inhibitory concentration
NACA	Network of Aquaculture Centres in Asia-Pacific
NHP	Necrotizing hepatopancreatitis
NSAAH	National Strategy for Aquatic Animal Health
OD	Optical density
OIE	World Organisation for Animal Health
OIRSA	Organismo Internacional Regional de Sanidad Agropecuaria
OTC	Oxytetracycline
PCR	Polymerase chain reaction
PCP	Progressive control pathways
Pir	*Photorhabdus* insect-related
PL	Postlarvae
PMP/AB	Progressive Management Pathway for Improving Aquaculture Biosecurity
qPCR	Quantitative polymerase chain reaction
R cells	Restzellen cells
RAS	Recirculating aquaculture system
SEAFDEC	Southeast Asian Fisheries Development Center
SPF	Specific-pathogen-free
SPT	Specific-pathogen-tolerant
TCBS	Thiosulfate citrate bile salts sucrose
TDH	Thermostable direct haemolysin
tRNA	Transfer ribonucleic acid
TSB	Tryptic-soy broth
TSB+	Tryptic-soy broth with added 2.5 percent NaCl
TSV	Taura syndrome virus
Vc_{AHPND}	*Vibrio campbellii* that causes AHPND
Vp_{AHPND}	*Vibrio parahaemolyticus* that causes AHPND

WGS	Whole genome sequencing
WHO	World Health Organization
WSD	White spot disease
WSSV	White spot syndrome virus

1. Introduction

This disease strategy manual focuses on acute hepatopancreatic necrosis disease (AHPND), which is an important shrimp disease, as the mortalities can reach up to 100 percent. AHPND is caused by pathogenic strains of bacteria belonging to the genus *Vibrio*, mainly *Vibrio parahaemolyticus* (abbreviated as Vp_{AHPND}) carrying specific toxin genes. Vp_{AHPND} affects penaeid shrimp, including the most commonly farmed species, the whiteleg shrimp (*Penaeus vannamei*) and the giant tiger prawn (*P. monodon*). AHPND was first seen in farmed shrimp in the People's Republic of China in 2010 and subsequent outbreaks occurred in Viet Nam, Malaysia, Thailand, the Philippines, Mexico and other Latin American countries, Bangladesh, the United States of America, Taiwan Province of China, South Korea and, in 2020, in Okinawa Prefecture of Japan. Losses due to AHPND were estimated to be more than USD 7 billion per year (Shinn *et al*., 2018a). Because it is extremely virulent and rapidly spreading, AHPND has been listed as a disease notifiable to the World Organisation for Animal Health (OIE) (OIE, 2019a). Responses of the aquaculture industry to the threat of this disease include the development of country-specific contingency plans.

Disease strategy manuals are part of technical plans, which are sets of instructions of manuals, required to support the various components of national contingency plan (Arthur *et al*., 2005). The outline of a disease strategy manual is based on the Australian Aquatic Animal Diseases Veterinary Emergency Plan (AQUAVETPLAN).

This disease strategy manual consists of the following major sections, namely:

(1) nature of the disease (etiology, susceptible species, global distribution)
(2) diagnosis of diseases (Levels I, II, III diagnosis, sample submission, bioassays and corroborative diagnostic criteria)
(3) prevention and treatment (farm management, antibiotics, bacteriophages, probiotics, development of AHPND-tolerant shrimp, shrimp immunity and vaccination)
(4) epidemiology (geographic distribution and prevalence, genotype, persistence in the environment, vectors and reservoir hosts, modes of transmission, factors influencing disease transmission and expression, impact of the disease)
(5) principles of control and eradication (methods for preventing spread and eliminating pathogen, quarantine and movement controls, tracing, risk factor analysis, surveillance, farming practices, destruction and disposal of diseases shrimp, disinfection of affected farms, vector control, environmental considerations, public awareness, control, containment and zoning, control and mitigation of disease, trade and industry considerations, domestic markets, exports markets
(6) policy and rationale (overall policy, AHPND-specific objectives, problems that need to be addressed, overview of response options, strategies for eradication and control, improve knowledge and capability and funding and compensation)

This manual can serve as a framework for the development of national contingency plans in anticipation of AHPND outbreaks. The aims are to greatly reduce the risk of production losses resulting from AHPND, allow expansion of existing producers as they gain confidence, and provide support for a robust global shrimp aquaculture industry.

2. The nature of acute hepatopancreatic necrosis disease (AHPND)

AHPND is a bacterial disease that has caused mass mortalities in farmed populations of whiteleg shrimp (Figure 1) and giant tiger prawn. Outbreaks of this disease have led to severe losses to shrimp producers in Southeast Asia and Latin America. The disease first appeared in shrimp farms in the People's Republic of China and Viet Nam in 2010 and was later found in several other countries in Asia. In the western hemisphere, AHPND was first reported in Mexico in 2013; and it subsequently spread to other Latin American countries and the United States of America. Mortality from AHPND occurs usually within 30–35 days, but as early as 10 days, after postlarvae (PL) are stocked in ponds. This characteristic led to the disease being initially referred to as early mortality syndrome (EMS). However, a chronic form could cause mortality throughout the culture period. Also, in Asia, AHPND-related mortalities occur primarily in grow-out; while in Latin America, the major impact is in postlarval and nursery stages. The difference in presentation may be related to the lower stocking densities typical of farms in Latin America.

Through isolation of bacteria from diseased shrimp and laboratory infection studies, the causative agent was identified as virulent strains of *V. parahaemolyticus* (Zhang *et al*., 2012; Tran *et al*., 2013) and was listed by the OIE as a notifiable disease in 2016 (OIE 2019a). In recent years, four other AHPND-causing *Vibrio* spp. were identified from affected shrimp populations. These include *Vibrio harveyi*, *V. owensii*, *V. campbellii* and *V. punensis* (Kondo *et al*., 2015; Liu *et al*., 2015, 2018; Ahn *et al*., 2017; Dong *et al*., 2017a, 2017b; Restrepo *et al*., 2018). AHPND can be caused by strains of several *Vibrio* spp. due to the fact that the toxin genes $pirAB^{vp}$ reside in a plasmid that contains two clusters of conjugative transfer genes and a mobB gene, which allow horizontal transfer between bacterial species (Xiao *et al*., 2017; Dong *et al*., 2019a, 2019b). The acute form of this disease can be considered a toxicosis rather than an infection. However, as the disease progresses, there is secondary bacterial colonization on the damaged tissues.

The toxins produced by the pathogenic *Vibrio* spp. affect the hepatopancreas (HP). Clinical signs include a pale-to-white atrophied HP (Figures 2 and 3), empty stomach and midgut, soft carapace, and sometimes the appearance of black spots or streaks (due to melanization) within the HP tubules in the chronic stage. However, these clinical signs are not specific and insufficient to make definite diagnosis.

Figure 1. The occurrence of a mass mortality due to AHPND in *P. vannamei* cultured in China.

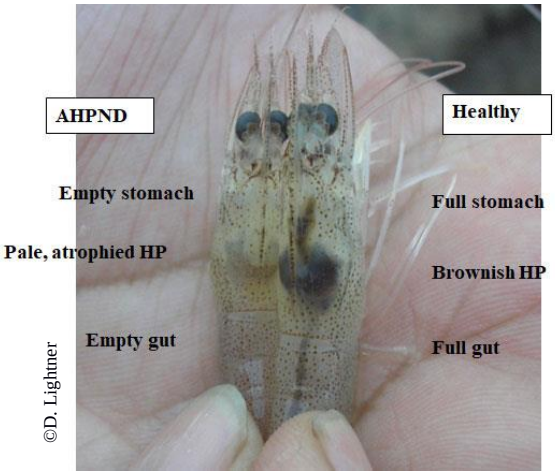

Figure 2. Clinical signs of AHPND-affected *P. vannamei* versus healthy shrimp.

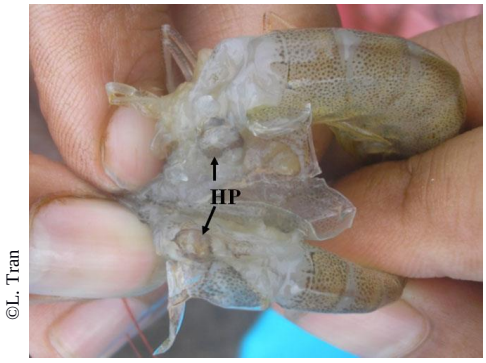

Figure 3. AHPND-affected shrimp. Note the pale and atrophied HP indicative of AHPND.

2.1 Etiology

The genomes of these AHPND-causing *Vibrio* spp. contain a 69–73 kb plasmid harboring genes that encode the *Photorhabdus* insect-related (Pir) toxin PirAB^{vp}. PirA^{vp} (12.7 kDa) and PirB^{vp} (50.1 kDa) are extracelluar proteins, acting together as a binary toxin. The plasmid was referenced as pVPA3-1 in Han *et al*. (2015a) and as pVA1 in Lee *et al*. (2015). To prove that this plasmid is responsible for the virulence of AHPND, knockout mutants (constructed in the laboratory) and a natural deletion mutant (named M2-36, isolated from the shrimp pond) of *pirAB*^{vp} showed the abolishment of the virulence in the laboratory infection studies. The virulence was recovered after the introduction of the *PirAB*^{vp} operon into the knockout mutant (Lee *et al.*, 2015).

Species of *Vibrio* are Gram-negative, rod-shaped bacteria found in marine and estuarine environments throughout the world. Some strains are important pathogens of aquatic species and some can affect humans as well (Madden, McCardell and Morris, 1989; Nash *et al.*, 1992; Thompson and Lida, 2004; Actis, Tolmasky and Crosa, 2011). *Vibrio* spp. are a normal part of the flora in shrimp ponds, but they are usually only known to be secondary, opportunistic invaders when associated with diseases of aquatic animals (Elston, Elliot and Colwell, 1982). AHPND-causing *Vibrio* spp. are different, as these are primary causal agents. They colonize the stomach of shrimp (Figure 4) and secrete the PirAB^{vp} toxin to the HP (the target organ), resulting in detachment and breakdown of epithelial cells, which then became substrates for the bacteria to replicate. Eventually, this results in the dysfunction and destruction of the HP.

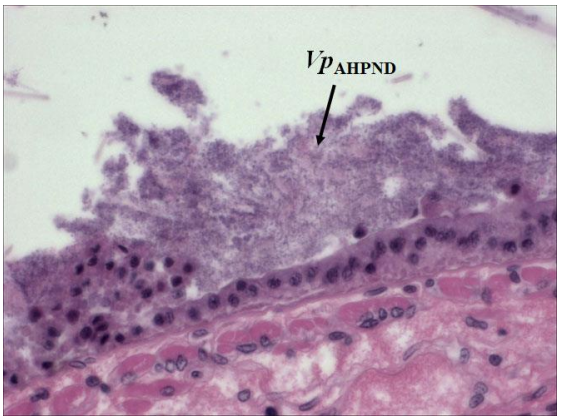

Figure 4. AHPND-affected shrimp. Vp_{AHPND} colonized in the stomach of *P. vannamei.*

In this manual, Vp_{AHPND} is used to refer to the causative *V. parahaemolyticus*, which was first identified in association with the disease and is more frequently found in diseased shrimp than other *Vibrio* spp. The information in this manual also applies to all other *Vibrio* spp. that cause AHPND.

The whole genomes of a number of strains of the AHPND-causing *Vibrio* spp. have been sequenced (Dong *et al*., 2019b). Among the strains of AHPND-*V. campbellii* (Vc_{AHPND}), their total genome sizes are 6.1 Mb (strain from the People's Republic of China) and 6.3 Mb (strain form Latin America). Each genome possesses two circular DNA chromosomes (Chr), Chr #1 (3.5–3.6 Mb) and Chr#2 (2.2 Mb); and four plasmids (63.9–204.5 kb). The size of the $PirAB^{vp}$ plasmid (named as pLA16-2) of the Latin American isolate is 73 kb, 3.4 kb larger than the that of the Chinese isolate (plasmid named as pVCGX1), supporting the geographical variation in the $PirAB^{vp}$ plasmids among AHPND-*Vibrio* isolates (Han, Tang and Lightner, 2015b). Both isolates have high copy numbers of transfer ribonucleic acids (tRNAs), 134 in the Chinese isolate and 133 in the Latin American isolate; this is of interest as tRNAs usually serve as integration sites for horizontal gene transfer.

Sometimes, from screening of Vp_{AHPND} populations in diseased shrimp or shrimp ponds, natural mutants have been found, such as deletions of $pirA^{vp}$ or $pirAB^{vp}$ genes (Lee *et al.,* 2015; Han *et al.,* 2017a). Such mutants are non-pathogenic to shrimp as determined through laboratory infection studies. The deletion events may be mediated by the inverted repeats of an insertion sequence (named as ISVal1) that flank the $PirAB^{vp}$ operon (Han *et al.,* 2017a). The ISVal1 may allow the $pirA^{vp}$ and $pirB^{vp}$ genes to be easily transferred within cells, through transposition, and between cells, via conjugation or plasmid uptake. The finding of AHPND non-pathogenic mutant strains (deletions and possible insertions) not only supports the crucial role of the $pirAB^{vp}$ in AHPND pathogenicity, but also is relevant to accurate diagnosis based on polymerase chain reaction (PCR). Most of the AHPND-diagnostic PCR methods target $pirA^{vp}$ or/and $pirB^{vp}$ sequences. These primers may react to some of the non-pathogenic mutants, if the primers' binding sites are still present, and generate false positives. Recently, two *V. parahaemolyticus* isolates were found to be $pirAB^{vp}$-positive determined by PCR, but they did not express $PirAB^{vp}$ toxin and did not cause AHPND pathology in the exposed *P. vannamei* in the laboratory infection studies (Vicente *et al*., 2019). Therefore, AHPND-PCR positive results need to be confirmed either through histology, immunodetection or laboratory infection (see Section 3).

2.2 Susceptible species

According to the OIE's *Aquatic animal health code* (Aquatic code, OIE, 2019a), species that fulfill the criteria as being susceptible to AHPND include: whiteleg shrimp (*P. vannamei*) and giant tiger prawn (*P. monodon*). The Chinese white shrimp (*P. chinensis*) and the kuruma prawn (*P. japonicus*) are listed as species for which there is incomplete evidence for susceptibility.

2.3 Global distribution

AHPND was first reported in populations of cultured *P. vannamei* in the People's Republic of China and Viet Nam in 2010, and has since been reported in Malaysia (2010), Thailand (2011), Mexico (2013), the Philippines (2014), South American countries (2014-2016), Bangladesh (2017), the United States of America (2017), the Taiwan Province of China (2018), South Korea (2019) and Okinawa Prefecture of Japan (2020) (Gomez-Jimenez *et al.*, 2014; Yang *et al.*, 2014; Kondo *et al.*, 2014, 2015; de la Peña *et al.*, 2015; Soto-Rodriguez *et al.*, 2015; Restrepo *et al.*, 2016; Dabu *et al*., 2017; OIE, 2017, 2019c; Kumar *et al*., 2018; Han *et al*., 2020a; OIE, 2020). AHPND is suspected to be present, although not reported, in other countries in both Asia and Latin America (Figure 5).

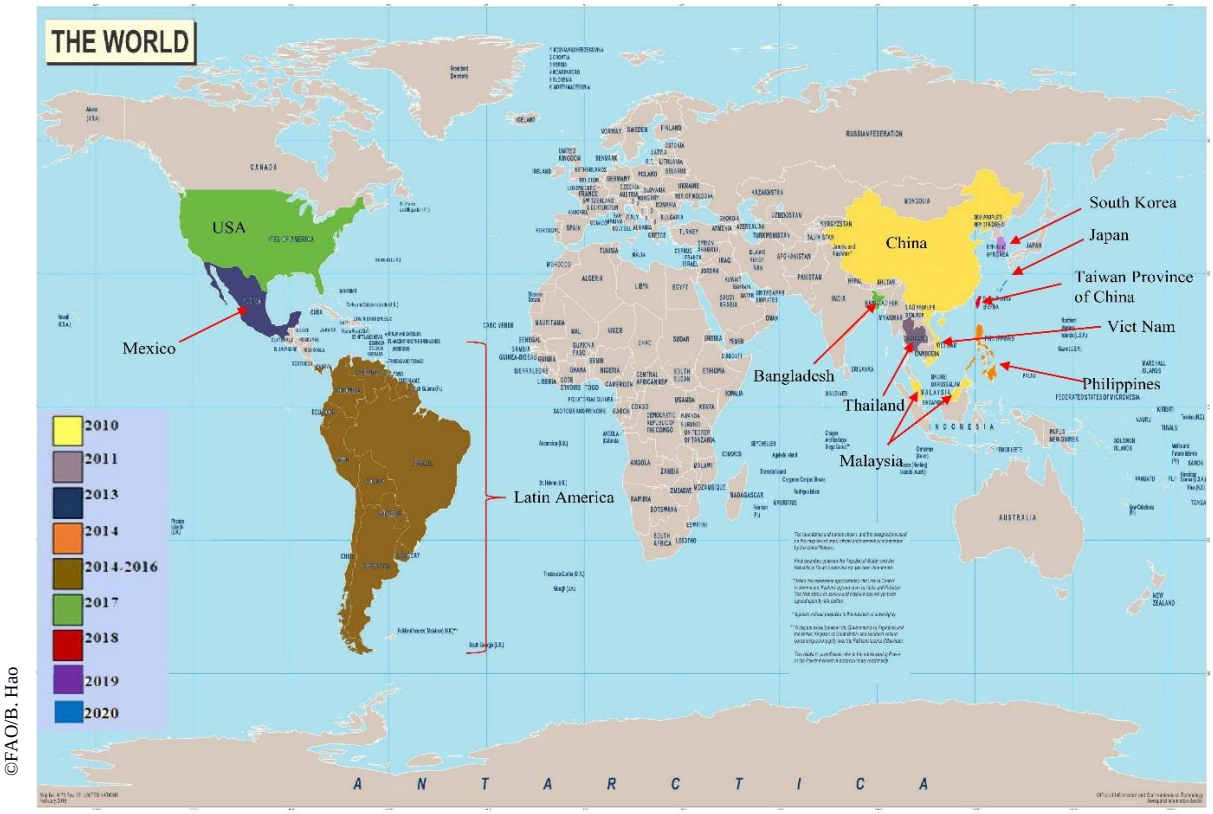

Figure 5. Reported distribution of AHPND and year of first report. China (2010), Viet Nam (2010), Malaysia (2010), Thailand (2011), Mexico (2013), the Philippines (2014), Latin American countries (2014–2016), Bangladesh (2017), United States of America (2017), Taiwan Province of China (2018), South Korea (2019), Okinawa Prefecture of Japan (2020). Map No. 4170 Rev. 17, United Nations, February 2019.

3. Diagnosis of disease

Shrimp affected by AHPND usually show early (<30–35 days after stocking) mortality, and exhibit distinctive gross clinical signs. Examination of histological sections of HP stained with haematoxylin and eosin (H&E) can provide a presumptive diagnosis of AHPND. However, for definitive diagnosis and to determine the status of pathogenicity, PCR (or quantitative polymerase chain reaction, qPCR), loop-mediated isothermal amplification (LAMP) testing and bacterial isolation followed by laboratory infection studies are recommended (OIE, 2019b). Immunoassays, such as dot-blot, Western blot and enzyme-linked immunosorbent assay (ELISA) are not used routinely by shrimp pathologists as these methods lack the sensitivity of PCR (and qPCR) and LAMP.

The following sections describe these means of diagnosis: observation of clinical signs, histopathology, PCR-based methods, LAMP methods, immunoassays targeting $PirA^{vp}$ and $PirB^{vp}$ proteins, and bacterial isolation and identification followed by laboratory infection study to verify isolates' pathogenicity. The diagnostic methods of aquatic animals can be categorized into three levels as described by Bondad-Reantaso *et al.* (2001). Level I activities include the observations of animals' behaviors and examination of gross clinical signs. Level II analyses are the isolation and examination of pathogens in parasitology, bacteriology and mycology and histopathological evaluations of infected hosts. Level III assays include bacterial/viral isolation, culture, electron microscope examination, and molecular techniques (PCR, LAMP, immuno-assays).

3.1 Gross clinical signs (Level I)

The onset of clinical signs and mortality usually starts within 30–35 days post-stocking in the intensive ponds. The mortalities range from 40–100 percent. Clinical signs include a pale-to-white, atrophied HP (Figures 2 and 3), empty stomach and midgut (Figure 2), black spots or streaks visible within the HP (due to melanized tubules) and soft shells at the chronic phase of disease. In addition, the HP does not squash easily between the thumb and forefinger (probably due to increased fibrous connective tissue and accumulation of haemocytes) (NACA, 2012). The gross signs, related to the chronic presentation, are not specific to AHPND; they are also associated with shrimp affected by necrotizing hepatopancreatitis (NHP, also known as infection with *Hepatobacter penaei*) and other *Vibrio* bacteria diseases.

3.2 Laboratory methods

3.2.1 Sample submission

At the first sign of the disease, clinical samples (shrimp HP, whole shrimp, faecal samples or bacterial isolates) should be sent to local (regional or state) aquatic animal diagnostic laboratories. Whenever possible, shrimp with clinical signs of disease should be selected for sampling (OIE, 2019b). If AHPND is diagnosed, the Competent Authority (CA) should be notified and the diagnosis confirmed through repeated testing with other methods and/or testing of additional specimens from the suspected AHPND-affected populations. The CA should be contacted directly to obtain information on what additional clinical materials may be required and how they should be collected, stored and transported to satisfy requirements for confirming the original diagnosis of AHPND.

(a) Shrimp samples

The number of individual samples required when screening for AHPND will depend on the sensitivity and specificity of the diagnostic protocol, the population size, the disease prevalence and the level of confidence desired (Lightner, 1996). Ideally, all laboratory procedures should comply with the OIE's *Manual of diagnostic tests for aquatic animals* (Aquatic manual, OIE, 2019b). The recommended sample size (i.e. number of individual shrimp) that should be collected for diagnosis when AHPND is present in the population at a prevalence of 2 percent is 149 as stated in the OIE Aquatic code, chapter 1.4 (OIE, 2019a). This is assuming that the sensitivity and specificity of the method used for diagnosis are both 100 percent. With some methods, individual shrimp can be pooled for collective diagnosis. In the State of Texas, United States of America, the local authority surveys the farms every two weeks, collecting 50 shrimp to examine for clinical signs. If five shrimp (10 percent) are suspected for AHPND, then the samples will be submitted to local/regional diagnostic laboratories for PCR and histological analyses.

For PCR (or qPCR) detection, 50 PLs or 5–10 HP (or stomach, midgut, hindgut) sampled from juveniles or adult shrimp, can be pooled into one sample for DNA extraction. The quality of the specimens is important, and they must be properly preserved, stored and transported to avoid DNA degradation. For histological evaluation, the shrimp should be fixed in Davidson's alcohol-formalin-acetic acid for 24–48 h (depending on the shrimp size) and then transferred to 70 percent ethanol for storage. It is best to split a sample into two parts, one for Davidson's fixation, with the other part either frozen (at -20 ^{o}C or lower temperature) or preserved in 70–95 percent ethanol. Fixed tissues can be processed for histology; the frozen or ethanol-preserved tissues can be used for PCR (or qPCR) analyses.

Preliminary enrichment culture for detection of *Vp*$_{\text{AHPND}}$ from fresh, subclinical samples (HP or PL) or environmental samples may be carried out through the use of any suitable bacteriological medium (e.g. tryptic-soy broth (TSB) or alkaline peptone water (APW) containing 2.5 percent NaCl supplement) incubated for 6 h (to begin with) at 28±2 °C with gentle shaking (100–150 rpm). Then, after letting any debris settle, the bacteria in the culture broth are pelleted by centrifugation. After discarding the supernatant, the DNA can be extracted from the bacterial pellet in preparation for PCR (qPCR) analyses.

(b) Faecal samples

Faecal strands can be used as PCR (qPCR) testing specimens as a non-invasive method for screening and monitoring valuable broodstock populations. The collected faeces can be used for DNA extraction directly, or, the *Vp*$_{\text{AHPND}}$ bacteria present in the faeces (fresh or chilled on ice) can be enriched through 6-h culturing in TSB with added 2.5 percent NaCl (TSB+) (Han *et al.*, 2017b). A loopful of broth can be streaked on thiosulfate citrate bile salts sucrose (TCBS) agar plates for isolation of *Vibrio* spp. and confirmation by laboratory infection studies, if needed.

(c) Water and pond sediment samples

An efficient method to detect the presence of *Vp*$_{\text{AHPND}}$ in water, sediment and pond environments is the incubation of pellet feed in a net bag for 6 h followed by DNA extraction of the pellets (see below). Alternatively, from pond water, a volume of 200 mL can be pre-filtered with 100 µm mesh and then filtered with a polycarbonate membrane (0.2 µm). The

membrane can be cut into pieces, then the retained bacterial DNA can be extracted with a standard procedure and analyzed by PCR (or qPCR). For enrichment, the samples can be homogenized in TSB+ at a ratio of 1:10 (wt/vol) and the broth incubated for 6 h at 28±2 ^{o}C. Alternatively, pond water can be concentrated with a Microsep Advance Centrifugal Device (PALL Corporation), desalted through a Tris buffer (10 mM Tris-Cl, pH 8.5), and the extracted DNA from the retained bacteria used as templates for PCR (or qPCR) analyses.

For pond sediment, a 1–10 g sample can be mixed with 2.5 percent saline and pre-filtered with 100 µm mesh to remove macro fauna, the filtrate clarified by centrifuging at 500 x g, and then the supernatant centrifuged at 16 000 x g for 15 min. The pelleted bacteria can then be extracted for DNA. For enrichment, the pond sediment sample can be resuspended in 5 mL TSB+ and cultured for 6 h at 28±2 ^{o}C. DNA can be extracted and analyzed by PCR (qPCR) analyses.

(d) Enrichment of *Vp*$_{\text{AHPND}}$ with shrimp feed

Because *Vp*$_{\text{AHPND}}$ only requires a relatively small number of bacteria to cause mortality in affected populations, the bacteria can replicate in shrimp feed suspended in the water. The increased bacteria in the colonized feed can then be used for diagnosis. To accomplish this, a net bag with a few feed pellets can be incubated in broodstock tanks, larval tanks, nurseries, or ponds for a period of 6 hours; then AHPND DNA can be extracted from the feed and followed by PCR (or qPCR) analysis.

(e) *Vp*$_{\text{AHPND}}$ isolates

From the AHPND-suspected (or AHPND-positive) shrimp, *V. parahaemolyticus* (or other *Vibrio* spp.) can be isolated from the HP (and/or guts) with a standard procedure (Tran *et al.,* 2013). These samples should be chilled on ice immediately after collection then analyzed as soon as possible. Direct contact with ice should be avoided to maximize the viability of *Vibrio* spp.

For direct isolation and culturing, the fresh HP (and/or guts) should be aseptically removed, minced and homogenized in saline water. One loopful of the lysate can be streaked onto a TCBS agar plate and incubated at 28±2 ^{o}C for 24 h. Green colonies are picked for isolation of the *V. parahaemolyticus* and *V. campbellii* (which cannot ferment sucrose and produce green colonies) (Figure 6A). AHPND-causing *V. owensii* appear as yellow colonies on a TCBS plate (Figure 6B). For enrichment, the HP lysate can be inoculated into flasks containing TSB+ to enrich the *Vp*$_{\text{AHPND}}$ by growing at 28±2 °C for 6–18 h (or overnight). These bacteria can be streaked on the culture media (trypticase soy agar with added 2.5 percent NaCl, or marine agar) to subculture for individual colonies. The pure bacterial isolates can be sent to a local (regional or state) diagnostic laboratory for determining if they possess the *pirAB*^{vp} genes or secrete the PirAB^{vp} toxin by PCR (qPCR) or by immunoassays, respectively.

For PCR (qPCR) detection, the bacterial DNA can be prepared by growing the bacteria overnight at 28±2 °C in TSB+. A 1.0 mL sample of culture can be centrifuged in a microcentrifuge tube for 3 min at 16 000 x g (or maximum speed). The pelleted bacteria can be washed twice with a buffer (e.g. Tris-HCl) and resuspended in 1.0 ml H_2O, boil for 10 min. This bacterial DNA solution can be stored at -20 °C until use. The detection limit of most conventional PCR methods is ~10^4 colony forming units (CFU)/mL of *Vp*$_{\text{AHPND}}$ pure culture (Tinwongger *et al.,* 2014; Lai *et al.,* 2015). The qPCR and LAMP methods are generally 100

times more sensitive than conventional PCR (Han *et al.*, 2015c; Kongrueng *et al*., 2015; Arunrut *et al.*, 2016).

From the AHPND-affected shrimp ponds, faecal strands, pond water and sediments can also be used for Vp_{AHPND} isolation using the pretreatment and enrichment procedures mentioned above. After enrichment, a loopful of top broth can be streaked onto TCBS agar plates for subculturing of individual colonies. *V. parahaemolyticus* and *V. campbellii* appear as round, opaque, green or bluish colonies 2 to 3 mm in diameter on a TCBS agar plate; *V. owensii* appears as yellow colonies (see Figure 6).

Identification of bacterial species can be carried out using the API Rapid NE test (bioMerieux Industry), 16S rRNA sequencing (Weisburg *et al.*, 1991) or PCR targeting species (*V. parahaemolyticus*)-specific genes (*ToxR* genes) (Kim *et al.*, 1999). These bacteria can be stored at -80 °C in TSB+ supplemented with sterile glycerol (20 percent vol/vol).

Following the bacterial isolation and identification, laboratory infection tests can be used to confirm their AHPND-associated pathogenicity; a highly susceptible host species, such as *P. vannamei*, should be used.

Viable Vp_{AHPND} can be isolated from frozen shrimp, this is contrary to a previous finding by Tran *et al*. (2013). Vp_{AHPND} survive freezing process but require a step of enrichment in APW for 6 h prior to plating in culture media.

The diagnosis depends on the gross signs, histopathology, PCR-based methods, immunoassays, Vp_{AHPND} isolation and laboratory infection. A flowchart of the procedure for diagnosis of AHPND is given in Figure 7.

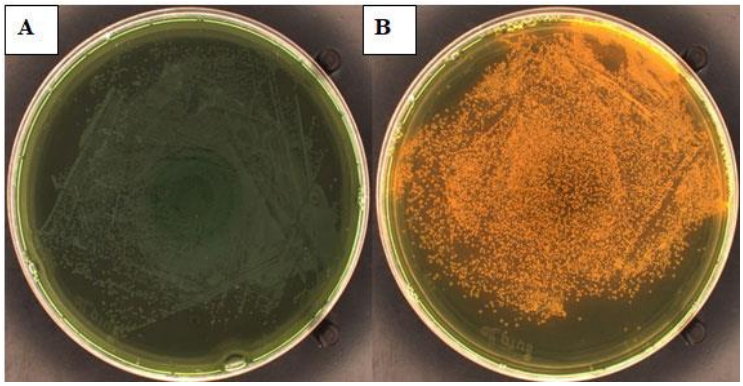

Figure 6. AHPND-causing *Vibrio* spp. isolated from *P. vannamei* and grown on TCBS agar plates. (A) *V. parahaemolyticus* produced green colonies, (B) *V. owensii* produced yellow colonies.

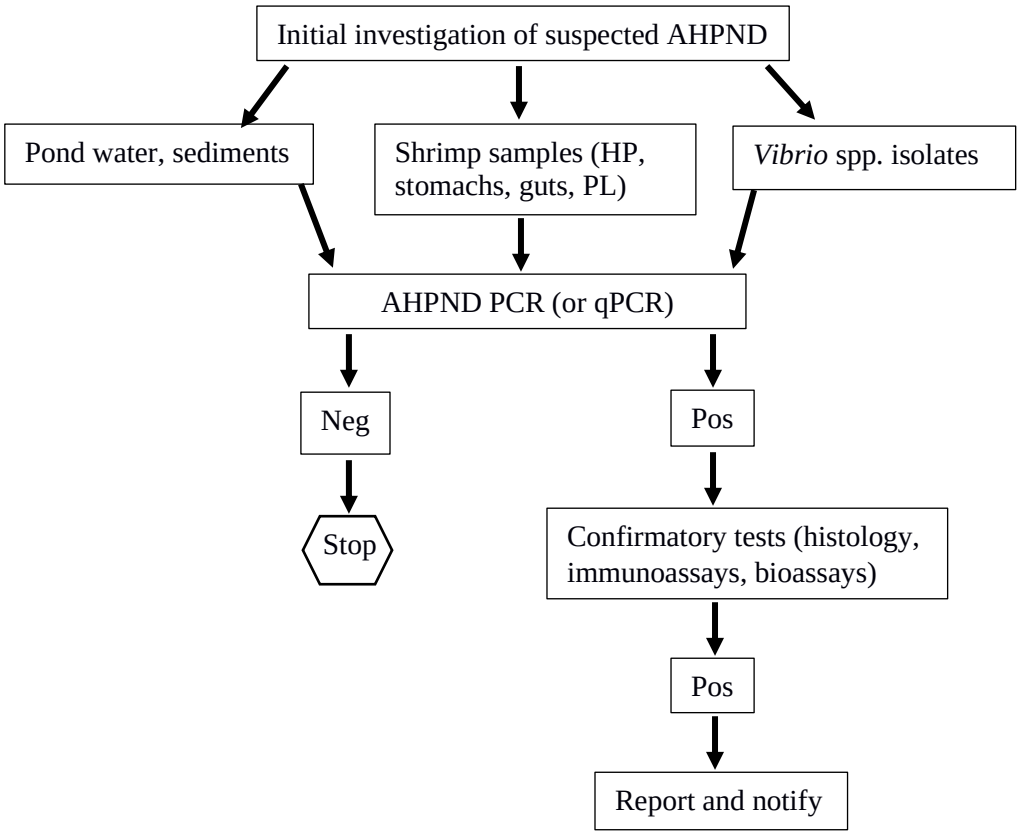

Figure 7. AHPND diagnostic flowchart. Pos: positive, Neg: negative.

3.2.2 Histopathology (Level II)

For histopathology, moribund PLs or whole shrimp (or samples of their HP) can be used. The samples should be preserved in Davidson's fixative, processed into paraffin blocks, and sectioned. The tissue sections should then be stained with H&E through the use of standard protocols (Lightner, 1996). Stained sections are then examined using a compound microscope. The normal histology of healthy shrimp HP is shown in Figure 8.

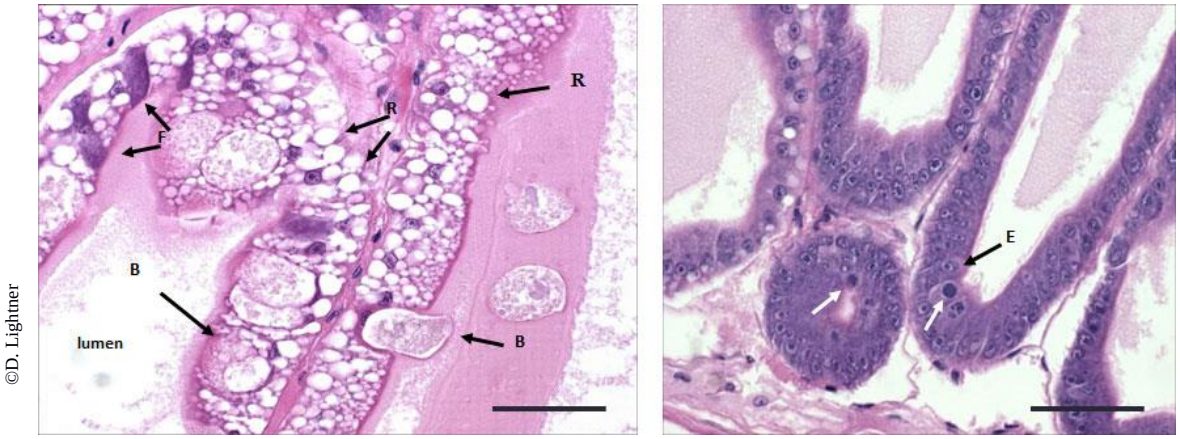

Figure 8. Normal HP showing the presence of embryonic or Embryozellen cells (E), fibrillar or Fibrillenzellen cells (F), resorptive/absorptive or Restzellen cells (R), and blister or Blastozellen cells (B) cells. White arrows: metaphase. Scale bars = 25 µm.

Table 1. Morphological and functional characteristics of HP cells of penaeid shrimp.

Cell type	Morphology	Function
R (Restzellen)	• the most abundant cell type in the HP • lines to the lumen of the HP tubules • contains variable-sized lipid vacuoles (multivacuolated cells) • has a large, round nucleus with a prominent nucleolus	• main nutrient reserve • food absorption • storing lipid droplets and glycogen • sequestering of mineral deposits, including calcium, magnesium, and others
F (Fibrillenzellen)	• fibrillar appearance, basophilic • contains large numbers of ribosomes, endoplasmic reticulum	• synthesizing proteins • storing the minerals
B (Blastozellen)	• contains a single large vacuole which occupies 80–90% of the total cell volume • a cluster of small vacuoles may sometimes be present between the large vacuole and the cell border • The nucleus lies proximal to the large vacuole and appears to be compressed	• producing digestive enzymes • responsible for digestion • concentrating the nutrients in the large vacuole • secreting the nutrients to the lumen
E (Embryozellen)	• a small undifferentiated columnar cell seen at the distal end of the tubule • contains an ovoid shape nucleus • undergoes differentiation to become F, B and R cells	• generating new cells

The HP is a large organ occupying the greater portion of the cephalothoracic cavity, with a thin layer of membrane. The HP is comprised of four types of epithelial cells: embryonic or Embryozellen cells (E cells), fibrillar or Fibrillenzellen cells (F cells), resorptive/absorptive or Restzellen cells (R cells), and blister or Blastozellen cells (B cells) (Caceci *et al.*, 1988; Al-Mohanna and Nott, 1989; Bondad-Reantaso, Tran and Hue, 2013). Their morphologies and functions are summarized in Table 1.

At the early stage of disease, histological examination shows that the HP tubules start to degenerate, rounding up and sloughing into their lumens. The sloughing caused by *Vp*$_{AHPND}$ is likely due to the effect of the PirAB^{vp} toxin on the cytoskeletal proteins (such as microtubules and actin filaments) involved in the cell's attachment to the basement membrane. Some epithelial cells exhibit markedly prominent enlarged nuclei (karyomegaly) (Figures 9 and 10); the inflammatory response is not evident at this stage.

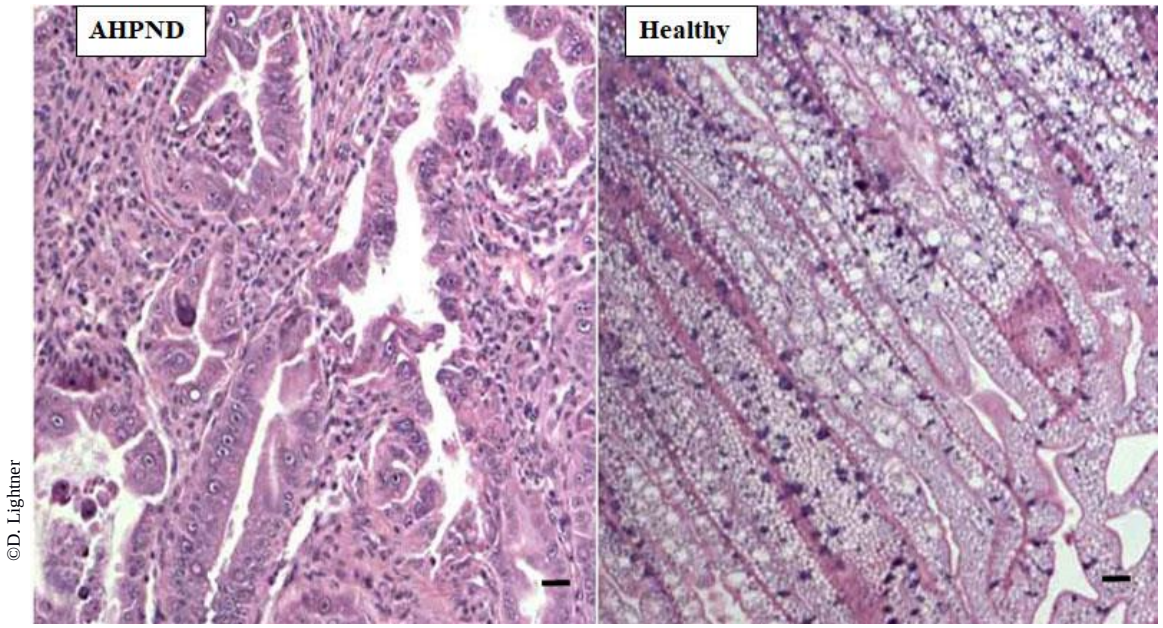

Figure 9. AHPND-affected vs healthy shrimp. Histological examination of the HP of *P. vannamei*. Left: early stage of diseased shrimp. Right: healthy shrimp. Scale bars = 25 µm.

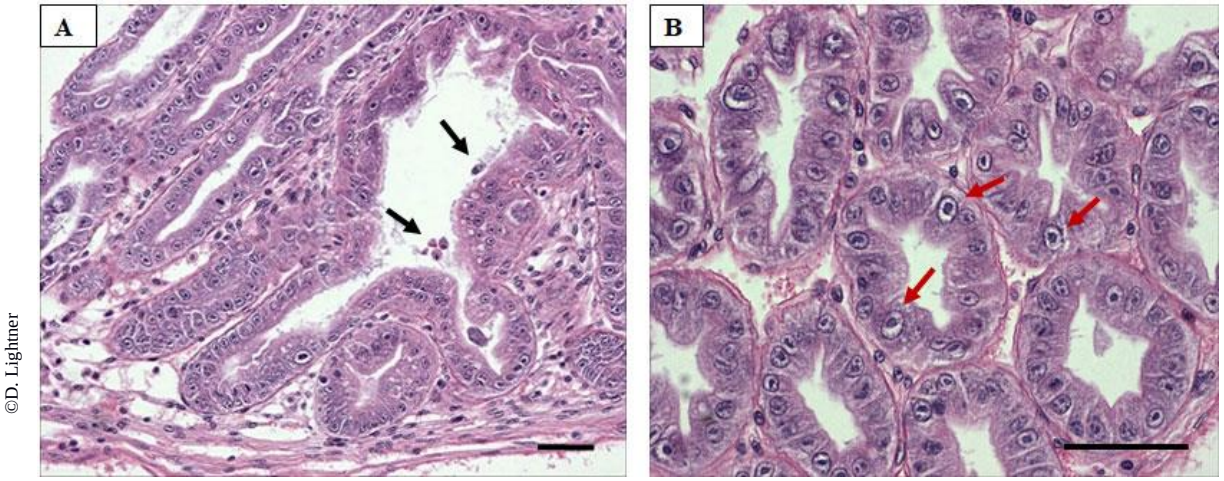

Figure 10. AHPND-affected shrimp. Early acute stage of diseased *P. vannamei.* (A) slight sloughing of epithelial cells (black arrows) examined at a low magnification, (B) the appearance of enlarged nuclei (karyomegaly, red arrows) under a higher magnification. Scale bars = 25 µm.

At acute phase, the pathology is characterized by a progressive degeneration of the HP tubules from proximal to distal, significant rounding and massive sloughing of HP epithelial cells into the lumens, dysfunction of B, F and R cells and lack mitotic activity in E cells (Figures 11A, B, C).

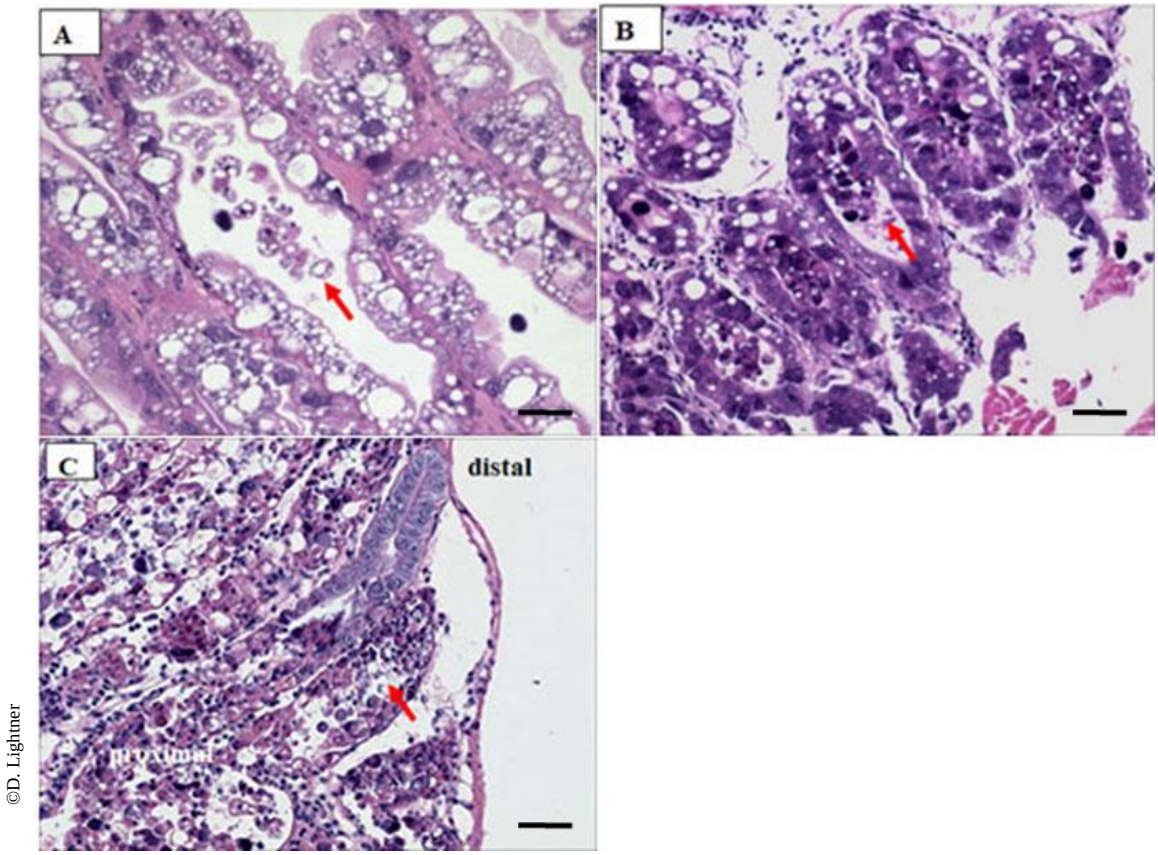

Figure 11. AHPND-affected *P. vannamei*, acute phase, sloughing of tubular epithelial cells (red arrows). Scale bars = 25 µm.

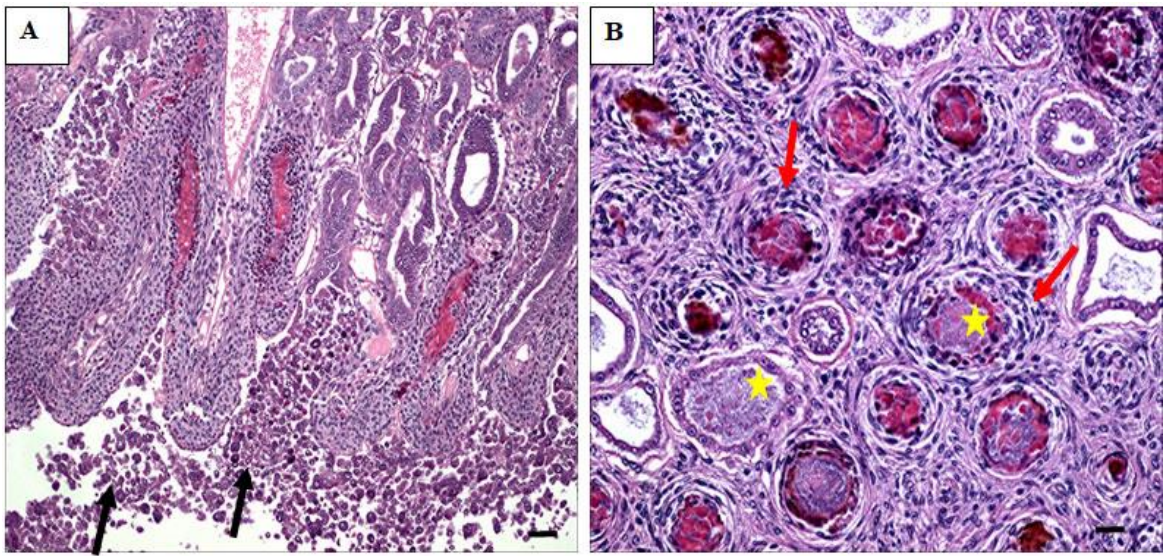

Figure 12. AHPND-affected *P. vannamei*, terminal phase. (A) Sloughing of tubule epithelium (black arrows), significant proximal haemocytic inflammation; most tubules are destroyed, some tubules with putative vibriosis. (B) Extensive haemocytic infiltration (red arrows), massive bacterial colonization in tubule lumens (yellow stars). Scale bars = 25 µm.

The terminal stages show extensive intertubular haemocytic aggregations, formation of melanized granulomas, and abundant secondary bacterial infections in tubule lumens (Figures 12A, B).

Although histopathology can provide a presumptive diagnosis of AHPND, specific molecular tests such as PCR (qPCR), LAMP, or immunoassays (dot-blot, Western blot analysis, ELISA, that detect PirAB^{vp} toxin) are required for confirmation of the disease (OIE, 2019b).

3.2.3 Molecular techniques (Level III)

(a) PCR-based method

Several PCR and qPCR protocols have been described for the specific and sensitive detection of AHPND toxin genes *pirA*^{vp} and *pirB*^{vp} in samples. Details on how to perform these tests can be found in the original publications and in the OIE Aquatic manual (OIE, 2019b). Commercial PCR and qPCR kits for detection of *pirA*^{vp} and *pirB*^{vp} genes are also available, for example, IQ2000 AHPND/EMS Toxin 1 Detection and Prevention System (conventional PCR); IQ REAL AHPND/EMS Toxin 1 Quantitative System (qPCR); and the IQ Plus AHPND/EMS plasmid and Toxin 1 kit for pond-site diagnosis.

PCR data should be interpreted with caution, particularly when shrimp without clinical signs are analyzed. Samples of shrimp that do not display clinical signs of disease can have low levels of amplified products (PCR amplicons) approaching the detection limit of the tests and lead to variable results. If fresh samples (HP, faecal strands, pond water, sediments) are available, the *Vp*$_{\text{AHPND}}$ can be enriched by culturing in TSB+ (see above), prior to DNA extraction and PCR analyses. A nested PCR (AP4 method) with increased sensitivity (100 times than single-step PCR) and specificity has been developed for AHPND, this method can be used to detect low-levels of *Vp*$_{\text{AHPND}}$ and environmental samples (Dangtip *et al*., 2015).

In addition, PCR is only capable of detecting parts of the *pirA*^{vp} and *pirB*^{vp} genes. Depending on the primers' sequences, some PCR primers cannot discriminate between pathogenic and non-pathogenic bacteria (such as deletion or insertion mutants) (Vincente *et al*., 2019). In most of cases, PCR should be used in conjunction with histopathology, immunoassays (detecting PirA^{vp} or PirB^{vp} proteins) or laboratory infection (using bacterial isolates) for AHPND diagnosis.

(b) Loop-meditated isothermal amplification (LAMP) method

The detection limit of most single-step PCR methods is usually approximately 10^4 CFU/mL *Vp*$_{\text{AHPND}}$ with pure bacterial culture. AHPND LAMP is shown to be 100 times more sensitive, as this method uses four primers (thus also providing a higher specificity than single-step conventional PCR) to generate large quantities of amplified products (Kongrueng *et al.*, 2015).

The AHPND LAMP-amplified products are usually detected by visualization with an agarose gel electrophoresis (Koiwai *et al.*, 2015); this method, however, has a high risk of contamination from opening the reaction tubes for electrophoresis. Alternatively, the LAMP can be combined with ssDNA probe labeled with gold nanoparticles, and this has a detection limit of 100 CFU of *Vp*$_{\text{AHPND}}$. This LAMP has the advantages of rapid assay (50 min) and high specificity, and is easy to perform (Arunrut *et al.*, 2016).

(c) Immunoassay

Monoclonal antibodies (MAb) have been produced against secreted PirA^{vp} and PirB^{vp} proteins present in the bacterial broth. These antibodies are proved to be specific to the PirAB^{vp} toxin

released from *Vp*$_{AHPND}$ as determined by dot- and Western-blotting analyses (Wangman *et al.*, 2017). For dot-blot analysis, the bacterial lysate can be dotted onto the nitrocellulose membrane followed by immunodetection using the PirAB^{vp} antibodies. Detection sensitivity of the MAb specific to ToxA (i.e. PirA^{vp}) is 3 ng per spot, which is equivalent to 250 µg of PirA^{vp} in 1 mL of bacterial lysate. The MAb against PirB^{vp} is even more sensitive and can detect 0.78 ng of protein per spot, equivalent to 180 µg of PirB^{vp} per mL of bacterial lysate. These MAbs can be used for detecting *Vp*$_{AHPND}$ (as low as 1 CFU/mL) in a dot-blotting format with the shrimp HP lysate after enrichment of bacteria in the TSB+ for 6 h.

3.2.4 Bioassays

Several laboratory infection protocols for testing the pathogenicity of *Vp*$_{AHPND}$ in healthy indicator shrimp have been described.

(a) Immersion infection study

Both liquid and solid media can be employed to grow the *Vp*$_{AHPND}$ bacteria for preparing inoculum for infection tests. The inoculum's density (CFU/mL) depends on the virulence of bacterial strains.

For liquid media, the inoculum can be prepared by inoculating the *Vp*$_{AHPND}$ isolate into a flask containing 30 ml (volume is variable, depending on the experimental design) of sterile TSB+ then incubating in a rotary shaker for 16–18 h (overnight) at 28±2 °C. After overnight incubation, the bacterial density can be determined using a spectrophotometer at optical density $(OD)_{600nm}$. An OD_{600nm} of 0.1 corresponds to ~10^4 CFU/mL (Lai *et al.*, 2015).

Bacteria grown on solid media can be scraped and resuspended into the saline water (2 percent NaCl). The concentration can then be determined by measuring OD_{600nm}.

The immersion procedure can be carried out by immersing healthy shrimp (indicator shrimp) for 15 min with aeration in a large container containing a solution of *Vp*$_{AHPND}$ at a density of approximately 10^8–10^9 CFU/mL. Following the 15 min immersion in the bacterial suspension, this bacterial broth can be added directly into an experimental tank containing clean seawater to obtain a final density of 10^5–10^6 CFU/mL tank water. Shrimp in the negative control group are immersed in sterile TSB+. The shrimp exposed to *Vp*$_{AHPND}$ usually exhibit 100 percent mortality within 72 h; morbidity or death can also occur earlier than 12 h, depending on the virulence of the *Vp*$_{AHPND}$ strain.

(b) *per os* infection study

Vp$_{AHPND}$ can be cultured in TSB+ at 28±2 °C for 16–18 h with gentle shaking to reach a density of 1 × 10^9 CFU/mL, then mixed with shrimp feed (ratio: 1 g feed to 1 mL of bacterial broth) for 5 min, and then fed to shrimp already stocked in the experimental tank. Shrimp in the negative control groups are fed shrimp feed mixed with sterile TSB+.

(c) Reverse gavage to deliver the PirAB^{vp} toxin to the hepatopancreas

The broth medium, after being inoculated with *Vp*$_{AHPND}$ and incubated for 16–18 h (see above), can be centrifuged at 3 200 × g for 5 min. The supernatant fluid (containing the PirAB^{vp} toxin) is filtered through a 0.2 µm filter, then a food colorant (for tracing if the inoculum reaches to

HP) is added into the filtrate. Each experimental shrimp in the reverse gavage treatment receives approximately 0.1 ml of the colored filtrate, dispensed with a micropipette, through the anal route. The negative control shrimp receive colored, sterile TSB+. The pathogenic effects of PirAB^{vp} toxin on the indicator shrimp can be monitored by the occurrence of mortality or by histopathology.
The comparison of the suitability of the different methods for surveillance and diagnosis of AHPND can be found in Aquatic manual, chapter 2.2.1 (OIE, 2019b). The advantages and disadvantages associated with commonly used laboratory tests for diagnosing AHPND are summarized in Table 2.

In a suspected AHPND outbreak, PCR (qPCR) should be used as the initial confirmatory test as it provides a rapid turnaround. The validity of the PCR (qPCR)-positive data should then be confirmed by histological evaluations. A definitive association can be made from the isolation of pathogenic *Vp*$_{\text{AHPND}}$ determined by laboratory infection studies or the presence of PirAB^{vp} toxin by immunoassays.

Table 2. Advantages and disadvantages of AHPND diagnostic methods

Diagnostic method	Advantages	Disadvantages
Histopathology (Level III)	• presumptive diagnosis • allows an evaluation of disease	• may not detect early stage of disease • needs 2–7 days preparation time • relies on the pathologist's expertise
PCR-based assay (Level III)	• highly sensitive & specific • able to test all life stages • specific to *pirAB*^{vp} genes • rapid results	• easy to have contamination problems • technically complicated • cannot discriminate pathogenic bacteria from non-AHPND mutants, in some tests
LAMP (Level III)	• highly sensitive & specific • able to test all life stages • Specific to *pirAB*^{vp} genes • rapid results • inexpensive • can be used for pond-site diagnosis	• easy to have contamination problems • cannot discriminate pathogenic bacteria from non-AHPND mutants, in some tests
Antibody-based assay (Level III)	• specific to the PirA^{vp} &/or PirB^{vp} • can be developed for pond-site diagnosis • can become a sensitive method when the bacteria are enriched through culturing	• generating antibodies is expensive & time consuming • Western blot &/or ELISA are laborious procedures • low sensitivity
Rapid methods from commercial kits (Level III)	• rapid results • field ready • highly sensitive	• need to purchase from commercial sources (maybe expensive) • some devices can only run limited numbers of samples, especially the pond-site devices
Vp$_{\text{AHPND}}$ isolation and laboratory bioassay (Level III)	• demonstrates the presence of *Vp*$_{\text{AHPND}}$	• requires bacteria isolated from fresh, or frozen HP/stomach • bioassay takes ~7 days & requires follow-up confirmation by histology, PCR &/or immunoassay • need wet-laboratory facility & healthy indicator shrimp

3.2.5 Corroborative diagnostic criteria

(a) Definition of suspect case

AHPND is suspected if at least one of the following criteria is met:

(1) mortality and clinical signs consistent with AHPND
(2) histopathology consistent with AHPND
(3) detection of *pirA*^{vp} and *pirB*^{vp} toxin genes by PCR (or qPCR)

(b) Definition of confirmed case

AHPND is considered to be confirmed if two or more of the following criteria are met:

(1) histopathology consistent with AHPND
(2) detection of *pirA*^{vp} and *pirB*^{vp} toxin genes by PCR and amplicon's sequence analysis
(3) detection of PirAB^{vp} toxin by immunoassays
(4) The *Vp*$_{\text{AHPND}}$ can be isolated from HP (or stomach, guts) of affected shrimp, then the disease is confirmed by performing the laboratory infection in conjunction with the diagnostic methods (histopathology, PCR, sequencing, immunoassays) for AHPND

4. Prevention and treatment

4.1 Farm management

Implementing good aquaculture practices to improve biosecurity on shrimp farms is very important for preventing the introduction and spread of *Vp*$_{\text{AHPND}}$ and other major shrimp pathogens during grow-out (see Section 6.1.5 for details). Before implementing a biosecurity action plan on a particular site, the epidemiology of AHPND should be thoroughly studied in order to understand the risk factors and pathways (entry points) associated with the introduction and transmission of *Vp*$_{\text{AHPND}}$ on a farm (see Section 5). AHPND can be introduced through five major pathways: affected PLs, water, fomites, vectors and fresh feed; control measures can be implemented based on this knowledge to potentially mitigate the risk of *Vp*$_{\text{AHPND}}$ introduction and spread on shrimp farms.

To reduce the risk of *Vp*$_{\text{AHPND}}$ introduction, new animals introduced onto a farm should be sourced from a reputable supplier and have a known health status (e.g. specific-pathogen free [SPF] stock; pathogen freedom certification). In recirculating aquaculture systems (RAS) or raceway systems, influent water should be clean and free of pathogens of concern; this can be achieved through sourcing pathogen-free water (e.g. spring water; well water) or treating influent water prior to entry into the system. In ponds, *Vp*$_{\text{AHPND}}$ may be found as biofilms in sediments or exist as free-living bacteria. Pond bottoms should be cleaned via disinfection (prior to water discharge), sludge removal, dry-out and liming prior to stocking to reduce the risk of *Vp*$_{\text{AHPND}}$ contamination. Equipment and vehicles can serve as fomites for *Vp*$_{\text{AHPND}}$ transmission and should therefore be disinfected prior to transfer between sites, ponds or tanks. Vectors, although not directly affected by *Vp*$_{\text{AHPND}}$, can serve as vehicles of transmission for the bacteria and include species of molluscs, crustaceans and seabirds (see Section 5.4). People

may also act as vectors by carrying the pathogen on their hands, footwear and clothing. Vectors can be controlled with fences around farm sites, nets above ponds or tanks, pond disinfection/dry-out, and predator fish (e.g. tilapia). Procedures should be in place for staff and visitors, such as access restriction (sign in/out), protective clothing, disinfection of footwear and handwash stations. Feed may serve as a source of *Vp*$_{AHPND}$ introduction and, therefore, should be purchased from a trusted supplier. Live feed (e.g. *Artemia* spp., rotifers) can act as vectors of the pathogen and should be certified free of *Vp*$_{AHPND}$.

The level of *Vp*$_{AHPND}$ in the pond environment will increase or decrease based on the organic load available. Therefore, control of overfeeding is key to keep *Vp*$_{AHPND}$ at lower numbers. Good and frequent pond drainage will facilitate the control of bacterial growth.

Stress has an immunosuppressive effect on animals, increasing their susceptibility to infection. Therefore, using good farm management practices on farms that limit stress and improve shrimp health are important for reducing the risk of disease spread. This includes maintaining optimal water quality parameters and stocking density, limiting handling and reducing the presence of predators.

4.2 Antibiotics

Traditionally, antibiotics have been considered for use as a therapeutic agent in aquaculture farms and hatcheries to control bacterial diseases (Baticados and Paclibare, 1992). Several antibiotics such as oxytetracycline (OTC), tetracycline, quinolones, sulphonamides and trimethoprim are permitted for aquaculture use in Asia (Yano *et al.,* 2014). The use of antibiotics cannot be indiscriminate; their effectiveness has to be evaluated through the susceptibility testing of *Vp*$_{AHPND}$ isolates in the laboratories.

The use of antibiotics, however, in general is problematic. Use in open systems can result in contamination of the environment, and use in closed systems can kill beneficial bacteria. In addition, excessive use of antibiotics generates a strong selective pressure that can result in the transfer of resistance genes associated with plasmids or transposons among bacterial species (Kehrenberg *et al.,* 2001). Antimicrobial resistance (AMR) is one of the most important problems in public health, veterinary medicine and aquaculture. Therefore, the use several antibiotics of importance to human medicine is banned or restricted in many countries. For example, chloramphenicol, fluoroquinolones (e.g. ciprofloxacin and enrofloxacin) and nitrofurans are banned by the government of Viet Nam (VMARD, 2016).

There is already evidence of antibiotic-resistant *Vp*$_{AHPND}$ strains. When four *Vp*$_{AHPND}$ strains were tested for antibiotic resistance (Lai *et al.,* 2015), most were found to be resistant (had minimum inhibitory concentrations (MICs) of ≥12.8 μg/mL) to five antibiotics (ampicillin, streptomycin, sulfamethoxazole, fosfomycin and bicozamycin). All four *Vp*$_{AHPND}$ strains showed strong sensitivity to enrofloxacin (MIC: 0.4 μg/mL) and ofloxacin (0.4 μg/mL), while three strains showed sensitivity to tetracycline (3.2 μg/mL) (Table 3). In a separate study, whose analysis was based on the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006), Han *et al*. (2015c) demonstrated that nine *Vp*$_{AHPND}$ strains from Mexico and Viet Nam were resistant to ampicillin, and two Mexican strains were resistant to tetracycline (30 μg) and OTC (30 μg). Dong *et al*. (2017c) reported *Vp*$_{AHPND}$ 20130629002S01 was susceptible only to florfenicol and resistant to 14 other antibiotics tested; the *Vc*$_{AHPND}$ 20130629003S01 also showed a wider spectrum of antibiotic resistance.

Table 3. MICs of selected antibiotics on *Vp*$_{AHPND}$[1]

Strain	Antibiotics[a] (µg/mL)											
	ABPC	**CP**	**KM**	**SM**	**TC**	**NA**	**SMZ**	**TMP**	**ERFX**	**FOM**	**OFLX**	**BCM**
Thv-1	12.8	3.2	6.4	12.8	0.4	12.8	≥12.8	3.2	0.4	≥12.8	0.2	≥12.8
Thv-16	12.8	3.2	3.2	12.8	3.2	1.6	12.8	6.4	0.1	≥12.8	0.2	≥12.8
5HP	12.8	0.8	12.8	≥12.8	0.4	0.8	≥12.8	12.8	0.1	≥12.8	0.4	≥12.8
M1-1	12.8	1.6	6.4	12.8	12.8	0.8	12.8	3.2	0.1	≥12.8	0.2	≥12.8

[1]Source: Lai *et al.* (2015)

[2]Antibiotics: ABPC (ampicillin), CP (chloramphenicol), KM (kanamycin), SM (streptomycin), TC (tetracycline), NA (nalidixic acid), SMZ (sulfamethoxazole), TMP (trimethoprim), ERFX (enrofloxacin), FOM (fosfomycin), OFLX (ofloxacin), BCM (bicozamycin)

The FAO's Code of Conduct for Responsible Fisheries (CCRF) Technical Guidelines on the Prudent and Responsible Use of Veterinary Medicines in Aquaculture (No. 5 Suppl. 8) provide recommendations and general guidance on the use of veterinary medicines in aquaculture to responsible government agencies, private-sector aquaculture producers and aquatic animal health professionals. These guidelines support the international aquatic animal health standards of the World Organisation for Animal Health (OIE), the food safety standards of the FAO/ World Health Organization (WHO) Codex Alimentarius and the One Health platform under the FAO/OIE/WHO Tripartite Collaboration on antimicrobial resistance (AMR)

4.3 Bacteriophages

Because of the problems of antibiotic use, phage therapy has become a promising alternative and natural method for controlling bacterial diseases. Phages (bateriophages) are viruses that destroy specific bacteria through effective bacteriolytic activity. They are abundant natural inhabitants of all aquatic ecosystems, and, therefore, easier to get regulatory approval for. Phage therapy uses phages that are specific to the targeted pathogenic bacteria. Among the advantages of using phages for disease control are that they are self-replicating, effect only targeted bacteria, relatively inexpensive and easily applied in the field (administered through feed or by direct release into water). Phage therapy does not cause any of the side effects associated with the use of antibiotics.

Phage therapy has a history of being successful in controlling shrimp diseases caused by pathogenic *Vibrio* and has shown potential for use in controlling AHPND. For example, in early studies, phages applied to pond water were successfully used to control luminous *V. harveyi* that was causing mortalities in farmed populations of *P. monodon* (Vinod *et al.,* 2006; Karunasagar *et al.*, 2007). Recently, phage pVp-1, a member of the family *Siphoviridae*, has been shown to have a broad host range and can infect 90 percent (20 of 22 strains analyzed) of *Vp*$_{AHPND}$ isolates (Jun *et al.*, 2016). In laboratory infection studies, shrimp treated with pVp-1 before (prophylaxis) and after (therapy) their exposure to *Vp*$_{AHPND}$ displayed significant protection, 25–50 percent mortalities, whereas the control groups (not treated with phage pVp-1, only exposed to *Vp*$_{AHPND}$) showed a 100 percent mortality (Jun *et al.,* 2018). Further studies are needed to evaluate the effectiveness of phage prophylaxis and therapy against AHPND in field trials.

4.4 Probiotics

Another approach has focused on the use of probiotics. Probiotics are microbial feed supplements or water additives administered to: (1) improve water quality, (2) enhance the physiological and immunological responses of aquatic animals, and (3) reduce the use of chemicals and antibiotics in aquaculture (Hai, 2015). These may be use alone or in combination with prebiotics (indigestible fiber, such as sweet potato starch) or immunostimulants (such as β-1,3-glucan). Successful probiotic agents used in shrimp aquaculture mostly belong to the genera *Bacillus*, *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Saccharomyces* and *Vibrio*. Non-pathogenic *Vibrio alginolyticus* is used in shrimp and salmon aquaculture. Probiotic supplements are generally either provided in the feed or applied directly to the water. Modes of action are primarily via competitive exclusion, the production of substances that inhibit pathogenic organisms, and immunomodulation; however, they may also have antagonistic activity and can improve health, growth of aquatic animals and water quality. Extensive studies have reported on probiotics in aquaculture (Martinez Cruz *et al.*, 2012; Hai, 2015). Tran, Hoang and Fitzsimmons (2018) reported that products that directly affect the bacterial population in the digestive tract are more efficacious at controlling the presence of *Vp*$_{\text{AHPND}}$; the challenge studies have shown that several gut probiotic products have a significant effect on the survival rate of shrimp affected by *Vp*$_{\text{AHPND}}$.

Two *Vibrio* antagonists, *Pseudoalteromonas* sp. CDM8 and CDA22, isolated from the hindgut of healthy shrimp were shown to significantly reduce AHPND-associated mortalities in laboratory infection studies (Wang *et al.*, 2018). When groups of shrimp fed with CDM8 or CDA22 for 21 days were exposed to *Vp*$_{\text{AHPND}}$, they had lower mortalities, 37 percent and 77 percent, respectively, than the similarly exposed group (97 percent mortality) fed with commercial feed. Both strains were able to produce antibacterial compounds against *Vp*$_{\text{AHPND}}$, which led to decreased total *Vibrio* bacteria in the shrimp digestive system.

Another approach is the use of bacteria that prey on or parasitize other bacteria. For example, isolates of *Bdellovibrio* sp. and *Bacteriovorax* sp. that have been isolated from water and sediment samples in Thailand can attack *Vp*$_{\text{AHPND}}$ as well as various other bacteria, both Gram-positive and Gram-negative (Kongrueng *et al.*, 2017). The optimal ratio for interaction between the *Bacteriovorax* isolate BV-A and *Vp*$_{\text{AHPND}}$ was determined to be 1:10. The capability of BV-A to reduce numbers of *Vp*$_{\text{AHPND}}$ was observed in co-culture after incubation for two days and continued until the end of the incubation period. In laboratory bioassays, BV-A was able to reduce mortality of shrimp PLs affected by *Vp*$_{\text{AHPND}}$. In addition, BV-A is shown to significantly reduce the biofilms formed by *Vp*$_{\text{AHPND}}$.

4.5 The development of AHPND-tolerant shrimp

Although shrimp have only a primitive immune system, disease tolerant shrimp lines have been successfully developed and used effectively in the control of shrimp viral diseases, such as Taura syndrome virus (TSV). TSV-tolerant shrimp were first generated from survivors of viral outbreaks on farms. The tolerant lines, developed through subsequent selective breeding, apparently restrict viral replication as lower levels of TSV were found in infected individuals (Srisuvan, Tang and Lightner, 2005). The physiological and genetic mechanisms underlying this disease tolerance have been described (Flegal, 2009, Whitfield *et al.*, 2017; Tasseto *et al.*, 2019). Although the mechanism of tolerance development may differ, a similar strategy for developing AHPND-tolerant lines could prove useful; shrimp lines could become tolerant to AHPND if they can either limit *Vp*$_{\text{AHPND}}$ colonization or become tolerant of the PirAB^{vp} toxin.

Currently, selecting breeding programmes for AHPND-tolerant *P. vannamei* are in progress in Mexico and Thailand. In particular, CP Thailand breeding program has reported an improved of survival under challenge conditions from 30% to 85% (R. McIntosh, personal communication).

Because shrimp innate immune systems are non-specific, it is possible that tolerance to one disease may also offer protection against other diseases, including AHPND. Preliminary results from a Mexican study show additive genetic variation for AHPND tolerance in an Ecuadorian shrimp line generated from a merging of several Ecuadorian lines with a history of white spot syndrome virus (WSSV) tolerance (Castillo-Juárez *et al.*, 2018). The Ecuadorian tolerant line was challenged with AHPND and showed greater survival times than a Mexican line bred for growth. Inbreeding was reported to have no negative effects on AHPND tolerance.

4.6 Shrimp immunity and vaccination

Shrimp possess an innate immune system that does not have a memory that would allow production of antibodies following exposure to pathogens. This precludes the use of traditional vaccines against specific pathogens for preventing shrimp diseases. The shrimp immune system, however, responds to harmful microorganisms through complex interactions of cellular and humeral processes (Smith and Chisholm, 1992). The cellular immune responses, occurring in haemocytes, involve phagocytosis, encapsulation, cell-mediated cytotoxicity, clotting and apoptosis (Söderhäll and Smith, 1983; Iwanaga and Lee, 2005; Lai *et al.*, 2005). Humeral responses, which occur in the haemolymph, involve the production of proteins, antimicrobial peptides and other compounds for combating bacteria, fungi and viruses (Heng and Lei, 1998). From AHPND-affected shrimp, comparative transcriptomic analyses have shown that many immune-related genes and signaling pathways are differentially expressed in the stomach, HP and midgut (Soonthornchai *et al*., 2016; Ge *et al*., 2017).

There are no vaccines for shrimp diseases. There are two lines of recent research, however, to obtain tolerance to AHPND. One is by exposure of shrimp to the inactivated *Vp*$_{\text{AHPND}}$ or to the recombinant PirA^{vp} protein, and the other is the use of antibodies, extracted from chicken egg yolk, in shrimp feed (Hirono *et al.*, 2016).

With regard to the inactivated pathogens, *Vp*$_{\text{AHPND}}$ bacteria were treated with formalin for 24 h at 4 ^{o}C, then heated at 60 ^{o}C. This inactivated *Vp*$_{\text{AHPND}}$ still contains functional PirAB^{vp} toxin. Shrimp (weighing 5–8 g) were exposed to the formalin-killed *Vp*$_{\text{AHPND}}$ through immersion, then the treated shrimp were challenged with viable *Vp*$_{\text{AHPND}}$; no mortality was observed in an 8-day bioassay. The results showed that formalin-killed *Vp*$_{\text{AHPND}}$ can confer a protective effect, but treatment with the formalin-killed *Vp*$_{\text{AHPND}}$ was not effective with smaller shrimp (0.8 g). Another study is to produce a recombinant PirA^{vp} protein in the *Escherichia coli* BL-21 and purify with immobilized metal affinity chromatography. In the laboratory challenge studies, shrimp exposed to the recombinant PirA^{vp} for 24 h showed protection from the challenged *Vp*$_{\text{AHPND}}$, resulting in only 1.7 percent mortality, whereas the shrimp in the control group (not administrated with the recombinant PirA^{vp}) were affected by *Vp*$_{\text{AHPND}}$ with a 48.3 percent mortality (Campa-Cordova *et al.*, 2017).

Based on laboratory studies, treatment with anti-PirAB^{vp} antibodies has great potential. Chick immunoglobulin IgY is a specific antibody, produced when a female chicken is immunized with the recombinant PirAB^{vp} toxin. The inoculated hen produces eggs with yolks containing high titers of immunoglobulin IgY against PirAB^{vp}. This IgY-enriched egg yolk used as a feed

additive has been demonstrated to be effective against AHPND in laboratory challenge studies. Large amounts of IgY can be prepared easily and at a low cost, making this method a promising tool for preventing and treating AHPND in the farms (Nakamura *et al.*, 2019). Developing an efficient delivery of IgY strategy in the field, present a bigger challenge.

5. Epidemiology

5.1 Geographic distribution and prevalence

AHPND, so far, has been reported from East, Southeast and South Asia and North and South America, occurring in the following ten countries:

In the **People's Republic of China,** AHPND, known locally as "covert mortality (偷死) disease", or "bottom death (死底) disease", first occurred in a small area of Guangxi Province in June 2010, but was of no concern to most farmers. By 2011, however, outbreaks had spread to other areas (Fujian, Hainan, Guangdong, Heibei) and were most serious in farms that had been in operation for more than five years and those that were close to the sea with ponds at higher salinities.

In **Viet Nam,** AHPND first appeared in the four southern coastal provinces in the region of the Mekong River Delta during 2010–2011: Tra Vinh, Soc Trang, Ca Mau and Bac Lieu, then spread to Tien Gang, Ben Tre and Kien Giang provinces; approximately 68.5% of the shrimp-producing area in Soc Trang, and 30 000 farming households in Bac Lieu and Ca Mau were affected by this disease (Dang, Pham and Phan, 2018). In 2012, AHPND spread to 19 provinces throughout Viet Nam, with an infected area of 46 093 ha (45.7% of the total culture area). In 2014, 8.72% of *P. monodon* ponds (i.e. 47 574 ha) and 32.48% of *P. vannamei ponds* (i.e. 18 966 ha) in the Vietnamese Mekong Delta were reported to be affected by shrimp diseases (Shinn *et al.*, 2018a); however, detailed data on the prevalence of AHPND is not available. In 2015, 5 875 ha of *P. monodon* ponds and 5 509 ha of *P. vannamei* ponds were reported to be affected by AHPND, respectively (Shinn *et al.*, 2018a). In 2017, the disease has since spread to 294 communes, belonging to 86 districts in 25 provinces throughout Viet Nam (Dang, Pham and Phan, 2018); histopathology results have confirmed the spread of AHPND to the northern part of Viet Nam and therefore nation-wide occurrence of the disease.

In **Malaysia,** AHPND was first reported in mid–2010, in the peninsular states of Pahang and Johor, on the country's east coast. The disease spread to the states of Perak, Pahang, Penang and Kedah in 2011 (NACA, 2012; Kua *et al*., 2018). Later, samples from the states of Sabah and Sarawak (2012), Terengganu (2013) and Melaka and Johor (2014) were also found positive for AHPND by histopathology. The annual prevalence rates of AHPND have been declining and were 50, 26, 34, 13 and 4 percent in 2011, 2012, 2013, 2014 and 2015, respectively. AHPND was also detected in *P. monodon* in 2014 (10 percent) and 2015 (5 percent). In 2016, AHPND still persisted in Malaysia, but at a lower prevalence.

In **Thailand,** AHPND was first observed during late August 2011 in a pond located in the eastern Gulf of Thailand (Chucherd, 2013; Putth and Polchana, 2016) and was subsequently reported in the provinces of Chantaburi, Rayong, Trat and Chachoengsao along the eastern coast of Thailand from January to April 2012. The disease then spread to the central and southern provinces of Nakorn-Patom, Chumphorn, Surat Thani, Nakorn-Srithammarat, Songkhla, Krabi and Phuket.

In **Mexico,** AHPND was detected in 2013 in farmed *P. vannamei* in Pacific coastal farms in Sonora, Sinoloa and Nayarit and, in 2014, spread to Colima and then to the Gulf of Mexico in Yucatan, Tamaulipas (south of Texas, United States of America), Campeche and Veracruz. In 2015, in Sinoloa, 16 401 out of 50 310 ha (5 647 ponds) of cultured area was affected by AHPND, and thus 74 percent of farms were affected by AHPND. Smaller shrimp (0.6 g) were affected the most.

In **Latin American countries,** a *Vp*$_{\text{AHPND}}$ was isolated from *P. vannamei* cultured in a South American country (Restrepo *et al.*, 2016), but the year and country are not described. An AHPND-*V. punensis* was also isolated in 2015 (Restrepo *et al.*, 2018). An AHPND-*V. campbellii* was isolated in an unspecified Latin American country in 2016 (Ahn *et al.*, 2017). Many countries in this region are reluctant to report the occurrence of AHPND due to possible impacts on trade.

In the **Philippines,** the first record of AHPND was documented in 2015 among *P. vannamei* and *P. monodon* in the regions of Luzon (Bulacan, Pampanga, Bataan and Batangas provinces), the Visayas (Cebu and Bohol provinces) and Mindanao (General Santos and Saranggani provinces) at a prevalence of 33, 21 and 5 percent, respectively (Dabu *et al.*, 2015). Government laboratories conducted a total of 2 606 analyses for AHPND during 2015. AHPND-positive samples totaled to 98, constituting 3.8 percent of the samples analyzed. Positive samples originated from 14 provinces: Pangasinan, Cagayan, Bulacan, Batangas, Negros Occidental, Bohol, Zambales, Pampanga, Cebu, Iloilo, Leyte, Oriental Mindoro, Marinduque and Davao del Sur (Apostol-Albaladejo, 2016).

In **Bangladesh,** *Vp*$_{\text{AHPND}}$ were isolated from *P. monodon* sampled from shrimp farms in districts of Satkhira and Bagerhat during June 2016 (Eshik *et al.*, 2018). The bacterial isolates were determined to be *Vp*$_{\text{AHPND}}$ by PCR using AP3 and AP4 methods (OIE, 2019b).

In the **United States of America,** AHPND was detected in *P. vannamei* cultured in three farms located in south Texas in 2017 (OIE, 2017).

In **Taiwan Province of China**, *Vp*$_{\text{AHPND}}$ was detected by PCR in *P. vannamei* farmed in Taitung city of Taiwan Province in November 2018 (OIE, 2019c).

In **South Korea**, AHPND outbreaks occurred in shrimp farms located on the Taean Peninsula (Chungnam Province) during September-October, 2019 (Han *et al.*, 2020a). Two representative *Vp*$_{\text{AHPND}}$ strains were isolated and characterized to have the Asia genotypes; these 2 strains were shown capable of causing AHPND in the laboratory infection studies. In addition, *Vp*$_{\text{AHPND}}$ was also detected by PCR in imported frozen *P. vannamei* (Han *et al.*, 2020b).

In **Okinawa Prefecture of Japan**, an AHPND outbreak occurred in a shrimp farm in October, 2020, the affected population had a 98% mortality (OIE, 2020). These shrimp *P. vannamei* were imported from Thailand in August, 2020.

5.2 Genotype

From the comparisons of whole genome sequences (WGS) of *Vp*$_{\text{AHPND}}$ strains deposited in the GenBank, there are two variable regions within the virulence plasmid (harboring *pirAB*^{vp} genes)

that exhibit a clear geographical variation: a 4243-bp Tn3-like transposon and a 9-bp small sequence repeat. The Tn3-like transposon is only found in the isolates from Mexico and other Latin American isolates, but not in Asian isolates from the People's Republic of China, Viet Nam, and Thailand (Han *et al.*, 2015b). A genotyping PCR targeting the Tn3-like transposon region was subsequently developed to distinguish among *Vp*$_{\text{AHPND}}$ strains collected from various geographic regions.

From a multilocus sequence typing method and a phylogenetic analysis, strains of *Vp*$_{\text{AHPND}}$ are shown to be diverse and not derived from a single lineage (Chonsin *et al.*, 2016). This genetic diversity may be also related to the presence of gene transfer elements residing in the virulence plasmid.

The WGS data were used to compare the relatedness between *Vp*$_{\text{AHPND}}$ isolates. An AHPND-causing *V. campbellii* (*Vc*$_{\text{AHPND}}$) and a *Vp*$_{\text{AHPND}}$ isolate were collected from the same AHPND-affected pond in the People's Republic of China. Both strains were positive for the virulence genes *pirAB*^{vp} (Dong *et al.*, 2017c). An immersion challenge test with *P. vannamei* indicated that these two strains possessed similar pathogenicity. Complete plasmid sequences comparison showed that the *pirAB*^{vp}-bearing plasmids in these two strains were highly homologous, suggesting the occurrence of horizontal transfer of the virulence plasmid pVA1 (Southeast Asia type). In addition, a study reported the presence of the pVA1 plasmid in many different serotypes of *Vibrio parahaemolyticus* from environmental samples, which suggested pVA1 transfer among bacteria is relatively frequent (Flegel and Sritunyaluucksana, 2018). Conjugation and DNA-uptake genes were found on their plasmids and the host chromosomes, respectively, which may facilitate the dissemination of *pirAB*^{vp} among *Vibrio* spp. These demonstrate that epidemiological information combined with WGS data can provide a means for in-depth investigations on the origins and dynamics of *Vp*$_{\text{AHPND}}$.

5.3 Persistence in the environment

Limited data are available regarding persistence of *Vp*$_{\text{AHPND}}$ in the environment. Strains of *Vibrio parahaemolyticus* show a wide tolerance to temperature, salinity and pH due to high metabolic diversity. This enables them to adapt and survive in almost all marine-estuarine environments where shrimp farms are located, which implies a high risk of outbreaks and rapid disease dispersion to free zones (Soto-Rodriguez *et al.* 2018). Pathogenic *Vp*$_{\text{AHPND}}$ strains are expected to possess properties similar to other strains of *V. parahaemolyticus* found in seafood, which have been shown to survive up to 9 and 18 days in filtered estuarine water and seawater, respectively, at an ambient temperature of 28±2 °C (Karunasagar *et al.*, 1987), and even longer in organic rich waters.

5.4 Vectors and reservoir hosts

Because *Vibrio* spp. are ubiquitous in the marine environment, there are numerous potential mechanical vectors or environmental reservoirs of *Vp*$_{\text{AHPND}}$. Species of *Vibrio*, including *V. parahaemolyticus*, exist as free-living bacteria and are often found in association with other aquatic organisms, including phytoplankton, zooplankton, molluscs, crustaceans, polychaetes and finfish that are common in brackish water and marine environments. In the ocean, *Vibrio* spp. are known to be able to attach to zooplankton that are carried long distances by ocean currents. On shrimp farms, the bacteria may be found as biofilms in pond sediments or on submerged equipment. Thus, these can be carriers for spreading *Vp*$_{\text{AHPND}}$ in farm environments. In shrimp hatcheries, *Vp*$_{\text{AHPND}}$ are likely to colonize brine shrimp (*Artemia* spp.), which is an

essential feed used in shrimp larviculture and maturation. Through PCR, *Vp*$_{AHPND}$ has also been detected in polychaetes (Desrina *et al.*, 2018) and bivalves used for live broodstock feed. Other potential, but unconfirmed, carriers include crabs, crayfish and other crustaceans.

In addition, *Vp*$_{AHPND}$ is likely to remain pathogenic in the gut and faeces of seabirds that feed on dead or dying shrimp at farms affected by AHPND. Studies in Japan and other countries have found *V. parahaemolyticus* to be present in faeces of seabirds. Thus, AHPND could be spread within and among farms through seabird faeces or regurgitated shrimp carcasses, similar to the situation found with TSV (Vanpatten, Nunen and Lightner, 2004).

5.5 Modes of transmission

Vp$_{AHPND}$ can be transmitted horizontally via co-habitation or oral ingestion of bacteria (OIE, 2019b). Once the bacteria are established in a pond, AHPND can be transmitted rapidly through cannibalism of sick and dead shrimp, or *Vp*$_{AHPND}$-colonized molts. However, the main source of infection has been identified as uneaten feed pellets colonized by *Vp*$_{AHPND}$. It has been observed that stopping feeding reduces mortality and while this is a very important observation to understand disease dynamics, it is not a practical measure to manage the disease. *Vp*$_{AHPND}$ is present in the digestive tract of infected animals. From *per os* bioassays, it is clear that individuals can become infected through ingesting sediments and pond water contaminated with the bacteria. Faecal-oral transmission was demonstrated by Han *et al*. (2017b).

There is no evidence of true vertical transmission (i.e. via infected eggs rather than contaminated egg surface) for *Vp*$_{AHPND.}$ To prevent possible transmission of *Vp*$_{AHPND}$ via the egg surface, appropriate methods for egg disinfection should be applied. A widely used method is given below. For fertilized eggs: rinse with running seawater for 1–2 minutes, immerse eggs in formalin (100 ppm) for 1 minute, immerse eggs in iodophor (0.1 ppm iodine) for 1 minute, rinse in running seawater 3–5 minutes, then transfer to disinfected larval rearing tank. For nauplii: use their phototaxic response to light to collect nauplii with netting or screen, rinse with running seawater for 1–2 minutes, immerse nauplii in formalin (400 ppm) for 30–60 seconds, immerse nauplii in iodophor (0.1 ppm iodine) for 1 minute, rinse in running seawater 3–5 minutes, and then transfer to disinfected tanks.

AHPND-affected broodstock can be cleared of, or to a reduced level of, *Vp*$_{AHPND}$ by keep them at 0 ppt for a minimum period of 7 days.

5.6 Factors influencing disease transmission and expression

The epidemiologic triad highlights the interaction between a pathogen (i.e. *Vp*$_{AHPND}$) and a susceptible host (i.e. susceptible species of shrimp) in a suitable environment (i.e. shrimp pond) that allows transmission of the pathogen and development of disease in that host (Figure 13). The pathogen must be present for the disease to occur, but its presence may not always result in disease. A variety of host-specific or environmental risk factors can influence the host's exposure or susceptibility to the pathogen.

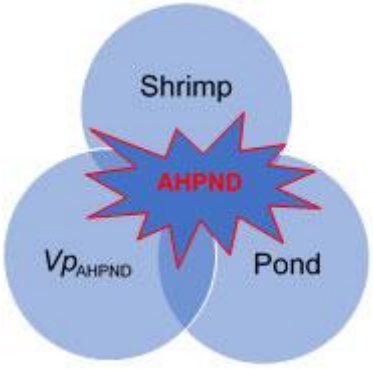

Figure 13. Epidemiological triad for AHPND.

Table 4. The general growth characteristics of *Vibrio parahaemolyticus*[1].

Growth condition	Optimum	Range
Temperature (°C)	30–37	20–44[2]
pH	7.8–8.6	4.8–9[2]
NaCl (%)	1.5–3.0 (=15–30 ppt)	0.5–10[2] (= 5–100 ppt)
Water activity (AW)	0.981	0.940–0.996
Atmosphere	aerobic	aerobic–anaerobic

[1]Source: ICMSF, 1996

[2]Data provided by Dr Sonia Soto Rodriguez, Investigación en Alimentación y Desarrollo, Mexico

Environmental conditions that affect both the pathogen (Vp_{AHPND}) and the shrimp host are important determinants of disease outbreaks. Environmental factors affect Vp_{AHPND} multiplication (Table 4) and play a role in the clinical manifestation of disease. Both the pathogen and host are affected by conditions such as temperature, salinity and water quality; shrimp are more susceptible to disease when stressed from suboptimal culture conditions. Environmental factors are known to promote AHPND outbreaks in shrimp farms include high concentration of nutrients (via fertilizers, molasses, etc.), high water temperature, high salinity (>5 ppt), high pH (>7), low water turnover and low planktonic biodiversity, and accumulation of organic matters (i.e. feed, shrimp carcasses, etc.) (Bondad-Reantaso, 2016).

Salinity is important as Vp_{AHPND} is halophilic. The effect of high salinity on AHPND has been demonstrated in laboratory infection studies where *P. vannamei* were exposed to Vp_{AHPND} (10^5 CFU/mL of tank water) for two days. The exposed shrimp had an 80 percent survival at a salinity of 5 ppt but suffered 100 percent mortality at 30 ppt. Field reports support the observation that AHPND appears to be more prevalent in shrimp ponds with higher salinities. Reports based on observations from farms indicate that the incidence of AHPND is usually higher during the hot season, with highest mortalities (>50 percent) seen from July to September. These environmental risk factors should be considered in the development of farm management protocols.

Boyd and Phu (2018) investigated potential environmental risk factors for AHPND during active outbreaks on farms in Soc Trang, Bac Lieu, and Ca Mau provinces in the Mekong River Delta, Viet Nam. High pH has previously been reported as a potential risk factor for AHPND; however, this was not supported in the results of this study. There was no significant difference in measured water quality parameters (i.e. salinity, temperature, pH, dissolved oxygen, nitrite, trace metals, pesticides, etc.) between ponds with and without the disease. It should be noted

that this study did not perform a multivariable analysis to take into account confounding or clustering.

Certain pond or farm level management practices can have an effect on the exposure or susceptibility of farmed shrimp to *Vp*$_{AHPND}$. These practices may affect the immunity of the shrimp (i.e. probiotics, stocking density), pathogen load (i.e. disinfection, water changes), and environmental conditions and water quality (i.e. feeding, dead shrimp removal, water additives). Boonyawiwat, Nga and Bondad-Reantaso (2018) conducted a cross-sectional study to identify risk factors associated with AHPND outbreaks related to farm practices on shrimp farms in operation from 2012 to 2013 in the Mekong Delta, Viet Nam. Farm-level risk factors associated with the occurrence of AHPND included having a large culture area, using the sun-dry sediment method for cleaning pond bottoms, and being in close proximity to other farms and using the same water source that is affected by AHPND. Pond-level risk factors included having a water depth equal to or less than 1.2 m, the occurrence of abnormal weather events during the first 35 days of culture (or until first signs of AHPND), and using fertilizers and probiotics for water treatment; however, this may be due to "reverse-causation" since the use of probiotics may occur in response to a pond having a history of outbreaks. Ponds that were treated with minerals and algaecides had a reduced risk of AHPND occurrence. Water quality data could not be included due to missing data.

A case-control study conducted by Boonyawiwat *et al.* (2017) identified the risk factors of AHPND related to management practices in shrimp ponds in four provinces of Thailand. The study included ponds affected with AHPND (cases) from August 2013 to April 2014; any shrimp ponds from the same farm found negative for AHPND were assigned as controls. High PL stocking density, PL source, use of predator fish to eliminate disease vectors in water preparation, chlorine treatment in water preparation, and culture of multiple shrimp species in the same farm were determined to be risk factors for AHPND occurrence in shrimp ponds. The following protective factors were identified (reduced the risk of AHPND occurrence): ageing water prior to use in ponds, polyculture, and delayed first day of feeding.

Co-infection is common in most cases of AHPND (Bondad-Reantaso, 2016). Infection by the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) has been shown to increase the susceptibility of shrimp to AHPND (Aranguren, Han and Tang, 2017). In laboratory exposure to *Vp*$_{AHPND}$, the EHP pre-infected shrimp had a higher mortality (52 percent) than the EHP-free shrimp (4 percent). EHP is an intracellular, spore-forming parasite that affects cultured shrimp (*P. vannamei* and *P. monodon*) in several Southeast Asian countries and in Venezuela (Tourtip *et al*., 2009; Thitamadee *et al.*, 2016; Tang *et al*., 2017). The dramatic effects of the co-infection are most likely explained by the synergistic actions of the microsporidian and *Vp*$_{AHPND}$ on the HP tissue.

5.7 Impact of the disease

Mortality in shrimp production facilities from AHPND can reach 100 percent within 35 days, or can present a chronic running mortality all along the production cycle. AHPND-associated production losses have had a substantial, large-scale impact on shrimp aquaculture. These losses are manifested in: (1) the production of farmed shrimp, including juveniles in the grow-out ponds, broodstock in the breeding centres and PLs in the hatcheries; (2) feed manufacturing; (3) processing facilities; (4) international marketing (because buyers are unlikely to purchase live or commodity shrimp from countries known to have AHPND); (5) employment (some 2 million people, including temporary and seasonal workers, are involved in shrimp farming and

related industries in Asia alone); and (6) government programmes such as farmers' compensation due to disease outbreaks and social welfare payments related to job losses.

In the **People's Republic of China,** in 2011, AHPND-related losses in shrimp production reached approximately 80 percent in the provinces of Fujian, Guangdong, Guangxi and Hainan (NACA, 2012).

In **Viet Nam,** in June 2011, unprecedented losses were reported on shrimp farms culturing *P. monodon* in Bac Lieu, Tra Vinh and Soc Trang. The economic losses were estimated to be over USD 60 million (NACA, 2012). In 2015, the combined AHPND-associated losses for both *P. monodon* and *P. vannamei* were estimated to be over US$ 25.98 million (Shinn *et al.*, 2018a).

In **Malaysia,** during 2011–2013, the mortality ranged from 40–100 percent in AHPND affected *P. vannamei*. The outbreaks resulted in a significant drop in the production of whiteleg shrimp, from 77 000 tonnes (2010) to 44 000 tonnes (2011), a 43 percent production loss. The Department of Fisheries estimated production losses at USD 0.1 billion in 2011. The total economic losses from the AHPND episodes in 2011–2014 reached to USD 0.49 billion (Kua *et al.*, 2016).

In **Thailand,** AHPND outbreaks resulted in shrimp production falling from a peak of 11.19 tonnes/ha in 2010 (prior to AHPND) to only 6.14 tonnes/ha in 2014, three years after the disease was first noted (Shinn *et al.*, 2018a). This also led to an estimated 22 percent drop in land use for shrimp culture. Annual production losses were severe, decreasing from 611 194 tonnes in 2011 to approximately 200 000 tonnes in 2014-15. Major contributors to these losses were a shortage of PLs and the reluctance of farmers to stock their ponds due to the emergence of AHPND. The production continued increasing in 2016 and 2017 to 300 000 tonnes. Over the period of 2012 to 2015, financial losses were estimated to be more than USD 5.01 billion, with an estimated 100 000 jobs lost.

In **Mexico,** the production in 2012 was 83 600 tonnes, which declined to 39 300 tonnes in 2013 due to AHPND outbreaks, a decrease in production of more than 50 percent. Production in 2014 was 44 000 tonnes, and with a slight recovery in 2015, increased to 59 000 tonnes.

In the **United States of America,** 3 Texas farms were affected. In the year prior to the outbreak (2016), the combined production of the 83 ha pond areas affected was 268 tonnes (3.2 tonnes per ha), with a survival estimated at 28 percent. During the emergence of AHPND in 2017, production was 75 tonnes from 34 ha of pond (2.2 tonnes per ha) with a survival of 25 percent. Survival values for these farms are considerably lower than is typical for other farms in Texas, which is usually around 60 percent. This may be also attributed to the presence of NHP, which was confirmed in Texas in 2014. Production in previous years was higher. In 2014, production was 4.5 tonnes per ha with a 40 percent survival; and in 2015, production was 3.5 tonnes per ha with a 31 percent survival. Thus, production in terms of tonnes per ha declined every year from 2014 to 2017 when the AHPND outbreak occurred.

Additional costs, not yet quantified, would include costs of treatment, diagnostic testing, facility disinfection, implementing control measures, monitoring of stocks, changes in management strategies and AHPND-related research. These control programmes are often difficult to discontinue due to the risk of new AHPND incursions. Even if a country is AHPND-free, there are ongoing costs due to efforts to prevent disease introduction, including surveillance and import controls.

6. Principles of control and eradication

This section provides basic information relevant to the development of management strategies in response to AHPND outbreaks in farmed populations of marine shrimp. The information is most relevant to the shrimp-farming countries with the occurrence of AHPND, but the measures described can also be applied to other countries. The disease is caused by pathogenic bacteria *Vp*$_{AHPND}$, and, although wild shrimp, such as *P. vannamei* (in Latin America), *P. monodon* (in Southeast Asia) and *P. chinensis* (in the People's Republic of China) could be affected, control measures for these wild populations would not be feasible.

The implementation of rapid and effective actions to contain and possibly eliminate an AHPND outbreak on a farm is important to prevent the further spread of disease. Depending on the disease scenario, one of the following broad control approaches (or a combination of these) may be used to respond to an emergency disease outbreak: (i) eradication; (ii) containment and zoning; or (iii) control and mitigation strategies (see Section 6.2 for details). The methods described in Section 6.1 may be employed on farms as part of these three major control strategies.

6.1 Methods for preventing spread and eliminating pathogen

When an AHPND outbreak occurs, the major goals to be achieved in response are: (1) to eradicate the disease where possible; (2) to prevent the spread of the disease; and (3) to prevent re-emergence. The specific methods employed in achieving these goals will depend on a variety of factors including the extent of the outbreak, farm management practices and marketing issues. These methods, along with the conditions in which they can be used most effectively, are discussed in the following subsections.

6.1.1 Quarantine and movement controls

Quarantine and movement restrictions should be implemented immediately upon suspicion of an AHPND outbreak. The CA should establish appropriate zone and compartment designations. Zoning and compartmentalization are management strategies to limit the spread of disease and facilitate international trade in shrimp and shrimp products. Zoning relies more on geographic barriers, and is usually under the responsibility of the CA; while compartments are within the production facilities and are managed through farm-level biosecurity programmes to maintain their health status. Detailed information regarding compartmentation and zoning can be found in the OIE Aquatic code (OIE, 2019a). Zoning is diagrammatically represented in Figure 14 and is described in detail below:

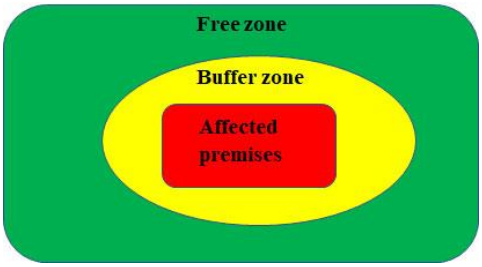

Figure 14. Designation of zone, area, and premise in the AHPND outbreak response.

- Affected premises (or area): the premises (e.g. farm) or area (e.g. geographically separated area) where the AHPND occurs, and its immediate vicinity. An affected premise or area is where a presumptive or confirmed positive case exists based on laboratory results according to international standards.
- Buffer zone: an area adjacent to the affected premises (or area).
- Free zone: the non-affected area.

A control area consists of the affected premises (or area) and the buffer zone.

Movement controls from the affected premises (or area) should include:

- Bans on the movement of live, fresh (chilled on ice) shrimp from the affected premises into AHPND-free areas.
- Bans on using live, fresh shrimp in the affected premises as baits for fishing.
- Bans or restrictions on releasing live shrimp and pond water from the affected premises into aquatic environments.
- Restrictions on discharging of processing plant effluent within the affected premises.
- Restrictions on harvesting and then transporting shrimp in the affected premises to off-site processing plants.
- Restrictions on the use and movement of equipment and vehicles between farms within the affected premises.
- Control of seabird and crab access to live and moribund/dead shrimp within the affected premises.
- Control of the disposal of diseased shrimp.

The implementing of these bans or restrictions will depend on the severity of the disease, the types of operation (such as farm location, farm size, pond system), and the response options chosen.

6.1.2 Tracing

Tracing refers to investigation of: (a) if the diseased shrimp have been moved to other areas; (b) if AHPND has spread to other areas; and/or (c) the origin(s) of the disease. Movements of the following from affected sites or premises might need to be traced:

- Live shrimp: for example, broodstock, PLs and other stocks, including those sold through bait shops.
- Fresh shrimp: shrimp intended for human consumption or for bait.
- Effluent and waste products from processing plants and farms: discharge into nearby coastal or inland waters.
- Vehicles: potentially contaminated transport vehicles, feed trucks, cars and boats.
- Farm materials: nets, buckets and other farm equipment.

6.1.3 Risk factor analysis

Risk factor analysis can coincide with an outbreak investigation and has been regularly used in the management of shrimp diseases in recent years (Corsin *et al.*, 2001; Tendencia, Bosma and Verreth, 2011; Piamsomboon, Inchaisri and Wongtavatchai, 2015; Boonyawiwat *et al.*, 2017). The analysis is conducted to find the factors associated with the occurrence of AHPND

outbreaks in a specific shrimp population. The study of risk factors of disease introduction, spread, or impact in shrimp farms is rather complex, as the aquatic animals are exposed to a wide range of environmental conditions and management practices. Cross-sectional or case-control studies are common approaches to collect and analyze data to explore potential risk factors associated with AHPND outbreaks among farms within a major epizootic area.

A cross-sectional study can occur during an active outbreak (outbreak investigation) or after outbreaks have already occurred on a farm. This is a useful study to determine the prevalence of disease. Data can be gathered from farms in the region of interest using a structured questionnaire (see an example in Appendix 1, prepared by Dr Pathrarpol Piamsomboon, Chulalongkorn University, Thailand) that addresses six classes of variables: (1) farm characteristics; (2) pondsite description and history; (3) disease information; (4) pond features; (5) pond and water preparations; (6) feed and other inputs. The selection of these variables is based on the different farming techniques implemented by farmers and on the measures suggested to prevent AHPND occurrence. If possible, data can also be extracted from existing farm records during the timeframe of interest, including disease diagnoses, water quality parameters (i.e. temperature, dissolved oxygen, salinity, pH, etc.), mortality data, or management practices (i.e. treatments, feeding, stocking density, etc.). Risk factor analysis based on cross-sectional data should be interpreted with caution due to problems with "reverse-causation", because it is often difficult to determine whether exposure to risk factors occurred before or after the AHPND outbreak occurred (Dohoo, Martin and Stryhn, 2009).

A case-control study begins after the AHPND outbreaks have ended, with a selection of known cases (e.g. ponds with a diagnosis of AHPND) and matching controls (e.g. ponds negative for AHPND in farms with the disease) from the same population included the study (population at risk for AHPND). As with cross-sectional studies, data can be gathered via a questionnaire or extracted from farm records during the timeframe of interest. The data is retrospective, which means it is based on events that occurred in the past and collected after the AHPND outbreaks have already ended within the study sample. The analysis of case-control data can determine the association between exposure to potential risk factors and the occurrence of AHPND.

6.1.4 Surveillance

Surveillance is an on-going systematic sampling of shrimp populations, pond water, sediments and monitoring the presence of *Vp*$_{\text{AHPND}}$. It can be used to detect the early occurrence of AHPND and determine its prevalence in populations and is used in the process of maintaining and certifying farms or areas as being AHPND-free. Detailed information on general requirements for surveillance to establish freedom from AHPND at various prevalence thresholds is provided in the OIE Aquatic code (OIE, 2019a).

In large shrimp ponds, which can be several hectares in size, monitoring the health of the shrimp populations is more difficult. Disease problems, such as mortality or the presence of shrimp displaying clinical signs, may go unnoticed. Even with rigorous periodic sampling, the probability of detecting disease issues in a large population is low until the problem has substantially progressed. Incubation of pelleted feed in a mesh bag in the suspected pond is recommended. Careful monitoring of the health of the shrimp populations, however, is important for detection of problems as early as possible so that strategies (e.g. culling, early harvest, pond disinfection and restocking) can be implemented to minimize economic losses.

Depending on the information needed, samples can be pooled prior to diagnostic analysis to reduce costs. Shrimp samples to be tested for AHPND can be groups of PLs or individuals' HP dissected from juvenile or larger shrimp. The prevalence of disease in the population, however, will not be determined because of the pooling. The samples can be fixed, frozen or preserved in ethanol or in other transportation solutions depending on the requirements of the specific diagnostic laboratory to which they will be sent. Surveillance could also be used to examine the presence of AHPND in wild populations, but statistical protocols for such studies would need to be developed on a case by case basis.

For microbiological analysis, potential AHPND-causing vibrios can be isolated on site. Alternatively, live shrimp can be sent to the local laboratories for bacterial isolation and *Vp*$_{\text{AHPND}}$ screening. For isolation of bacteria from pond water, sediments, or faecal strands, samples should be chilled with ice during transportation. If needed, *Vp*$_{\text{AHPND}}$ can be enriched by a 6-h culturing in TSB+, followed by subculturing on TCBS agar plates.

(a) Diagnosis

If an outbreak of AHPND is suspected, it is important to obtain an accurate and rapid diagnosis. The diagnosis of AHPND in PL or HP (or stomach, guts) is usually performed via a series of procedures after a preliminary diagnosis based on clinical signs. These include histological demonstration of lesions; however, diagnosis via histological methods is usually too slow to allow practical management decisions by shrimp farmers. Molecular diagnostic methods are much faster and can provide immediate information to shrimp farmers. The molecular detection of *Vp*$_{\text{AHPND}}$ has been successfully developed, with PCR (and qPCR) having been widely used for the rapid and specific detection of *Vp*$_{\text{AHPND}}$ (OIE, 2019b). PCR (and qPCR) detection kits targeting the *pirAB*^{vp} genes are also available commercially.

(b) Rapid on-site diagnostic assays

There are no vaccines or reliable treatments for shrimp diseases that can be applied to farms, and thus the shrimp farming industry relies heavily on good farm-level biosecurity programmes to prevent the introduction of *Vp*$_{\text{AHPND}}$ to facilities. Optimal on-site detection systems should be rapid, inexpensive, sensitive and easy to maintain and operate by non-specialists. In addition, the reagents should be provided in a format that allows easy shipping and storage. There are a range of different technologies currently available for rapid diagnosis of AHPND:

- LAMP is a simple method involving the heating of DNA templates and a set of *Vp*$_{\text{AHPND}}$-specific primers at a constant (isothermal) temperature (e.g. 65 ^{o}C) for 45–60 min. This method is rapid, specific and highly sensitive (Koiwai *et al.*, 2015; Arunrut *et al.*, 2016). LAMP does not need complex instrumentation, such as a thermocyler as for PCR, nor trained professionals.
- A commercial *Vp*$_{\text{AHPND}}$ PCR detection assay, based on insulated isothermal PCR (using the TaqMan-based qPCR principle), is also available for on-site detection of AHPND (Chang, Lee and Su, 2018). The analyzer is called POCKIT™ (manufactured by GeneReach Biotechnology Corp.) and has been certified by OIE. The assay can be completed within 60 min. A hand-held device is also available for pond-site detection of AHPND *pirAB*^{vp} genes (Chung, Lee and Ma, 2015).

These types of rapid diagnostic test, validated with standard methods, need to be evaluated by policy-makers and then can eventually be incorporated into the surveillance plan.

6.1.5 Farming practices

To reduce the risk of AHPND during grow-out, many producers favor the use of semi-closed or recirculating aquaculture systems. Components of these systems for maintaining water quality, such as reservoir ponds, biological filters and water storage may take up as much as 60 percent of the total farm area. Since the production area is reduced, farmers compensate by increasing stocking densities by two–three times, and by the use of deeper, lined ponds. These systems need vigorous aeration to maintain the high dissolved oxygen levels required for production. To avoid toxic conditions from the build-up of sediments, the daily removal of sludge from the culture ponds is widely practiced. A system known as "shrimp toilet", allows the removal of waste through a central drainage which is automatically activated every few minutes keeping the pond bottoms clean. These more intensive production systems require higher initial investments and are dependent on the quality of PL used for stocking.

Some farmers still use the old, less intensive, culture methods. They may take a two-phase grow-out approach. They stock PLs in small nursery ponds (or in tanks) for a few days up to 45 days for monitoring before transferring them to the grow-out ponds. In these nursery areas, any AHPND-related mortalities, which occur within a short time following exposure, should become evident and the diseased shrimp can be destroyed. Once the larger grow-out ponds are stocked, monitoring becomes more difficult and the effects of AHPND become costly. Farmers try to keep the shrimp in the grow-out pond for a maximum of 60 days, then decide whether to transfer them to a new pond, or make a partial harvest before the pond water and soil deteriorate.

A strategic goal of pond management is to maintain the density of Vp_{AHPND} (if present) to $<10^4$ CFU/mL pondwater (the density can vary depending on the strain's virulence), as no mortality is observed at these low bacterial levels. It is possible that the production of virulence factors is triggered by high Vp_{AHPND} densities. Quorum sensing is a form of chemical communication used by some pathogenic bacteria, including some *Vibrio* spp., in which genes controlling the production or the release of virulence factors are activated only at high densities. Although there is no direct data to support this mechanism, *per se*, for Vp_{AHPND}, laboratory challenge studies have shown that Vp_{AHPND} density needs to reach a threshold, around 10^4 CFU/mL in tank water (dependent on the virulence of the specific isolate) in order to cause mortality in groups of *P. vannamei*. Shrimp farmers now adopt management practices to eliminate or reduce the buildup of organic matter in ponds, as this provides a substrate for bacterial growth. Through avoiding overfeeding, removing sludge and increasing water exchange, the bacterial density in ponds can be kept below the threshold that results in mortality.

Since the emergence of AHPND, shrimp producers in Southeast Asia and Latin America have changed farm designs and operation to facilitate management of this disease (Table 5). Such changes include the use of: (i) smaller, lined ponds; (ii) central drains (i.e. shrimp toilets); (iii) pre-filtered clean water; (iv) the use of tilapia for removal of sediments; (v) increased aeration; (vi) frequent feeding regimes to reduce uneaten feed; and (vii) probiotics applied to the ponds. These management strategies seem to have been effective, as global shrimp production has shown a gradual recovery since 2016.

Table 5. Design and operation of traditional shrimp ponds and new ponds[1]

Pond Design	Traditional intensive pond	New intensive pond
Size (area)	1+ ha	1 000–4 000 m^2
Shape	Rectangular	Square
Depth	1.0–1.5 m	1.5–2.5 m
Bottom	Earthen	Lined (high density polyethylene)
Aeration	20–40 hp/ha	55–75 hp/ha
Discharge location	Side gate	Center drain
Water exchange	<50% over cycle	300%+ over cycle
Polyculture (reservoirs)	None	Tilapia (tolerant to high salinity)
Feeding	4–5 times (daytime)	300+ times/12–24 h
Kg/m^2/crop	1–2 kg/m^2 (before AHPND)	3–4 kg/m^2

[1]Source: Mr David Kawahigashi, VANNAMEI101, Thailand

(a) Shrimp screening prior to stocking

PL must be of appropriate age and development for stocking ponds. Minimum PL10 (postlarvae stage 10) shrimp are recommended for direct stocking into grow-out ponds**,** while PL12 are recommended for low-salinity (<5 ppt) ponds (Limsuwan and Ching, 2013). The age of PL can be determined by the number of spines on the rostrum (Figure 15). Most farmers require that hatcheries provide a report of PL health status prior to purchasing. The general requirements for PL>10 are listed in Table 6. These include, among other factors: fully developed gills and musculature and a healthy HP (Figure 16). Samples of PL intended for stocking should also be screened, through PCR (or qPCR) analyses, for the presence of any major shrimp pathogens, including AHPND. Some hatcheries also provide a report on the numbers of *Vibrio* spp. present in the HP; results should show <1 000 CFU/g of total *Vibrio* spp. in TCBS and <100 CFU/g of green-colony *Vibrio* spp. As seen in Figure 6, the AHPND-causing *V. owensii* are yellow colonies, thus the screening based on colony colour may underestimate the numbers of AHPND-*Vibrio* present. A stress test may be also performed by placing PL in freshwater for 30 min and then returning them to salt water (30 ppt) for another 30 min; the survival in these stress tests should be greater than 95 percent. Addressing these issues will insure that farmers receive high-quality PL.

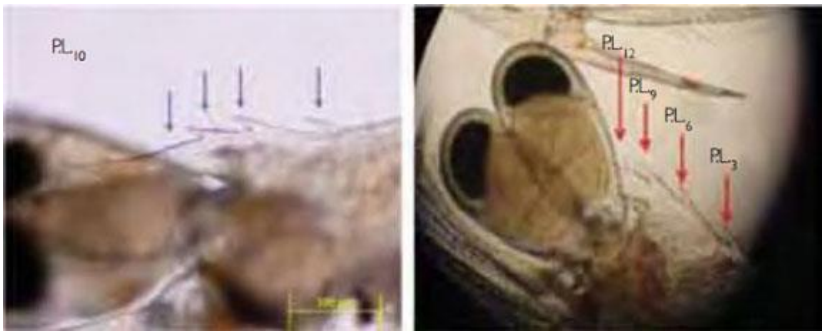

Figure 15. Postlarval stages of *P. vannamei*. Left photo illustrates that the three rostral spines and small spine bud identify this specimen as a PL10 shrimp. Right photo illustrates that each completely formed spine on the rostrum represents one of the three larval stages.

Table 6. Recommended tests of *P. vannamei* postlarvae prior to stocking into ponds

Test	Category	Requirement
Microbiological	*Vibrio* counts in HP	• Total *Vibrio*: <1 000 CFU/g • TCBS green colonies: <100 CFU/g
PCR-based	AHPND and other major pathogens	• Not detected
Visual evaluation	Larval activity	• Suspend aeration in the tank for a couple of minutes to look for active swimming against the water current
Light microscopic examination	HP	• Dark brownish colour • Abundant lipid droplets
	Digestive tract	• Peristaltic movement in the intestine and a muscle:gut ratio of 4:1 (Figure 16)
	Screening for parasites	• Lack of fungi and ciliated protozoans in the gills and body of larvae
Osmotic stress test	Sudden changes in salinity	• >95% survival

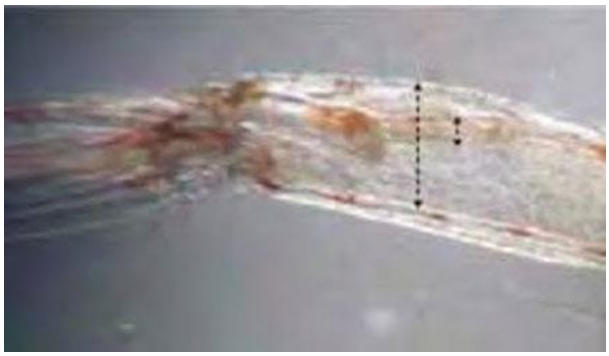

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Figure 16. A muscle:gut/ratio of 4:1 (arrows) is an indication of healthy shrimp.

(b) Pond water preparation

For pond water preparation, the source should not be shared with farms having AHPND outbreaks. Intake water should be best well water, stored in the reservoir ponds, conditioned for 10–15 d, and then fertilized to ensure a stable bloom before stocking. The water should be properly treated (i.e. ozone) to avoid the loss of diversity; *Vibrio* spp. may proliferate and dominate in the pond.

Application of biofloc technology in shrimp farming ponds is now being considered a good way to contain AHPND. One study showed that biofloc can protect *P. vannamei* from Vp_{AHPND} and the management of biofloc in aquaculture ponds can assist in controlling bacterial infections (Shinn *et al*., 2018b). However, the key risk factor of this system is the organic load.

(c) Pond bottom preparation

To prepare the pond prior to stocking, the topsoil (10–20 cm for an earthen pond) should be removed; or the pond should be dried (for at least two weeks), plowed and apply the liming substances before stocking.

For AHPND, one of the most important risk factors is the sludge on the pond bottom, which consists of organic wastes, uneaten feed, dead shrimp parts, molts, dead algae, settled biofloc, etc. As sludge becomes a substrate for the growth of *Vp*$_{AHPND}$, a high priority for pond management is to keep the pond bottoms clean. For this purpose, pond size is reduced from 1 ha to 1 000–5 000 m^2 and a central drain is included at a size of 5–7 percent of the entire pond area. This drain is connected to a pump to remove the sediments to a sludge collection pond (Kawahigashi, 2018).

6.1.6 Destruction and disposal of diseased shrimp

Any chemical agents used to disinfect or destroy AHPND-affected shrimp must be approved for that use by the CA. The relevant state or local CA should also be consulted for advice before the use of chemicals.

The slaughter of diseased shrimp should be both hygienic and humane, and avoid spillage or escape to the environment. To prevent the mechanical spread of *Vp*$_{AHPND}$ during the destruction process, scavengers, such as seabirds, should be kept away by the use of electric fence barriers or netting.

AHPND-affected moribund and dead shrimp contain *Vp*$_{AHPND.}$ Therefore, they and other possible infectious wastes or sources of bacteria, such as potential aquatic carriers, must be destroyed and disposed of immediately and appropriately to reduce risks of disease. Burial is the least expensive and a practical method for large amounts of diseased shrimp. The burial site should be remote from shrimp cultural areas to reduce the risk of *Vp*$_{AHPND}$ entering natural waters or susceptible species being exposed to these bacteria. As heat effectively destroys bacterial pathogens, incineration can also be used. However, the incinerator must be capable of handling the water content involved. See the OIE Aquatic code (OIE, 2019a) for details of disposal methods.

6.1.7 Disinfection of affected farms

Disinfection of a farm affected by AHPND involves destruction of stocks and disinfection of all components of the facility. Many disinfectants are used in shrimp farming to disinfect water prior to stocking shrimp in ponds and disinfect make up water used to replace evaporation and seepage in ponds. Chlorination is widely used to disinfect municipal waters supplies, and there have been a few studies that showed its efficacy for disinfecting shrimp farm water supplies at 20 to 30 parts per million (ppm) (equivalent to mg/l). The effective dose rates of disinfectants commonly used in shrimp farming can be established through microbial bioassays tests (FAO, 2013). As *Vp*$_{AHPND}$ remain viable in pond water, the water should be disinfected through the addition of chlorine to a concentration of 50 ppm, with the water held for four days prior to discharge. Pond sediments should then be sun dried thoroughly for two months and may also be treated with a minimum of 6 tonnes/surface ha of lime ($CaOH_2$). Equipment (e.g. nets, boots, aerators, pipes, etc.), and the surfaces of tanks (and surrounding facilities) need to be disinfected thoroughly, as *Vp*$_{AHPND}$ can form biofilms on their surfaces.

6.1.8 Vector control

A wide range of aquatic plants and animals, including phytoplankton and zooplankton can serve as vectors for *Vp*$_{AHPND}$, allowing the attached bacteria to be dispersed within shrimp

ponds and to be transported to, and throughout, other aquatic ecosystems. These bacteria can colonize and form biofilms on pond sediments and other surfaces, such as those on equipment, pipes and tanks in aquaculture facilities. In the ponds, when these bacteria become detached, are dispersed and re-attach to other substrate-rich surfaces. Farm management strategies to reduce the proliferation of *Vp*$_{AHPND}$ include controlling vectors by the use of predator fish (such as tilapia), disinfecting the pond water prior to use, preventing accumulation of pond sediments and drying the pond bottoms between production cycles.

Seabirds are known to be important carriers for shrimp pathogens. They are commonly found around shrimp farms, where they feed on the dead and moribund shrimp at the pond edges. They often disgorge diseased shrimp in other ponds, thus spreading the bacteria to unaffected ponds. In addition, the scavenging seabirds defaecate in the surrounding waters and ponds, thus contaminating unaffected areas. Therefore, access of seabirds to AHPND-affected shrimp ponds must be controlled. Netting the farm sites and the use of deterrents such as polythene bags, thermocol and burning crackers are effective in this regard.

Where possible, contact between crabs and AHPND-affected shrimp should also be prevented. Crab intrusion can be prevented by building fences around the farm sites.

6.1.9 Environmental considerations

A management plan that addresses the spread of *Vp*$_{AHPND}$ to wild populations and natural environments should be developed as soon as possible after diagnosis of AHPND in the farmed shrimp. The assessment will require information on the density and distribution of the susceptible species and reservoirs, and appropriate management principles should be applied to reduce the chance of *Vp*$_{AHPND}$ spreading from farms to the wild. Any environmental impacts associated with the destruction and disposal of diseased dead shrimp or contaminated materials should be minimized.

6.1.10 Public awareness

In order to reduce the potential of AHPND spreading to other areas, information regarding the disease and its management must be made available to the general public. AHPND is included on the list of shrimp diseases that are required to be reported to the OIE. CA and industry professionals will need to know that live, fresh, frozen shrimp imports, locally, regionally or internationally, from diseased areas (or countries) need to be avoided. Producers in neighbouring areas need to know what steps to take to prevent the spread of AHPND to their facilities.

Information regarding the status of an AHPND outbreak and the actions being taken to control and eradicate the disease can be disseminated in a variety of forms such as farm visits, providing brochures to farmers, local and social media, agency technical reports, industry bulletins, aquaculture workshops and seminars, and symposia.

It is important to note that *Vp*$_{AHPND}$ are not human pathogens. The virulence factors affecting humans, thermostable direct haemolysin (TDH, known as the Kanagawa phenomenon), TDH-related haemolysin and type III secretion system II-related genes, are not detected in *Vp*$_{AHPND}$ strains (Chonsin *et al.*, 2016). Thus, the *Vp*$_{AHPND}$ isolates characterized so far pose no threat to human health (Bondad-Reantaso and Arthur, 2018).

6.2. Control, containment, and eradication options

The feasibility of containing an AHPND outbreak among aquaculture facilities will depend on prompt diagnosis, the extent of the outbreak and the rapid response. The options may include:

- *Eradication*. Eradication of AHPND from a country will be possible if *Vp*$_{AHPND}$ have not spread to wild populations of shrimp or other aquatic reservoirs. Eradication is most likely to be successful if the outbreak has been rapidly detected and is localized.
- *Containment and zoning*. In areas where *Vp*$_{AHPND}$ has entered into coastal waters and become established in wild populations, country-wide eradication may not be possible. Measures can be taken, however, to prevent further spread of the disease through containment to affected premises (areas) where control efforts are continued. Most important action would be the control in the movement of PLs and broodstock.
- *Control and mitigation***.** This measure involves the implementation of management practices that reduce the incidence and severity of AHPND outbreaks.

6.2.1 Eradication

The complete eradication of AHPND from some Southeast Asian and Latin American countries is viewed as impractical because the wild shrimp (e.g. *P. monodon* in Southeast Asia and *P. vannamei* in Latin America) are susceptible to *Vp*$_{AHPND}$ and these bacteria are already present in the water. Eradication of this disease, however, is feasible for production facilities that are under strict management. Eradication at such facilities will entail implementing procedures that have proven effective with other shrimp diseases, such as outbreaks of white spot disease (WSD) in Australia, East Africa, Spain and the United States of America (State of Hawaii). If possible, the origin of the *Vp*$_{AHPND}$ should be determined to prevent its re-entry into the disinfected areas.

Disinfected farms could resume production, provided that strict measures are taken to prevent re-emergence of AHPND, especially through the use of SPF PL or broodstock obtained from reliable sources (Alday-Sanz, 2018). After disinfection and an appropriate period of fallowing (OIE, 2019a) of the production facilities, farmers should test whether or not the process was effective. This can be accomplished by incubating pelleted feed in the water for a period of 6 h and performing PCR (or qPCR) testing with feed, or stocking small areas with healthy shrimp that are highly susceptible to AHPND. The use of bivalves (e.g. oysters, clams) as sentinels has also been suggested for this purpose. Although they are not affected by AHPND, as a result of their filter feeding, these molluscs can act as mechanical vectors; *Vp*$_{AHPND}$ can thus be detected in these organisms by PCR (or qPCR). These sentinel organisms must be acquired from AHPND-free areas. These could potentially be used to test, prior to restocking, if the facility disinfection was effective. Protocols for the use of sentinel organisms in aquaculture facilities, however, have not been developed.

After restocking the production facility, the stocks should be inspected weekly, for at least 45 days, for the appearance of any clinical signs of AHPND. Then, samples should be taken in accordance with approved protocols and tested for the presence of *Vp*$_{AHPND}$ by PCR (or qPCR).

6.2.2 Containment and zoning

If eradication of *Vp*$_{AHPND}$ is not feasible following an outbreak, zoning and associated disease control measures should be implemented to prevent the spread of the pathogenic bacteria to

AHPND-free areas. The containment of the disease to specific areas can potentially be accomplished through restricting the movement of live shrimp, fresh shrimp products, water or other potentially contaminated items from affected premises and through the establishment of buffer zones (see Section 6.1.1). Conceptually, these buffer zones should undergo routine surveillance with the intent of detecting any spread of the *Vp*$_{AHPND}$ from the affected premises. There are no established guidelines, however, for defining the size of or procedures for monitoring of such buffer zones, so these procedures would have to be developed by the appropriate governmental agency on a case-by-case basis.

6.2.3 Control and mitigation of disease

If the disease cannot be eradicated from natural waters or effectively contained to specific areas, then measures to control AHPND at the facility level and mitigate production and economic losses will need to be developed. Most critical measure will refer to prevent overfeeding and minimizing wastes. Depending on the situation, this may entail adopting new pond designs (i.e. smaller grow-out ponds, use of central drains, see Table 5), the use of nursery phases and the stocking of good quality PL (Table 6). More stringent systems might need to be implemented for the filtration of intake water, increased water exchange or recirculation capacity.

In addition, outbreaks of AHPND may be triggered when the farmed populations are under increased stress; therefore, reducing sources of stress may help prevent low levels of bacteria from rising to densities where PirAB^{vp} toxin is produced. Stress may come from a variety of sources such as high stocking density, poor water quality or other less optimal environmental conditions (e.g. suboptimal temperature or salinity). To ameliorate these problems, farmers should attempt to maintain good pond conditions through increasing aeration and water exchange, carefully monitoring and adjusting the feeding regime, applying prebiotics and probiotics and removing the sludge.

6.3 Trade and industry considerations

Areas that have problems with AHPND may suffer economically due to the possibility of exports of shrimp products to international markets being curtailed and these commodities limited to local markets. Associations of local and regional shrimp producers have highly invested interests in eliminating any shrimp disease that negatively affects production or product values. Such associations could provide valuable information on the design and implementation of biosecurity programmes to prevent AHPND outbreaks and in the development of appropriate response strategies.

6.3.1 Domestic markets

Production from AHPND-affected farms would not be desirable in domestic markets outside of the affected premises unless the products are dried, canned, processed, cooked or value-added (such as breaded, battered or marinated). Agencies regulating commerce may place restrictions on the transporting or marketing of live, fresh shrimp products between affected and disease-free areas.

6.3.2 Export markets

AHPND is a serious disease of penaeid shrimp and is listed by the OIE as a notifiable disease (OIE, 2019a). Thus, countries where AHPND is not present should establish import conditions,

such as requiring imported shrimp to be certified as free of *Vp*$_{AHPND,}$ their testing for *Vp*$_{AHPND}$ upon arrival and the rejection of any batches testing positive. In AHPND enzootic countries, the CA is responsible for conducting the health certification of all exports of live shrimp and shrimp products, and should consult the CA of the intended country of destination for information about its current importation requirements.

The safety of shrimp products is addressed in the OIE Aquatic code (OIE, 2019a). The criteria are that: **either there is no *Vp*$_{AHPND}$ present in the product or**, **if present, that it has been inactivated through processing**. To determine if *Vp*$_{AHPND}$ is present, the product should be analyzed with diagnostic procedures (OIE, 2019b). Processing treatments that inactivate the bacteria include: (1) physical treatments (e.g. heating, drying, smoking); (2) chemical treatments (e.g. pH, salt); and (3) biological treatments (e.g. fermentation).

Processed shrimp products, such as cooked, dried, canned or smoked shrimp, as well as shelf-stable products, aquaculture feed, etc. would be considered safe. If a processing treatment is in doubt with regard to the inactivation of *Vp*$_{AHPND}$, isolation of *Vp*$_{AHPND}$ and/or bioassays can be performed through feeding the suspected *Vibrio* bacteria or shrimp product in question to healthy (*Vp*$_{AHPND}$-free) indicator shrimp in the laboratory for a duration of 1–2 weeks. At the end of the bioassay, if the indicator shrimp are not affected by AHPND, then the shrimp product does not contain viable *Vp*$_{AHPND}$.

7. Policy and rationale

7.1 Overall policy

Addressing biosecurity requires significant resources, strong political will and concerted international action and cooperation. National strategic planning for aquatic animal health and biosecurity is vital; without it, a country can only react in a piecemeal fashion to new developments in international trade and serious transboundary aquatic animal diseases, and its aquaculture and fisheries sectors will remain vulnerable to new and emerging diseases. The Food and Agriculture Organization of the United Nations (FAO) encourages Member Countries to develop and formalize National Strategies for Aquatic Animal Health (NSAAH) and health management procedures (FAO, 2007).

A NSAAH is a broad yet comprehensive strategy to build and enhance capacity for the management of national aquatic biosecurity and aquatic animal health. It contains the national action plans at the short–, medium– and long–terms using phased implementation based on national needs and priorities. The NSAAH outlines the programmes and projects that will assist in developing a national approach to overall management of aquatic animal health and includes an Implementation Plan that identifies the activities that must be accomplished by government, academia and the private sector. The draft framework of the NSAAH should be discussed with and accepted by key stakeholders via a public-private partnership. The final document should be distributed to national policymakers, aquaculturists, other private stakeholders and the general public; and the NSAAH should be endorsed by the Competent Authority as an official policy document.

The development of a NSAAH includes a gap analysis (achieved through a self-assessment survey and strengths weaknesses opportunities and threats analysis) conducted by national and regional focal points, a committee or a task force, a working group on aquatic animal health or

any structure that fits the country. The technical elements that may be considered in the strategic framework will vary depending on an individual country's situation, and thus may not include all the programme elements listed below (alternatively, additional programmes may be identified as having national and/or regional importance and thus need to be included):

i. Policy, Legislation and Enforcement
ii. Risk Analysis
iii. National Aquatic Pathogen List
iv. Health Certification, Border Inspection and Quarantine
v. Disease Diagnostics
vi. Farm-level Biosecurity and Health Management
vii. Use of Veterinary Drugs and Avoidance of AMR
viii. Surveillance, Monitoring and Reporting
ix. Communication and Information Systems
x. Zoning and Compartmentalization
xi. Emergency Preparedness and Contingency Planning
xii. Research and Development
xiii. Institutional Structure (Including Infrastructure)
xiv. Human Resources and Institutional Capacity
xv. Regional and International Cooperation
xvi. Ecosystem Health

The usefulness or application of the development of the NSAAH has now expanded to fit as a critical component of the Progressive Management Pathway for Improving Aquaculture Biosecurity (PMP/AB). The PMP/AB was developed by FAO and partners to guide countries towards achieving sustainable aquaculture biosecurity and health management systems through a bottom-up approach, with strong stakeholder involvement to promote the application of risk management at sector and national levels. The PMP/AB is an extension of the "Progressive Control Pathways" (PCP) stepwise approach which has been internationally adopted to assist countries in developing and monitoring national strategies for the reduction, elimination and eradication of important transboundary diseases of livestock and zoonotic diseases (Bondad-Reantaso and Arthur, 2018; FAO, 2018, 2019b, 2020). Whereas most PCPs focus on control of single diseases or disease complexes, the PMP/AB focuses on building resilience to aquaculture biosecurity vulnerabilities (i.e. threats), which includes pathogens, poor management practices and lack of capacity in public and private institutions. The PMP/AB is risk-based, progressive and collaborative. It is intended to be flexible and can be applied by any country to manage risks in any aquaculture sector, no matter the current national approach for aquaculture biosecurity in place. The pathway is comprised of four stages and countries decide themselves how far and how fast it is appropriate for them to progress to each stage. Responsibilities must be shared among key national, regional and international stakeholders from government, the production sector and academia, as well as other players in the value chain, building on each other's strengths towards a common goal. The development of a risk-based NSAAH is an important element and end-goal of Stage 1 of the PMP/AB. This AHPN disease strategy manual can serve as an important toolkit, as part of contingency planning and the wider emergency preparedness and response element of the NSAAH and PMP/AB.

7.2 AHPND-specific objectives

AHPND is a bacterial disease that causes substantial mortalities, reaching to 100 percent in farmed penaeid shrimp. The accumulated losses due to AHPND since 2010 are estimated to be more than USD 7 billion per year (Shinn *et al*., 2018a), making strategies to prevent catastrophic losses to the shrimp farming industry increasingly important. Following the initial diagnosis, the Director of the Competent Authority and the Chief Veterinary Officer of the country will select the most appropriate strategy for responding to outbreaks of AHPND. These will involve selection of the most appropriate control option: 1) eradication of AHPND from the shrimp farms; 2) containment and zoning; or 3) control and mitigation (see Sections 6.2 and 7.4). The Competent Authority's objectives should include:

- to eliminate the *Vp*$_{\text{AHPND}}$ from the country when possible;
- to prevent the spread of the disease to farmed or wild populations through surveillance and tracing;
- to prevent re-emergence of the disease;
- to minimize the economic impacts of the disease, including loss of domestic and international markets; and
- to ensure that stakeholders and the public are informed of the issues involved in preventing the introduction or spread of *Vp*$_{\text{AHPND}}$ through improper importation or movement of shrimp products.
- to ensure that stakeholders and the public are informed of the issues involved in preventing the introduction or spread of *Vp*$_{\text{AHPND}}$ through improper importation or movement of shrimp products.

7.3 Problems that need to be addressed

An outbreak of AHPND in commercial shrimp farms can result in severe economic losses due to high mortality in affected populations and subsequent reduced production. Without a proper response to disease emergence, *Vp*$_{\text{AHPND}}$ can rapidly spread, through a variety of means, to other farms, and can also become established in wild shrimp populations and the aquatic environments.

Government programmes need to be developed to protect and help individuals and communities that may be at risk from outbreaks of AHPND, especially those communities that are largely dependent on shrimp aquaculture. Shrimp farming, in general, constitutes a substantial social and economic activity, especially for rural areas of developing countries. Shrimp aquaculture generates incomes and creates employment opportunities in local communities. Thus, AHPND outbreaks can result in dramatic losses both to producers and to local economies, especially those depending almost entirely on incomes derived from shrimp farming.

Responses to the emergence of AHPND must be rapid. Initial efforts should be to identify the *Vp*$_{\text{AHPND}}$ quickly through routine diagnostic procedures and then to institute procedures immediately to eradicate (where possible) or prevent the spread of the disease.

7.4 Overview of response options

Responses range from efforts to eradicate the disease from the country to the development of strategies for individual farms to manage production with potential low levels of *Vp*$_{\text{AHPND}}$ in their ponds. The actions are outlined in the following chart (Figure 17). Country-wide eradication strategies (Option 1) can usually only be successful if an initial entry and outbreak

of *Vp*AHPND in an aquaculture facility is quickly discovered, before it has had a chance to become more widely distributed or established in wild penaeids. Where eradication is impossible, containment of the disease to certain zones (Option 2) may be possible, especially in countries where production facilities are widely separated. If the AHPND becomes enzootic in wild shrimp populations or widespread among farms, however, then farm-level management (Option 3) will be the only effective means to reduce economic losses.

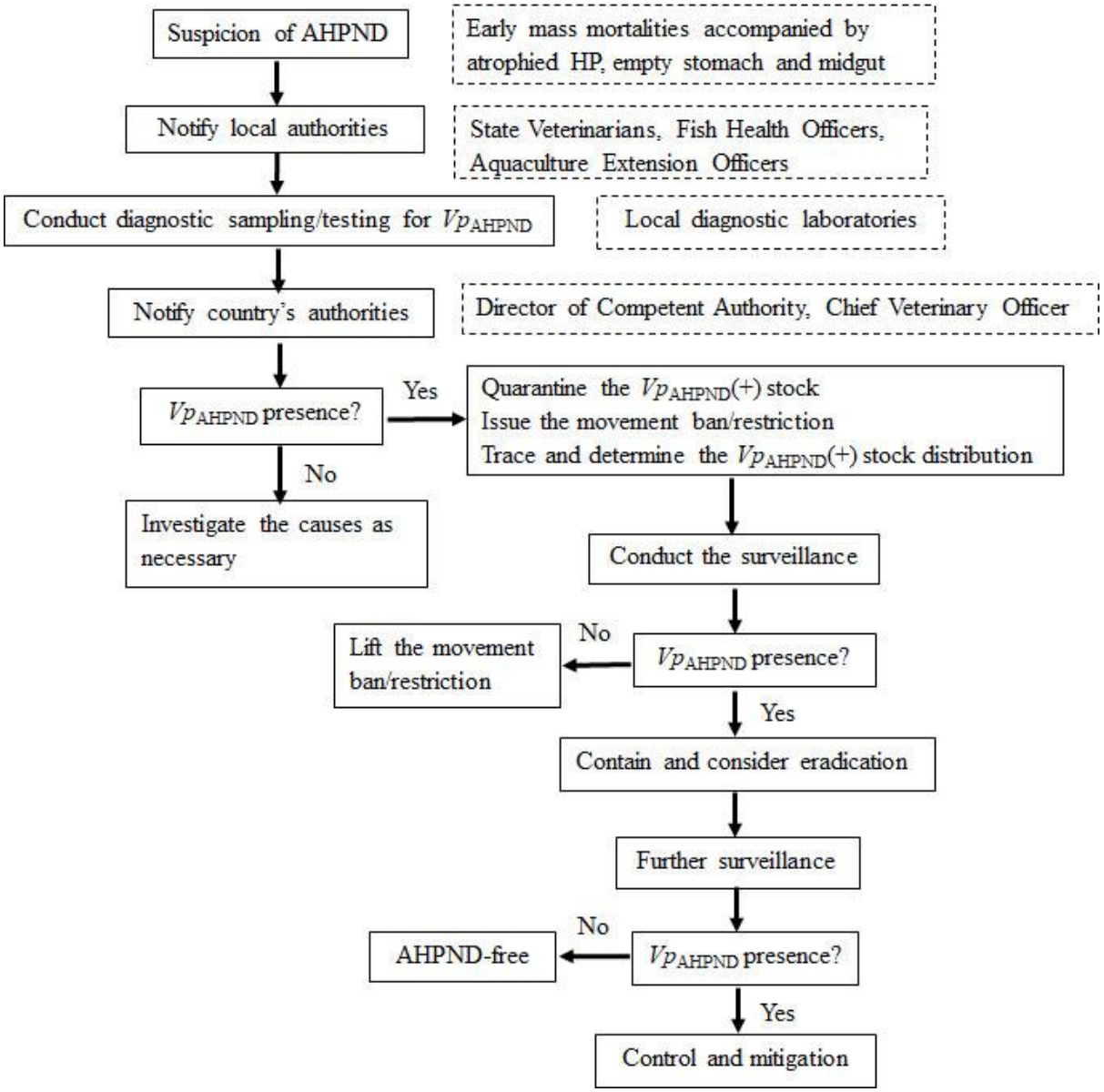

Figure 17. AHPND responses flowchart.

7.5 Strategies for eradication and control

7.5.1 Eradication from production facilities

There is now a long history in the global aquaculture industry of responding to shrimp diseases. Producers, government agencies and industry organizations have developed and successfully employed protocols for eradication of pathogens from shrimp farms. Many producers have met this challenge and have remained active in spite of the ongoing costs of maintaining disease-

free status. Eradication of AHPND from an infected production facility will involve the following steps:

- ***Diagnosis*** – Obtaining an accurate diagnosis is the first step in disease management. Diagnosis requires specimens of, and samples of tissues and/or bacterial isolates from, moribund shrimp or live shrimp with clinical signs of disease. Specimens, tissue samples and bacterial isolates are sent to a competent diagnostic laboratory. Upon confirmation of the diagnosis of the disease, the prevalence of the disease in the farmed populations can be determined through surveillance.
- ***Quarantine and movement restrictions*** – To limit the spread of Vp_{AHPND} from the infected facility, quarantine and movement restrictions must be implemented on the affected premises immediately upon suspicion or diagnosis of an AHPND outbreak.
- ***Destruction and disposal of diseased stock and disinfection*** – To eradicate Vp_{AHPND} from the facility, all diseased stocks must be removed by emergency harvesting or destroyed. Disposal of wastes should be carried out immediately in a manner that prevents access by seabirds, crabs or other scavengers. Usually such wastes are disposed of by burial; the facility should then be thoroughly disinfected.
- ***Restocking with SPF/specific pathogen tolerant (SPT) stocks*** – Once the facility is disinfected, it can be restocked with stocks certified to be SPF (in particular, free of Vp_{AHPND}) and/or SPT, (AHPND-tolerant). There are numerous sources of SPF PL and broodstock of *P. vannamei* available. The AHPND-tolerant lines are under development in several Asian and Latin American countries, with some being ready to commercialize.
- ***AHPND-free declaration*** – Pathways for OIE declaration as an AHPND-free country, zone or compartment can be found in the OIE Aquatic code (OIE, 2019a). A country, zone, or compartment may make a self-declaration of freedom from AHPND for the country, zone or compartment if it meets one of the following criteria:

  1. **Absence of susceptible species**: There are no susceptible species (e.g. *P. vannamei. P. monodon*) present.
  2. **Historically free**: Susceptible species are present but the area is considered free of AHPND provided that:
     a. either there has never been a substantiated occurrence of AHPND in any farmed or wild population, or the disease has not been detected for at least ten years and basic biosecurity conditions have been continuously met during that time; or
     b. a susceptible species is introduced from an AHPND-free area, where basic biosecurity conditions are in place, that previously had no susceptible species present.
  3. **AHPND has been eliminated**: <u>This would apply if susceptible species are present and either an AHPND incident occurred within the previous ten years or the current disease status is unknown.</u> Proof of AHPND-free status should be based on a series of surveys of farmed and wild populations of susceptible species over at least a two-year period. In addition, basic biosecurity conditions should be in force continuously for the past two years. Surveys should be for at least two years, and follow the surveillance protocols recommended (OIE, 2019b). There should be two surveys per year, to be conducted three or more months apart, and these should be designed to provide a greater than 95 percent confidence with a prevalence of 2 percent or lower. Shrimp to be sampled are preferred to display any clinical signs. Such surveys should not be based on a voluntary submission; they should involve the CA of the country.

7.5.2 Containment and movement control

It may be possible to contain the AHPND outbreaks to certain areas or zones. This may be effective under some circumstances where AHPND-free and affected production facilities are not in a close proximity. Appropriate government regulations will need to be developed in support of this strategy, including: (a) restrictions on movement of shrimp products, (b) restrictions on water discharge, (c) prevention of spread by seabirds or other wildlife, and (d) surveillance.

7.5.3 Management and mitigation

If efforts for widespread eradication or containment are impractical or likely to fail, producers may still operate successfully if farms are managed properly. Two scenarios for successful production under these circumstances are to: 1) restock affected farms that have eradicated AHPND and have been disinfected; or 2) manage farms that have AHPND at a low prevalence. Farms in the first category can, through continued surveillance over a period of two years without re-emergence of the disease, be considered AHPND-free. Farms in the second category can continue to produce shrimp, but they may have to prohibit exports of live or fresh shrimp to areas/countries that are AHPND-free.

Effective mitigating measures geared at curbing AHPND outbreaks in shrimp farms include strict adherence to biosecurity and good aquaculture practices.

- ***Management of facilities restored to AHPND-free status*** **–** Farms that have been restored to AHPND-free status will have to implement strict management protocols in order to maintain this status. This will entail stocking the facility with SPF shrimp and ensuring high levels of biosecurity. In general, a biosecurity programme includes:

    - establishing physical structures and barriers, such as fences and gates, and ensuring that all buildings are secured to prevent unauthorized entrance;
    - having procedures in place to control visitors' activities (e.g. requiring sign in/out, wearing protective clothes, disinfection of footwear, providing hand wash stations);
    - refusing entry to individuals who have been at a disease-affected facility within the past three days;
    - disinfecting intake water supplies with filters, and ozonation for indoor facilities to inactivate shrimp pathogens, including *Vp*$_{\text{AHPND}}$;
    - implementing sanitation procedures in the facility, such as avoiding the movement of equipment from one tank (or pond) to another;
    - cleaning and disinfecting (e.g. with chlorine for 30 min) the rearing units before and after production cycles;
    - excluding *Vp*$_{\text{AHPND}}$ vectors during pond preparation, by measures such as the use of predator fish;
    - using quarantine and SPF certified stocks;
    - using live or fresh feeds (*Artemia* cysts, biomass; polychaetes, prebiotics, probiotics) that are SPF, from a trusted source; and
    - performing routine health inspections of all stocks.

- ***Mitigation*** **–** Farms that are not AHPND-free will have to develop management protocols that prevent AHPND from becoming problematic. These management protocols should be aimed at reducing other forms of stress to farmed stocks such as by maintaining optimal environmental conditions, ensuring good water quality and the use of SPF stocks. For *P. vannamei* and *P. monodon*, optimal temperatures for grow out range from 25–35 ^{o}C (Ponce-

Palafox, Martinez-Palacios and Ross, 1997) and optimal salinities range from 15–25 ppt (Boyd, 1989). Water quality can be maintained by water exchanges, careful feeding practices, pond aeration and the application of probiotics. Pond management is being used successfully in several Southeast Asian countries, including Thailand. Production in Thailand was severely impacted by AHPND outbreaks from 2011 to 2014, resulting in substantial economic losses; however, through development of better management practices production has returned to levels that existed prior to the outbreaks.

7.6 Improving knowledge and capability

AHPND is an OIE listed disease and, therefore, OIE Member Countries are obligated to comply with notification requirements as specified in the OIE *Aquatic Animal Health Code* (Articles 1.1.3 and 1.1.4). Early detection and notification by the CA will help to minimize the spread of Vp_{AHPND} within the country and assist in achieving better control of the disease worldwide.

Countries affected by AHPND should continue to build awareness and capacity at the national level and throughout the value chain, including farmers, extension service providers and consumers. Countries that culture shrimp and have not detected Vp_{AHPND} outbreaks should remain vigilant by enhancing capacity in emergency preparedness and surveillance. Communication and capacity building activities should be a collaborative effort between key stakeholders of industry, government and academe through the development and implementation of a NSAAH, as part of the PMP/AB approach. By adopting the PMP/AB, a country will progressively build capacity in aquatic animal health expertise, diagnostic testing, surveillance, emergency preparedness, research, and more, to work towards achieving sustainable biosecurity and health management systems.

A number of international workshops focusing on AHPND (Table 7) have been organized by international and regional agencies such as FAO, the Network of Aquaculture Centres in Asia Pacific (NACA), Department of Agriculture, Forestry and Fisheries (Australia) (DAFF), the Association of Southeast Asian Nations (ASEAN), the Organismo Internacional Regional de Sanidad Agropecuaria (OIRSA) and the Southeast Asian Fisheries Development Center (SEAFDEC) and attended by aquatic animal health professionals and stakeholders to improve their knowledge and capabilities in the control of AHPND in the Southeast Asia and Latin America regions.

Table 7. Technical workshops, symposia and meetings focused on AHPND

Sponsors (Project)	**Title and report (Website)**	**Location /year**
NACA/DAFF	Emergency Regional Consultation on EMS/AHPNS of Shrimp (https://enaca.org/?id=719)	Thailand /2012
FAO (TCP/VIE/3304)	Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (http://www.fao.org/docrep/018/i3422e/i3422e00.htm)	Viet Nam /2013

FAO (TCP/INT/3502) /OIRSA	International Technical Seminar/Workshop "EMS/AHPND: Government, Scientist and Farmer Responses" (http://www.fao.org/fi/static-media/MeetingDocuments/WorkshopAHPND/PresentationsList.html) (for a summarized report, see Bondad-Reantaso (2016)	Panama /2015
ASEAN/ SEAFDEC	Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia (https://repository.seafdec.org.ph/handle/10862/3096)	Philippines /2016
FAO (TCP/INT/3501 and 3502)	Technical Workshops on AHPND, This Is the Way Forward (http://www.fao.org/fi/static-media/MeetingDocuments/WorkshopAHPND/PresentationsList.html)	Thailand /2016

The output of these workshops includes:

- identification of the causative agent for AHPND;
- case definition for AHPND and OIE disease card publication;
- conduct of AHPND-targeted research on various themes such as pathogenicity and virulence, epidemiology, diagnostics, non-antimicrobial control measures and polyculture technologies;
- improved shrimp farm biosecurity and good aquaculture practices through training courses to farmers;
- development of a strategy to support long-term improvement of aquatic biosecurity governance capacity;
- building of a knowledge sharing and communication network through NACA;
- development of a regional emergency fund for member countries of NACA; and
- publication of 23 scientific and technical papers on AHPND and related subjects in a special issue of Asian Fisheries Science.[1]

7.7 Funding and Compensation

Expenses for various aspects of the response to an AHPND outbreak can be substantial, the individual producer and shrimp grower associations would fund some of which, while others may be more appropriate for funding through regional agencies or national governments.

[1] Available at: https://www.asianfisheriessociety.org/publication/archivedetails.php?id=152&q=1.

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APPENDIX 1

Survey questionnaire form[2]

Acute hepatopancreatic necrosis disease (AHPND) risk factors associated with shrimp farming practices and geographical location in [name of State or Province], [Country name]

The questionnaire is a part of a research project of the [name of laboratory], [country name], and is used for research purposes only. All information regarding farm owners will remain confidential.

Thank you for your cooperation.

[name]

1. General information

1.1 Owner's name: _____; Tel: _____
1.2 Owner's address:
House No. _____; Village name: _____
Sub-district _____ District _____ Province _____
1.3 Farm's coordinates:
Latitude _____ Longitude _____

2. Farm characteristic

2.1 Farm area _____
2.2 Culture area _____
2.3 Number of ponds _____
2.4 Reservoir water pond: ☐ Yes, water reserve area_____☐ No
2.5 Sludge pond: ☐ Yes, sludge area _____☐ No
2.6 Water management system:
☐ Open; ☐ Semi-closed; ☐ closed; ☐ Recirculation
2.7 Water recycling in farm (use water from previous crop for the next crop)
☐ 100% recycle
☐ Partial recycle
☐ Discharge all the water at shrimp harvest
2.8 Do you treat water before discharge?
☐ Yes, method: _____ ☐ No
2.9 Where does the water used for shrimp culture come from?
☐ Sea _____ ☐ River_____ ☐ Irrigation canal _____
☐ Underground water _____ ☐ others_____
2.10 Personnel who operate the farm
☐ Yourself; ☐ Yourself and workers; ☐ Appointed manager and workers

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[2] Courtesy of Dr Pathrarpol Piamsomboon, Chulalongkorn University, Thailand.

2.11 Do you have other farms in your care?

☐ Yes, Number of farms you own .....

☐ No

2.12 AHPND outbreaks occurred in farm located next to yours? ☐ Yes ☐ No

2.13 Is your farm fenced? ☐ Yes ☐ No

2.14 Do you allow non-related personnel to enter the farm freely? ☐ Yes ☐ No

2.15 Do you have any pets roaming freely in farm:

☐ Yes, what kind of pet _____; ☐ No

2.16 Do you apply any disinfection practice for vehicles entering a farm?

☐ Vehicle body spray ☐ Tire bath ☐ None

2.17 Do you have hand- and foot-disinfection bath for personnel entering a farm?

☐ Yes ☐ No

2.18 Do you separate work and equipment for each pond (or cluster of ponds)?

☐ Yes ☐ No

2.19 How many crops do you produce per year? _____

2.20 Do you practice polyculture?

☐ Yes. what is another cultured species _____; ☐ No

2.21 Does the adjacent farm conduct routine diagnostic testing?

☐ Yes ☐ No

3. Pond history of AHPND

3.1 Pond No: _____ Date of disease occurrence: _____

3.2 Cultured species: ☐ Pacific white shrimp; ☐ se shrimp; ☐ others_____

3.3 Laboratory confirmation

PCR confirmation of AHPND ☐ Yes ☐ No (Skip to article 4, pond features)

3.4 PCR detection of AHPND in sediment? ☐ Yes ☐ No

3.5 PCR detection of AHPND in pond water? ☐ Yes ☐ No

3.6 Observed clinical signs:

- Percent mortality (%) _____
- Empty stomach? ☐ Yes ☐ No
- Empty gut? ☐ Yes ☐ No
- Soft shells? ☐ Yes ☐ No
- Swimming lethargically? ☐ Yes ☐ No
- Swimming around the edges of ponds? ☐ Yes ☐ No

3.7 Laboratory examination

- Hepatopancreas (HP) fresh smear ☐ Normal
  ☐ Shrinkage (atrophied)
  ☐ Presence of melanization
  ☐ black spots or streaks
  ☐ does not squash easily
- External parasite ☐ Found _____
  ☐ Not found
- Bacterial culture from HP ☐ Green colony _____ CFU/g
  ☐ Yellow colony_____CFU/g

3.8 Day of culture when initial mortality is observed: _____

3.9 Stocking density (no. PL/m^2): _____

3.10 Source of PL:

☐ Name of hatchery _____Location of hatchery_____

3.11 Source of broodstock

☐ domestic broodstock ☐ imported broodstock

3.12 Age of PLs at date of stocking: _____

3.13 Are these PL submitted for AHPND screening before stocking? ☐ Yes ☐ No

3.14 Weather condition during the AHPND outbreaks:

☐ Normal; ☐ Hot (temperature ___); ☐ Rain ☐ Cold (temperature _____)

3.15 Water quality during the AHPND outbreaks:

Dissolved Oxygen: _____; pH: _____; Ammonia: _____

Nitrite: _____;

3.16 Other pathogens/diseases found: At the same time? ☐ Yes ☐ No

☐ EHP; ☐ IHHNV; ☐ WSSV; ☐ IMNV; ☐ YHV; ☐ TSV;

☐ White feces syndrome;

☐ other pathogen(s): _____

3.17 Has this pond ever been affected with other disease?

☐ EHP; ☐ IHHNV; ☐ WSSV; ☐ IMNV; ☐ YHV; ☐ TSV

☐ White faeces syndrome;

☐ other pathogen(s): _____

3.18 Has this pond ever been affected by AHPND? ☐ Yes ☐ No

3.19 AHPND outbreaks occurred in ponds located next to your farm? ☐ Yes ☐ No

4. Pond features

4.1 Are the ponds lined with a polyethylene sheet?

☐ Yes ☐ Whole pond ☐ Slope ☐ No

4.2 Pond biosecurity

☐ Bird-proof netting; ☐ Crab-proof fencing;

☐ Hand- and foot-disinfectant baths; ☐ None

5. Pond and water preparation

5.1 What is the salinity (ppt) of water used for shrimp culture? _____

5.2 Is the sludge (soil at the bottom of the pond) removed after each harvest?

☐ Yes ☐ No

5.3 Do you dry the pond before use?

☐ Yes, how long? _____; ☐ No

5.4 Do you apply lime to the pond bottom?

☐ Yes, concentration _____☐ No

please describe the method:

_____

5.5 Is the water filtered through a trawling net before entering culture ponds?

☐ Yes, size of mesh? _____ How many layers? _____☐ No

5.6 How many days to keep water in the reservoir pond before use: _____

5.7 Do you use animal (chicken/pig) manure or cow dung to fertilize a pond?

☐ Yes ☐ No

5.8 Do you use inorganic fertilizer?

☐ Yes, chemicals: _____; ☐ No

5.9 Water treatment, which are the following substance(s) that you use?

☐ Insecticide (Trichlorfon, Dichlorvos); ☐ Copper compounds; ☐ Tea seed;
☐ Chlorine; Iodine; ☐ Saponin; Quaternary ammonium compounds (QACs);
☐ Probiotics (licensed producer, not-licensed); ☐ phage therapy;
☐ Other _____

Please describe how you prepare the water for shrimp culture:

_____

5.10 Supplement during culture

Probiotic_____ ; how to apply: ☐ feed; ☐ add to water

Vitamin _____ ; Other _____

6. Feed management

6.1 Feed

☐ Commercial feed ☐ Live feed _____

6.2 Feed supplementation

☐ Probiotics (license, non-license) Type _____

☐ Immuno-stimulant _____

☐ Other _____

Please describe how you apply feeding supplementation

_____

6.3 Feeding on the date of stocking: ☐ Yes; ☐ No

6.4 Feeding ratio (%) and frequency (per day):

_____

Other note or comment:

_____

End of the questionnaire

APPENDIX 2

Group photographs

An emergency regional consultation on EMS or AHPNS held in Bangkok, Thailand during August 9-10, 2012

Technical Workshop on EMS or AHPNS of Cultured Shrimp held in Hanoi, Vietnam during June 25-27, 2013 under FAO project TCP/VIE/3304

First Inter-regional Workshop on EMS/AHPND Risk Management and Risk Reduction Strategies at National and Regional Levels, 25-27 June 2015, Panama City, Panama under FAO project TCP/INT/3502

Second International Technical Seminar/Workshop on Acute hepatopancreatic necrosis disease (AHPND): There is a way forward (FAO TCP/INT/3501 and TCP/INT/3502), 23-25 June 2016, Bangkok, Thailand

The Acute Hepatopancreatic Necrosis Disease Strategy Manual provides supporting information for the development of contingency plans for countries, producers and other stakeholders with regard to outbreaks of acute hepatopancreatic necrosis disease (AHPND) in farmed marine penaeid shrimp. This manual is based, in large part, on the Food and Agriculture Organization of the United Nations (FAO) project TCP/INT/3502 Second Interregional Workshop: "Acute hepatopancreatic necrosis disease". The project contributes to the FAO Strategic Programme to increase resilience of livelihoods to threats and crises (SP5), particularly two outcomes, namely: Outcome 5.2 – Countries made use of regular information and early warning against potential, known and emerging threats; and Outcome 5.4 – Countries prepared for and managed effective responses to disasters and crises.

The document describes current information relevant to reducing the risk of, and economic losses from, outbreaks of AHPND. Included are: current knowledge of AHPND and its susceptible host species; detailed diagnostic methods; epidemiology of the AHPND; methods of prevention and management of the disease; planning of national strategies for aquatic animal health (NSAAH) and biosecurity implementation; and discussions of past and potential economic impacts. The manual will also assist countries by providing a framework for the development of national strategies for responses to AHPND outbreaks.

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